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Paul Spearman
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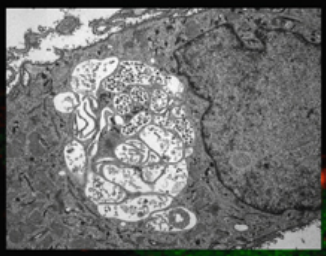
HIV Interactions with Host Cell Proteins



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HIV Interactions with Host Cell Proteins

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Cover motif: Images depict HIV infection of human macrophages. Top: Confocal image showing human macrophage infected with HIV, stained for p24 antigen (green) and CD63 (red). Bottom: Electron micrograph showing HIV-infected macrophage with a large intracellular compartment containing viral particles. Surface staining with ferritin is evident as dark stain on cell surface. Images contributed by Hin Chu and J.J. Wang from the Spearman laboratory at Emory University.

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Preface

The study of viruses necessarily involves dissecting the intimate details of cellular pathways. Viruses have often been employed as tools in studying cellular pathways, as was done by early retrovirologists such as Peyton Rous in attempting to understand the mechanism of cellular transformation and oncogenesis. On the other side of the coin, virologists seek to define those cellular elements interacting intimately with their virus of interest in order to better understand viral replication itself, and in some cases to develop antiviral strategies. It is in the intersection of virology and cell biology that many of us find the most rewarding aspects of our research. When a new discovery yields insights into basic cellular mechanisms and presents new targets for intervention to fight a serious pathogen, the impact can be high and the excitement intense.

HIV has been no exception to the rule that viruses reveal many basic aspects of cellular biology. In recent years, in part because of the importance of HIV as a major cause of human suffering, numerous cellular processes have been elucidated through work on processes or proteins of this human retrovirus. The excitement in this field is especially well illustrated by the discovery of new innate means of resisting viral replication, such as the work on APOBEC3G, TRIM5α, and BST-2/tetherin presented in this volume. Important insights have also been made through the discovery of the role of cellular factors required for integration (LEDGF/p75) and through the identification of host factors involved in trafficking of the HIV structural proteins during assembly.

In this volume, we have attempted to bring together a series of reviews from leading experts in the field focused on the topic of HIV's interactions with cellular proteins and pathways. In some cases, the story starts from a particular viral protein (such as Vpu or Vpr); in others, it begins with the study of a relevant cellular molecule (TRIM5α). We hope the reader will find this a useful basis from which to understand the intricate ways in which HIV has evolved to usurp a variety of cellular machinery to achieve its own ends. In addition, we hope that readers will be able to place the cellular interactions described here in the context of important parts of the life cycle that remain poorly understood. Perhaps the

work presented here will stimulate new investigators to discover new facets of the interaction of HIV with cells that will be of broad value to both virologists and cell biologists.

Summer 2009 Paul Spearman, Eric O. Freed

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Host Restriction of HIV-1 by APOBEC3 and Viral Evasion Through Vif

Anna Maria Niewiadomska and Xiao-Fang Yu

Contents

Abstract The arms race between virus and host is a constant battle. APOBEC3 proteins are known to be potent innate cellular defenses against both endogenous retroelements and diverse retroviruses. However, retroviruses have developed their own methods to launch counter-strikes. Most primate lentiviruses encode a protein called the viral infectivity factor (Vif). Vif induces targeted destruction of APOBEC3 proteins by hijacking the cellular ubiquitin-proteasome pathway. Here we review the research that led up to the identification of A3G, the mechanisms by which APOBEC3 proteins can inhibit retroelements, and the counter-mechanisms that HIV-1 Vif has developed to evade its antiviral activities.

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1 Introduction

Great strides have been made in the research of the Human Immunodeficiency Virus (HIV) since it was first identified as the causative agent of Acquired Immunodeficiency Syndrome (AIDS). However, it was not until a few years ago that the function of the viral protein, viral infectivity factor (Vif), was revealed. Vif was found to overcome the host antiviral protein, APOBEC3G (A3G) (Sheehy et al. 2002). The A3G protein belongs to a family of human cytidine deaminases that can inhibit the replication of Vif-deficient viruses. A3G is packaged into newly formed virions of the defective virus, and upon infection of new cells, induces C→U mutations in the minus strand of the newly synthesized viral cDNA (Sheehy et al. 2002; Bieniasz 2004; Rose et al. 2004; Goff 2003; Navarro and Landau 2004; Turelli and Trono 2005; Zhang et al. 2003; Mangeat et al. 2003; Lecossier et al. 2003; Harris et al. 2002, 2003; Mariani et al. 2003; Chiu and Greene 2008; Bishop et al. 2008). In addition, A3G may also have other antiviral activities that have not yet been fully characterized (Newman et al. 2005). Vif, however, counteracts this potent antiviral element of our immune system by hijacking components of the cellular degradation pathway, and targeting A3G for proteasomal degradation (Yu et al. 2003). This prevents A3G from being packaged into budding viruses, and allows them to successfully infect new cells. In this review, we seek to analyze recent data in the field and address areas of research that still need to be tackled.

2 Non-Permissive Cells and the Identification of A3G

HIV-1 Vif was first identified in 1986 as small 192 residue protein, with a molecular weight of 23 kD. It was termed "sor" for short open reading frame protein and categorized as an accessory protein, since Vif deletion viruses were still able to replicate in certain cell lines (Kan et al. 1986; Lee et al. 1986; Sodroski et al. 1986; Strebel et al. 1987). These cell types were called permissive cells. However, it was found that in other cell types (non-permissive cells) virions produced in the absence of Vif were approximately 1,000 times less infectious than those produced from wild-type HIV-1 (Sheehy et al. 2002; Madani and Kabat 1998; Gabuzda et al. 1992; Simon et al. 1998). Permissive cell types included HeLa, HEK 293T, SupT1, and CEM-SS cell lines. On the other hand, primary human T-lymphocytes, macrophages, H9, and CEM cells were shown to be non-permissive, and thus incapable of producing infectious virions (Gabuzda et al. 1992; Simon et al. 1998; von Schwedler et al. 1993). Eventually, cell fusion experiments showed that this non-permissive phenotype was dominant. Permissive 293T cells were fused with the non-permissive HUT78 cells, resulting in virions that were less infectious than those produced from 293T cells alone (Madani and Kabat 1998; Simon et al. 1998). However, the particular restriction factor in these so-called non-permissive cells was not identified until 2002, when Sheehy et al. performed further experiments with two

genetically related cell lines, the permissive CEM-SS line, and the parental non-permissive cells CEM T-cell line it was generated from (Sheehy et al. 2002). Comparison of cDNAs generated from these two cell lines consistently produced an approximately 1.5-kb cDNA segment that was expressed in all non-permissive cell lines tested, but not in permissive cell lines. This protein was identified as CEM15 or human cytidine deaminase apolipoprotein B mRNA-editing catalytic polypeptide-like 3G (APOBEC3G, A3G), a member of the APOBEC family of cytidine deaminases. Later studies showed that A3F (Bishop et al. 2004; Liddament et al. 2004; Wiegand et al. 2004; Zheng et al. 2004; Dang et al. 2006), A3B (Bishop et al. 2004; Dang et al. 2006; Yu et al. 2004a; Bogerd et al. 2006a, b) A3DE (Dang et al. 2006), and A3H (Dang et al. 2006; Tan et al. 2008; OhAinle et al. 2008), related deaminase proteins, are also capable of restricting Vif-deficient HIV-1.

3 The APOBEC Family of Cytidine Deaminases

The APOBEC family of cytidine deaminases is a large family of proteins with a conserved zinc-coordinating deaminase motif, H-X-E-$(X)_{27–28}$-P-C-$X_{2–4}$-C, that is capable of converting cytosine to uracil in RNA or DNA. This family includes APOBEC1, expressed mainly in the small intestine (Teng et al. 1993), and activation-induced cytidine deaminase (AID), a B cell-specific protein, both of which are found on chromosome 12. The family also includes APOBEC2 on chromosome 6, which is highly expressed in muscle tissue (Liao et al. 1999) and APOBEC4 on chromosome 1, reported to be expressed primarily in testes (Rogozin et al. 2005). APOBEC1 is responsible for the C-to-U editing of apolipoprotein B (apo-B) mRNA, and is catalyzed by a multiprotein complex that recognizes an 11-nucleotide sequence downstream of the editing site (Turelli and Trono 2005; Harris and Liddament 2004). AID on the other hand edits single-stranded DNA, and is required for somatic hypermutation (SHM), class switching recombination, and gene conversion of immunoglobulin genes (Turelli and Trono 2005; Harris and Liddament 2004). The cellular functions of the APOBEC2 and APOBEC4 proteins are poorly understood. Much is known on the other hand about the 7 members of the APOBEC3 family: A3A, A3B, A3C, A3DE, A3F, A3G, and A3H. These proteins are found on chromosome 22 (Jarmuz et al. 2002), and are thought to have been generated by multiple, and relatively recent, duplication events. In fact, rodents possess only one APOBEC3 gene which has been shown to be inessential for mouse development, fertility or survival (Mikl et al. 2005). It is thought that this single APOBEC3 gene expanded in primates 40–100 million years ago (Jarmuz et al. 2002). It is significant that several APOBEC3 genes appear to be affected by positive selection pressure, as evidenced by the accumulation of non-synonymous mutations (Sawyer et al. 2004; Zhang and Webb 2004; Conticello et al. 2005). One explanation for this high level of selection pressure is that these proteins evolved as a defense against endogenous retroelements, since the expansion of APOBEC3 proteins in primates appears to coincide with a sharp decline in retrotransposition

Table 1 A comprehensive list of viruses and retroelements that have been found to be affected by various APOBEC family members

Member	HIV	SIV	HTLV	EIAV	MLV	RSV	HBV	AAV	HPV	Ty1	IAP/MusD	HERV-K	LINE-1	Alu
AID	No (Bishop et al. 2004; Yu et al. 2004a)	No (Yu et al. 2004a)			No (Bishop et al. 2004)		Yes (Jost et al. 2007)							
APOBEC1	No (Bishop et al. 2004)				No (Bishop et al. 2004)		Yes (Jost et al. 2007)							
APOBEC2	No (Yu et al. 2004a)	No (Yu et al. 2004a)												
APOBEC3A	No (Bishop et al. 2004; Wiegand et al. 2004; Dang et al. 2006; Bogerd et al. 2006a; Bogerd et al. 2006b; Chen et al. 2006)				No (Bishop et al. 2004) (Dang et al. 2006)	Yes (Wiegand and Cullen 2007)		Yes (Chen et al. 2006)	Yes (Varta-nian et al. 2008)		Yes (Bogerd et al. 2006a; Bogerd et al. 2006b; Chen et al. 2006)	Yes (Lee et al. 2008)	Yes (Bogerd et al. 2006b; Chen et al. 2006; Mucken-fuss et al. 2006; Niewia-domska et al. 2007)	Yes (Bogerd et al. 2006a; Tan et al. 2008)
APOBEC3B	Yes (Bishop et al. 2004; Dang et al. 2006; Yu et al. 2004a; Bogerd et al. 2006a; Bogerd et al. 2006b)	Yes (Yu et al. 2004a)	Yes/No (Sasada et al. 2005; Derse et al. 2007; Mahieux et al. 2005; Ohsugi and Koito 2007)		No (Bishop et al. 2004) Yes (Dang et al. 2006)	Yes (Wiegand and Cullen 2007)	Yes (Bonvin et al. 2006; Suspene et al. 2005; Zhang et al. 2008c)				Yes (Bogerd et al. 2006a; Bogerd et al. 2006b; Chen et al. 2006) No (Esnault et al. 2006)	Yes (Lee et al. 2008)	Yes (Bogerd et al. 2006b; Chen et al. 2006; Stenglein and Harris 2006; Mucken-fuss et al. 2006; Niewia-domska et al. 2007)	Yes (Bogerd et al. 2006a)

APOBEC3C	Yes (weak) (Bishop et al. 2004; Wiegand et al. 2004; Dang et al. 2006; Yu et al. 2004a; Bogerd et al. 2006a; Bogerd et al. 2006b)	Yes (Yu et al. 2004a)	Yes/No (Sasada et al. 2005; Derse et al. 2007; Mahieux et al. 2005; Ohsugi and Koito 2007)	No (Bishop et al. 2004) (Dang et al. 2006)	Yes (Wiegand and Cullen 2007)	Yes (Suspene et al. 2005; Baumert et al. 2007)	Yes (Vartanian et al. 2008)	Yes (Dutko et al. 2005)	Yes (Bogerd et al. 2006a; Chen et al. 2006; Esnault et al. 2006)	No (Lee et al. 2008)	Yes (weak) (Bogerd et al. 2006b; Chen et al. 2006; Mucken-fuss et al. 2006; Niewia-domska et al. 2007)	
APOBEC3DE	Yes (Dang et al. 2006)	Yes (Yu et al. 2004a)		No (Dang et al. 2006)						No (Lee et al. 2008)	Yes (Niewia-domska et al. 2007)	Yes (Tan et al. 2008)
APOBEC3F	Yes (Bishop et al. 2004; Wiegand et al. 2004; Dang et al. 2006; Bogerd et al. 2006a; Bogerd et al. 2006b)	Yes (Yu et al. 2004a)	Yes/No (Sasada et al. 2005; Derse et al. 2007; Mahieux et al. 2005; Ohsugi and Koito 2007)	No (Bishop et al. 2004; Dang et al. 2006)	Yes (Wiegand and Cullen 2007)	Yes (Bonvin et al. 2006; Suspene et al. 2005; Rosler et al. 2005)		Yes (Dutko et al. 2005; Schu-macher et al. 2005)	Yes (Bogerd et al. 2006a; Chen et al. 2006; Esnault et al. 2006)	Yes (Lee et al. 2008; Lee and P.D. 2007)	Yes (Bogerd et al. 2006b; Chen et al. 2006; Stenglein and Harris 2006; Mucken-fuss et al. 2006; Niewia-domska et al. 2007)	

(continued)

Table 1 (continued)

Member	HIV	SIV	HTLV	EIAV	MLV	RSV	HBV	AAV	HPV	Ty1	IAP/MusD	HERV-K	LINE-1	Alu
APOBEC3G	Yes (Bishop et al. 2004; Dang et al. 2006; Yu et al. 2004a; Bogerd et al. 2006a; Bogerd et al. 2006b; Chen et al. 2006)	Yes (Yu et al. 2004a)	Yes/No (Sasada et al. 2005; Derse et al. 2007; Mahieux et al. 2005; Ohsugi and Koito 2007)	Yes (Mangeat et al. 2003)	Yes (Harris et al. 2003) (Mangeat et al. 2003) (Bishop et al. 2004) (Dang et al. 2006) (Kobayashi et al. 2004)	Yes (Wiegand and Cullen 2007)	Yes (Bonvin et al. 2006; Suspene et al. 2005; Rosler et al. 2005; Noguchi et al. 2005; Turelli et al. 2004a; Tanaka et al. 2006)			Yes (Dutko et al. 2005; Schumacher et al. 2005)	Yes (Bogerd et al. 2006a; Chen et al. 2006; Esnault et al. 2006)	No (Lee and P.D. 2007) weak (Lee et al. 2008)	No (Bogerd et al. 2006b; Chen et al. 2006; Stenglein and Harris 2006; Muckenfuss et al. 2006; Niewiadomska et al. 2007; Turelli et al. 2004b)	Yes (Tan et al. 2008; Chiu et al. 2006)
APOBEC3H	Yes/No (Dang et al. 2006; Tan et al. 2008; OhAinle et al. 2008)	No (Dang et al. 2006)			No (Dang et al. 2006)				Yes (Vartanian et al. 2008)			No (Lee et al. 2008)	No (Muckenfuss et al. 2006) Yes (Tan et al. 2008; OhAinle et al. 2008)	Yes (Tan et al. 2008)

rates in humans (Waterston et al. 2002). Indeed, it has been shown that several APOBEC3 members are capable of inhibiting a variety of endogenous retroelements as well as various retroviruses.

Although A3G was the first anti-HIV-1 deaminase identified, A3F, A3B, A3DE, and A3H can all inhibit Vif-deficient HIV-1. And although A3C has little effect on HIV-1, it is extremely effective against Vif-deficient simian immunodeficiency virus (SIV) (Yu et al. 2004a). In addition, several APOBEC3 proteins can also inhibit a variety of retroviruses such as mouse mammary tumor virus (MMTV), murine leukemia virus (MLV), human T-cell leukemia virus (HTLV), equine infectious anemia virus (EIAV), Rous sarcoma virus (RSV), Feline leukemia virus (FIV), as well as various foamy viruses (FV). Several APOBEC3 proteins can also inhibit the partial double-stranded DNA virus, Hepatitis B (HBV) ,as well as the single-stranded DNA virus, adeno-associated virus (AAV). And finally a recent study presents evidence that may extend the antiviral repertoire of APOBEC3 proteins to double-stranded DNA viruses, such as human papillomavirus (HPV). Several APOBEC3 proteins can also inhibit various retroelements, including both long terminal repeat (LTR) retrotransposons such as Ty1, IAP, MusD, and HERV-K, as well as non-LTR retroelements such as human LINE-1 and Alu. Table 1 offers a comprehensive list of the APOBEC family of proteins and their known effects on various microorganisms.

4 The Antiviral Mechanism of APOEBC3 Proteins: Deaminase Dependent

APOBEC3 proteins have been reported to have various inhibitory effects on multiple viruses, as mentioned above. For the purpose of simplicity, we will focus mainly on the antiviral effects of A3G on HIV-1, the most well-studied virus. Specifically, A3G has been reported to inhibit: (1) the accumulation of viral DNA (Mangeat et al. 2003; Mariani et al. 2003; Bishop et al. 2008; von Schwedler et al. 1993; Bishop et al. 2006; Holmes et al. 2007; Simon and Malim 1996); (2) the accumulation of two-LTR circle DNA (Luo et al. 2007; Anderson and Hope 2008); and (3) proviral DNA formation (Luo et al. 2007; Miyagi et al. 2007) (Fig. 1).

APOBEC3 proteins, like AID, prefer to edit single-stranded DNA. In particular, A3G edits the newly reverse transcribed minus-strand DNA of HIV-1, where it induces C-to-U mutations (Zhang et al. 2003; Mangeat et al. 2003; Lecossier et al. 2003; Harris et al. 2003; Mariani et al. 2003; Yu et al. 2004b; Suspene et al. 2004). Uracil-DNA glycosylases, such as UNG2 or SMUG1, may then generate abasic sites, which in turn may inhibit plus-strand DNA synthesis (Cai et al. 1993; Klarmann et al. 2003; Yang et al. 2007a), or even trigger DNA degradation by cellular endonucleases such as apurinic-apyrimidinic endonucleases (Harris et al. 2003; Yang et al. 2007a). Several laboratories have shown that host UNG-2 can be packaged into HIV-1 virions, via its interaction with other HIV-1 proteins, such as the viral protein R (Vpr) (Mansky et al. 2000) or integrase (IN) (Willetts et al. 1999;

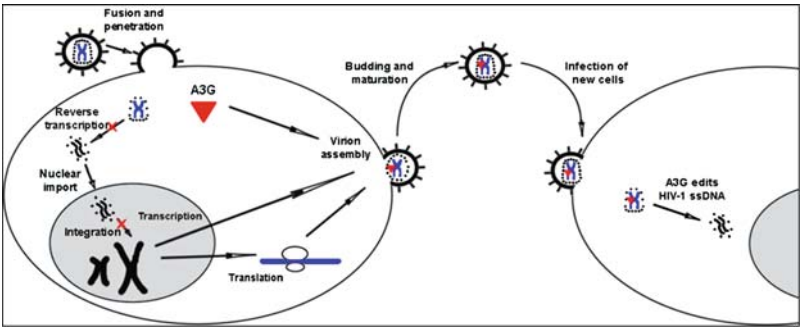

Fig. 1 A3G in the cell. A3G can be packaged into newly budding virions, which when new infections take place in target cells (*right*), can edit newly reverse transcribed ssDNA. A3G may also have deaminase-independent functions in producer cells (*left*), such as inhibition of reverse transcription, strand transfer interactions, and DNA strand elongation

Priet et al. 2003). Alternatively, if the minus-strand uracil-containing viral DNA serves as a template for synthesizing the sense-strand DNA, the G-to-A-hypermutated viral DNA may generate premature stop codons or heavily mutated nonfunctional viral proteins.

Others, however, believe that UNG-2 is dispensable for the antiviral activity of A3G. One group used a bacteriophage protein, uracil DNA glycosylase inhibitor (UGI), in order to demonstrate that the levels of HIV-1 infectivity and A3G restriction were not affected, regardless of the presence or absence of UNG catalytic activity (Kaiser and Emerman 2006; Mbisa et al. 2007). Similar results were obtained with HBV and RSV systems (Nguyen et al. 2007; Langlois and Neuberger 2008). Nevertheless, it cannot be ruled out that Vpr may degrade UNG2 and SMUG1 (Schrofelbauer et al. 2005), thus overcoming the problem of abasic site generation. Alternatively, two other glycosylases, MBD4 and TDG, exist, both capable of acting on dsDNA (Barnes and Lindahl 2004). Neither of these proteins has been studied in the context of A3G antiviral activity. However, deamination may not be the only antiviral mechanism of APOBEC3 proteins.

5 The Antiviral Mechanism of APOEBC3 Proteins: Deaminase Independent

Several groups have shown that A3G and A3F deaminase mutants still retain some anti-HIV-1 activity (Newman et al. 2005; Bishop et al. 2006; Holmes et al. 2007). The first of these deaminase-independent mechanisms is inhibition of reverse transcription (Guo et al. 2006, 2007; Yang et al. 2007b). One group showed that much of the early inhibition of viral DNA production induced by A3G correlated with the inhibition of early minus-sense strong stop DNA, and the inability of $tRNA_3^{Lys}$ to prime reverse transcription (Guo et al. 2006). Later, the same group

also used an in vitro system to show that A3G decreased the ability of $tRNA_3^{Lys}$ to bind to viral RNA and initiate reverse transcription (Guo et al. 2007). Similar results were obtained with A3F (Yang et al. 2007b).

The second mechanism described is the inhibition of strand transfer reactions (Mbisa et al. 2007; Li et al. 2007). Li et al. reported that A3G-induced inhibition of both minus- and plus-strand transfer in reverse transcription was responsible for the majority of the reduction in late DNA synthesis. Mbisa et al. showed that a deaminase-defective A3G resulted in defects not only in plus-strand DNA transfer and integration, but also in primer tRNA processing.

Finally, it was also shown that A3G can inhibit reverse transcriptase-catalyzed DNA elongations (Iwatani et al. 2007). Iwatani et al. used purified A3G, NC and RT in an established in vitro system to study reverse transcription. They found that A3G could inhibit all reverse transcriptase (RT)-catalyzed DNA elongation reactions, but did not have any effect on RNase H activity or NC's activities as a nucleic acid chaperone.

Interestingly, although A3G normally exerts its effects by inhibiting new infections after being packaged into newly budded viruses, it may also exert a post-entry block for HIV-1 in resting T cells (Chiu et al. 2005) and dendritic cells (Kreisberg et al. 2006). While activated CD4+ T lymphocytes are highly permissive to wild-type HIV-1 infection, resting PBMCs appear to be resistant to infection (Chiu et al. 2005). Chiu et al. showed that A3G in activated CD4+T cells exists in high-molecular mass ribonucleoprotein complexes (HMM), while A3G in resting CD4 + T cells is predominantly found in an enzymatically active low molecular mass (LMM) form. This LMM form of A3G is thought to cause a post-entry block early in the HIV-1 replication cycle, by impairing reverse transcription (Chiu et al. 2005). These results were reproducible in both naive and memory T cells (Kreisberg et al. 2006), as well as macrophages and dendritic cells (Stopak et al. 2007). Activation of these resting cells with various mitogens and cytokines caused a shift in A3G from LMM to HMM complexes, which also correlated with increased susceptibility to HIV-1 infection (Chiu et al. 2005; Stopak et al. 2007). That this post-entry block is caused by A3G is further supported by data showing that siRNA directed against A3G in unstimulated CD4+ T cells increased cell permissivity to HIV-1 (Chiu et al. 2005).

In addition, sequencing of reverse transcripts from infected cells showed only low levels of G-to-A hypermutation, suggesting that this early restriction is deaminase-independent. Nonetheless, this restriction is not absolute, as newly synthesized viral DNA was detected in resting CD4+ T cells after a 24- to 48-h delay (Chiu et al. 2005). Inactivated T cells was also reported by others to be permissive to HIV-1 infection in some cases (Stevenson et al. 1990; Watson and Wilburn 1992).

In addition, several APOBEC3 proteins appear to inhibit HBV, AAV, IAP, MusD, and LINE-1 in a deaminase-independent manner, which has yet to be described (Bogerd et al. 2006a; Chen et al. 2006; Stenglein and Harris 2006; Muckenfuss et al. 2006), supporting the idea of alternative, and deaminase-independent inhibition mechanisms.

However, there is still no clear consensus on these deaminase-independent activities, with some arguing that these effects may be just a result of protein overexpression to levels far above normal physiological concentrations. One group showed that active site mutants of A3G had no antiviral activity when expressed at levels similar to those observed in primary human T cells (Holmes et al. 2007; Miyagi et al. 2007; Mbisa et al. 2007; Schumacher et al. 2008). Another showed that the catalytic site of A3G and DNA cytosine deamination is important in inhibiting Ty1, MusD, and HIV-1 when expressed at near-physiologic levels (Schumacher et al. 2008). Nevertheless, this does not rule out the deaminase-independent mechanisms described above, as the active site mutants tested may also block important protein functions such as protein–protein or protein–DNA/RNA interactions.

6 A3G Structural Features

To better understand the structural features of APOBEC3 proteins that are important for relationships with viral proteins such as HIV-1 Vif and NC, and in order to inhibit these interactions and thus achieve our ultimate goal of preventing disease, it is essential to have high resolution structures of these proteins.

Three recent reports on the structure of the C-terminal cytidine deaminase domain (CDD) have highlighted several of these structural features. The first structure was resolved by nuclear magnetic resonance (NMR) (Chen et al. 2008). The second more recent structure was resolved by x-ray crystallography (Holden et al. 2008). The second report confirmed many of the structural features and characteristics first reported by Chen et al. but also reported several important differences. While the crystal structure had a five β-sheet core structure surrounded by six α-helices, the NMR structure was reported to have the same core β-structure, but was surrounded by only five α-helices. Furthermore, the A3G crystal structure was reported to have a long and well-defined β2 strand, while the same stretch of amino acids in the NMR structure was short, and interrupted by a 6-residue bulge. In addition, the two active center loops located near the active site were found at different positions. However, the most important difference was in the proposed nucleic acid substrate-binding area. While the crystal structure had a deep, well-defined groove that ran alongside the AC loops and an active site where the cytosine substrate is thought to be deaminated, the NMR structure did not have this groove. Instead, the authors noticed several positively charged residues surrounding the active site region. Several of these amino acids overlap with residues found in the crystal structure groove. These structural differences could be attributed to several mutations made in the A3G construct that was used to obtain the NMR structure. However, these differences may also be a result of the different methodologies used for isolating the protein and/or structural analysis.

Finally, the most recent report (Furukawa et al. 2009), also presents the structure of the wild-type CDD of A3G, resolved in solution by NMR. While this latest

structure shares some similarities with the crystal structure (such as the presence of a sixth alpha helix), it also has some features in common with Chen et al.'s NMR structure (such as the second interrupted beta strand). The convergence for the two active center loops near the active site was poor and these elements are not well defined in this structure. In addition, the authors presenting this third structure identified yet another position for the binding of the ssDNA substrate. They propose a model where the ssDNA is positioned along the two alpha helices α1 and α2. Clearly, additional experimental studies, in particular mutational assays will be necessary to determine which, if any, of these three models is correct.

Future studies in this field will most assuredly include attempts to crystallize the N-terminal deaminase domain of A3G, as well as the entire molecule. Furthermore, molecular modeling studies of other deaminase proteins based on this structure may shed light on their different substrate-binding and antiviral specificities.

7 Packaging of APOBEC3 Molecules

In order for APOBEC3 molecules to be effective in inhibiting HIV-1, they need to be effectively packaged into newly budded virions. Studies of A3G packaging requirements revealed that A3G binds to the HIV-1 Gag protein, and in particular to the nucleocapsid (NC) region (Cen et al. 2004; Khan et al. 2005; Schafer et al. 2004; Alce and Popik 2004; Zennou et al. 2004; Luo et al. 2004). Several groups have reported that HIV-1 Gag is necessary and sufficient for A3G packaging; however, these experiments were performed with virus-like particles rather than whole virus, which may have different requirements (Cen et al. 2004; Schafer et al. 2004; Alce and Popik 2004; Svarovskaia et al. 2004; Douaisi et al. 2004). Most groups, however, agree that the efficiency of A3G packaging is significantly enhanced by RNA interactions (Svarovskaia et al. 2004). Although A3G appears to edit only ssDNA, it is nevertheless capable of strongly binding to RNA (Jarmuz et al. 2002; Yu et al. 2004b; Khan et al. 2005; Iwatani et al. 2006; Khan et al. 2007; Wang et al. 2007a; Tian et al. 2007). The NC region of Gag is also important for this interaction with RNA, and, together with A3G, may form a protein:RNA complex (Iwatani et al. 2006; Burnett and Spearman 2007).

In the case of A3G, it is the N terminal cytidine deaminase that is thought to be responsible for the nucleic acid binding properties that contribute to packaging, while the C terminal domain is generally thought to be responsible for deamination (Newman et al. 2005; Iwatani et al. 2006; Navarro et al. 2005; Hache et al. 2005). Several groups have identified specific residues in the N-terminal region of A3G that were shown to affect packaging, in particular, W127 (Huthoff and Malim 2007), which was also shown to be important for RNA-binding.

Although many agree that an RNA interaction is important for efficient packaging, the nature of this RNA is still highly debated. One proposed candidate is the HIV-1 viral genomic RNA, where studies showed that, although A3G could be successfully packaged in the absence of viral RNA, these A3G molecules were not

associated with the viral cores of the virions (Khan et al. 2005, 2007). A3G molecules were able to be re-associated with the viral cores by the addition of viral RNA in trans. Another RNA candidate is a cellular RNA, and a component of signal recognition particles (SRP), 7SL RNA (Wang et al. 2007a). Indeed, 7SL RNA has been reported to be both abundantly and selectively packaged in HIV-1 virions (Khan et al. 2007; Wang et al. 2007a; Onafuwa-Nuga et al. 2006). Both A3G and NC mutants that were known to have packaging defects, also showed a reduced ability to bind to and package 7SL RNA (Wang et al. 2007a; Bach et al. 2008). Experiments involving overexpression of SRP19, a known 7SL-binding protein, in increasing concentrations in order to reduce the level of free 7SL RNA found in the cell were performed. This overexpression was associated with reduced levels of A3G packaging (Wang et al. 2007a), further supporting the idea that 7SL RNA contributes to the A3G packaging process. Others, however, believe that, although 7SL RNA can bind both A3G and NC, it is not an essential factor for the A3G packaging process (Bach et al. 2008). And although regions in both NC and A3G have been shown to be important for A3G packaging, it is not known whether this interaction is direct or if their interaction could be mediated by other molecules, such as 7SL RNA. This field is still far from a consensus, and more research is needed in order to resolve these discrepancies.

In addition to the mechanism of packaging, the location of packaging is also an important factor for antiviral activity. A3A, another member of the APOBEC3 family, has a potent inhibitory effect on several retroelements (Bogerd et al. 2006b; Chen et al. 2006), but has no effect on HIV-1 (Bishop et al. 2004). However, when A3A was targeted to the viral core, by creating a Vpr-A3A fusion protein, this cytidine deaminase was able to restrict both HIV-1 and SIV in a Vif-independent manner (Aguiar et al. 2008).

However, not all APOBEC3 proteins are packaged by the same mechanism. APOBEC3C, which efficiently restricts SIV but not HIV (Yu et al. 2004a), can nevertheless be packaged into both. A3C also interacts with HIV-1 Gag protein, but unlike A3G, A3C is packaged through a RNA-dependent and NC-independent fashion (Wang et al. 2008). Thus, it appears that individual APOBEC3 proteins have evolved to use different mechanisms for targeting retroviruses, possibly to broaden the range of viruses targeted.

8 Vif Targets APOBEC3 Proteins for Proteasome-Mediated Degradation

Although APOBEC3 proteins may be a formidable weapon in the cell's antiviral arsenal, several retroviruses have developed their own counter measures that inhibit HIV. The SIV and HIV-1 lentiviruses encode the Vif protein, which can induce the polyubiquitination and degradation of multiple APOBEC3 molecules (Yu et al. 2003; Mehle et al. 2004; Stopak et al. 2003; Marin et al. 2003; Conticello et al. 2003; Sheehy et al. 2003; Liu et al. 2004, 2005). Vif proteins assemble with Cul5,

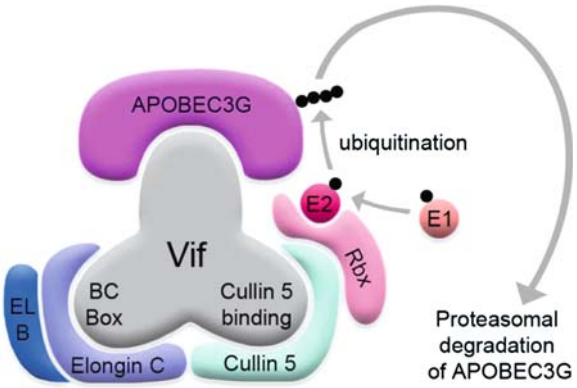

Fig. 2 The HIV-1 Vif-Cul5 E3 ubiquitin ligase complex; a model of Vif-induced APOBEC3 degradation. Vif binds ElonginC through a viral specific BC-box; ElonginC also interacts with ElonginB. The Vif-ElonginC-ElonginB complex also binds to Cul5, forming the Cul5-EloB/C-Vif E3 ligase. Cul5 also binds Rbx which recruits the E2-ubiquitin conjugating enzyme. Ubiquitin activated by E1 enzyme is transiently transferred to the E2 enzyme, then to the APOBEC3 target molecule. Polyubiquitination of APOBEC3 molecules takes place, followed by degradation by the 26S proteasome

ElonginB, ElonginC, and Rbx1 proteins to form an E3 ubiquitin ligase (Yu et al. 2003; Mehle et al. 2004; Liu et al. 2005; Yu et al. 2004c; Luo et al. 2005; Kobayashi et al. 2005) (Fig. 2).

E3 ubiquitin ligases are critical for regulating cellular processes such as mitosis and the cell cycle through targeted protein degradation (Pickart 2004). It is members of the E3 ubiquitin ligase family, such as the cullin-based E3 ligases that mediate protein degradation specificity. These cullins can then form a scaffold on which other E3 ligase protein components assemble and convey the substrate to the E2 ubiquitin-conjugating enzyme. The Vif binding cullin, Cul5, is commonly associated with the ElonginC and ElonginB adaptor molecules. ElonginC recognizes substrate receptor proteins containing a BC-box. The SLQxLA motif in Vif is highly conserved in lentiviral Vifs, and forms a virus-specific BC-box motif that mediates the interaction with ElonginC, which in turn interacts with both ElonginB and Cul5 (Figs. 2 and 3). Vif in turn binds to Cul5 through two other highly conserved sites, the $Hx_5Cx_{17\text{-}18}Cx_{3\text{-}5}H$ motif (Luo et al. 2005; Xiao et al. 2007; Mehle et al. 2006) and a LPx_4L motif downstream (Stanley et al. 2008) (Fig. 3). The first motif is responsible for binding zinc, which stabilizes the molecule, and the second is a highly conserved hydrophobic interface that mediates Cul5 selection.

In terms of Vif binding to APOBEC3 proteins, several regions have been described in both A3G and A3F, and will be further discussed below. It is also of interest that A3C and A3DE, two APOBEC3 proteins with weaker effects on HIV-1, are also ubiquitinated and degraded by Vif (Dang et al. 2006; Zhang et al. 2008a). And although A3B may also inhibit HIV-1, it is not degraded by Vif, possibly because of its low expression level in T cells.

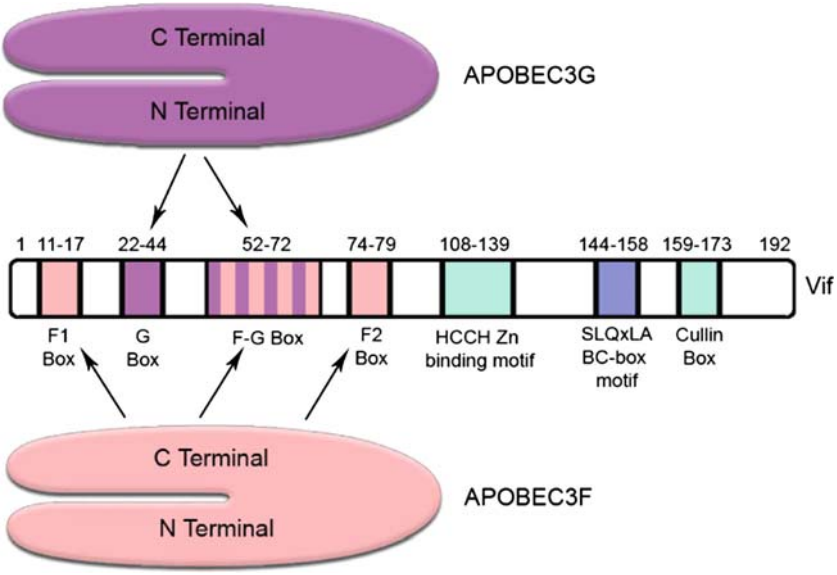

Fig. 3 APOBEC3 and Vif interaction domains. Various domains for interaction of Vif and APOBEC3 with each other as well as other proteins have been mapped. A3F and A3G have different binding requirements on Vif

9 Vif May also Inhibit A3G by Degradation-Independent Mechanisms

Several lines of evidence point to the existence of degradation-independent mechanisms of A3G inhibition in Vif. Recently, a degradation-resistant variant of A3G was identified (Opi et al. 2007). An A3G mutant with a single point mutation at position 97 was found to be defective in multimerization, and at the same time to be impervious to Vif-induced proteasomal degradation, although still capable of binding to Vif (Opi et al. 2007). Surprisingly, although this A3G mutant was not degraded, Vif was still able to prevent its encapsidation into HIV-1 virions, as well as inhibit its antiviral activity (Opi et al. 2007). In fact, one report suggests that Vif's abilities to establish the production of infectious virus and to degrade APOBEC3 proteins are completely separable functions (Kao et al. 2007). In this study, several different forms of tagged HIV-1Vif were studied. It was found that although Vif expressed from a proviral vector was less efficient at degrading A3G than Vif expressed from codon-optimized vectors, it still was able to efficiently inhibit A3G activity (Kao et al. 2007). In addition, although a YFP-tagged form of Vif was able to efficiently degrade A3G, it was unable to restore viral infectivity (Kao et al. 2007). Further support for this theory comes from Vif mutational studies. Although a point mutation of a serine at position 144 in Vif resulted in reduced levels of viral

infectivity, this mutant was found to be able to degrade A36 effectively and efficiently (Mehle et al. 2004). Moreover, HIV-1 is capable of inhibiting the deamination activity of both A3G (Santa-Marta et al. 2004) and AID (Santa-Marta et al. 2007) in bacterial *E. coli* systems, which do not contain any proteasomal-degradation machinery.

The details and mechanism of this degradation-independent inhibition are not yet known. Two theories that have been proposed are that: (1) Vif competitively binds to a common Vif/A3G binding element that interferes with A3G packaging (Goila-Gaur et al. 2008a); or (2) Vif promotes the shift of A3G from LMM to HMM states (Goila-Gaur et al. 2008a, 2008b). A recent paper also notes that although newly synthesized A3G and stable pre-existing A3G are packaged with the same efficiency into virions, HIV-1 will preferentially degrade newly synthesized A3G (Goila-Gaur et al. 2008b). It is thus possible that Vif may have to use alternative methods to inhibit the action of pre-existing A3G in the cell. Again, this is another area of the Vif:APOBEC field that is ripe for further exploration.

10 Specificity of the Vif:APOBEC Interaction

As discussed above, several important structural elements exist in Vif that allow interactions with various molecules. In addition to the structural elements of Vif necessary for interaction with APOBEC3 proteins, there also exist certain domains on APOBEC3 proteins, important for interactions with Vif. For example, it is known that it is the N-terminal cytidine deaminase domain of A3G which interacts with Vif (Conticello et al. 2003) (Fig. 3). In contrast, it was noticed that adding tags to the C-terminal of A3F induced resistance to degradation by Vif, which was later shown to be due to reduced interaction with Vif (Tian et al. 2006). In fact, the C-terminal of A3F alone is sufficient for interaction with, and degradation by, Vif (Zhang et al. 2008b). Recently published data further specify the region of A3F necessary for interaction with Vif, and map it to an area between amino acids 283–300 (Russell et al. 2008). Furthermore, the authors also show that this stretch of amino acids was sufficient for both interaction with, and degradation by the Vif protein. Therefore, although interaction with Vif maps to the N-terminal of A3G, it appears to be the C-terminal domain that is important for this interaction in A3F (Fig. 3).

Studies to map the specific regions in Vif that are important for A3G and A3F binding revealed that various amino-terminal domains of HIV-1 Vif molecules are involved in distinct substrate APOBEC3 recognitions (Marin et al. 2003; Goila-Gaur et al. 2008b; Tian et al. 2006; Simon et al. 2005; Schrofelbauer et al. 2006; Russell and Pathak 2007; Mehle et al. 2007; He et al. 2008). A region in HIV-1 Vif, spanning a stretch of amino acids from 22 to 44, was found to be important for the suppression of A3G but not A3F (Simon et al. 2005; Russell and Pathak 2007; Mehle et al. 2007). This region is known as the G-box (Fig. 3). On the other hand, several amino acids from 11 to 17 and 74 to 79 of HIV-1 Vif were found to be

important for the suppression of A3F but not A3G (Tian et al. 2006; Simon et al. 2005; Schrofelbauer et al. 2006; Russell and Pathak 2007; He et al. 2008) (Fig. 3). Yet another region, from amino acids 52 to 72, was found to be important for both A3G and A3F suppression (He et al. 2008) (Fig. 3). In addition to A3G and A3F, other APOBEC3 family members, such as A3A, A3B, A3C, and A3DE, can also bind to HIV-1 Vif (Dang et al. 2006; Zhang et al. 2008a; Marin et al. 2008). And although A3A and A3B are not thought to be normally degraded by HIV-1 Vif even though they are re-localized by it, certain Vif variants from different HIV-1 strains were capable of degrading them (Marin et al. 2008). However, little is known about how these proteins are recognized by HIV-1 Vifs of different HIV-1 strains, and further exploration is needed.

In addition to the many Vif regions that determine specificity of binding to various APOBEC3 and cellular proteins, various Vifs can also exert species-specific selectivity on the proteins they degrade. For example, HIV-1 Vif can effectively inhibit human A3G, and African green monkey SIV (SIV_{Agm}) Vif is able to inhibit Agm A3G. However, HIV-1 Vif is unable to recognize and inhibit simian (Agm or rhesus macaque) A3G. Vice versa, SIV_{Agm} Vif cannot inhibit human or macaque A3G. On the other hand, the Vif protein from macaque-specific SIV (SIV_{Mac}) can counteract all three A3Gs from humans, Agms, and rhesus macaques (Mariani et al. 2003). It was shown that this species specificity was conferred by a single amino acid, an aspartate at position 128 in A3G. Several independent studies found that this single amino acid residue at position 128 in human A3G was responsible for the species specificity. Changing this residue to its equivalent in Agms (D128K) was enough to reverse the specificity of HIV-1 and SIV_{Agm} Vifs for the binding and degradation of their respective human and Agm A3Gs (Bogerd et al. 2004; Schrofelbauer et al. 2004; Mangeat et al. 2004; Xu et al. 2004). In contrast, mutations at the equivalent position in A3F, at amino acid 127, had no influence on Vif suppression (Liu et al. 2005).

Finally, it is of interest that phosphorylation of both Vif and A3G can play a role in modulating these interactions. Vif can be phosphorylated at several serine and threonine residues: T96, S144, and T188 (Yang and Gabuzda 1998; Yang et al. 1996). Mutation of these conserved residues does not affect Vif-A3G binding, or A3G degradation (Mehle et al. 2004); however, mutations preventing phosphorylation of S144 in the Vif BC-box significantly decreased Vif function by inhibiting the Vif-ElonginC interaction (Mehle et al. 2004), and thus hindering the process of proteasomal degradation. In addition, a recent report has proposed that A3G is also capable of being phosphorylated (Shirakawa et al. 2008).

Shirakawa et al. showed that protein kinase A (PKA) can bind to and phosphorylate A3G at a threonine at position 32. Phosphorylation of A3G at this position appears to reduce Vif–A3G interaction, thus reducing levels of A3G ubiquitination and degradation. Computer modeling and mutagenesis studies were also used to study an interaction between two amino acids in A3G (T32 and R24) that are proposed to be important for binding to and subsequent degradation by Vif. Thus, phosphorylation may be yet another mechanism whereby protein interaction between Vif, A3G, and proteasomal degradation machinery can be regulated.

11 Vif and Cell Cycle Inhibition

In addition to the antiviral effects Vif exerts through degradation of APOBEC3 proteins, several reports describe a Vif-induced cell cycle delay (Sakai et al. 2006; Wang et al. 2007b; Dehart et al. 2007). Vpr is normally thought to be the HIV-1 protein responsible for the G_2 cell cycle arrest that has been observed in CD4+ T cells (He et al. 1995; Jowett et al. 1995; Stewart et al. 1997). Unexpectedly, though, studies in HIV-1-infected cells in the absence of Vpr showed an accumulation of cells at the G_2 phase, and further studies in the presence or absence of Vif confirmed that this phenotype was Vif dependent (Sakai et al. 2006). Additional experiments showed that expression of the Vif protein alone in the absence of HIV-1 infection was enough to increase the ratio of G_2:G_1 cells (Wang et al. 2007b). Moreover, another recent report showed that the G_2 arrest phenomenon was actually a cell cycle delay, and that Vif uses the same machinery to induce cell cycle delays that it does for APOBEC3 degradation (DeHart et al. 2008). DeHart et al. showed that a Cul5 E3 ligase was required for this phenotype, regardless of the presence or absence of APOBEC3 proteins. However, although the presence of Vif resulted in an accumulation of cells in the G_2 phase, when followed over a period of several days, it was shown that Vif did not inhibit cell division or reduce the number of dividing cells (DeHart et al. 2008). The degraded substrate responsible for the $G2_2$ disruption has not yet been identified.

12 Other Roles for Vif

In addition to APOBEC3 and proteasomal-degradation machinery proteins, several laboratories have reported that Vif also binds to many other cellular proteins. Ku70, a cellular protein involved in DNA double-strand break repair, and a component of HIV-1 pre-integration complexes (Li et al. 2001), was shown to bind to HIV-1 Vif in a yeast two-hybrid screen of a human lymphocyte cDNA library (Madani et al. 2002). Ku70, together with another protein, Ku80, form a complex (Ku70/80) that can function as a single-stranded DNA-dependent helicase. Therefore, Vif may recruit Ku70 early in the HIV-1 replication cycle to aid in the integration process of the HIV-1 PIC.

In another experiment, a glutathione S-transferase (GST) pull-down assay used to identify Vif-binding partners, showed that Vif bound specifically to the SH3 domain of Hck, a Src family tyrosine kinase (Hassaine et al. 2001). This group further showed that Hck inhibited both the production and the infectivity of HIV-1 viruses in the absence of Vif, and that expression of Hck in Jurkat cells rendered these cells less permissive to infection by Vif deletion viruses. Hck is present in monocytes but not in primary restrictive T cells (Hassaine et al. 2001). Interestingly, Hck also binds to the HIV-1 Nef protein (Saksela et al. 1995), and dominant negative forms of Hck have been shown to block HIV-1 Nef-induced MHC class I downregulation (Chang et al. 2001).

SP140, the nuclear speckle factor, is yet another cellular protein that has been reported to bind to Vif (Madani et al. 2002). SP140 was found to be expressed in non-permissive cells, but absent in permissive cells; however, expressing SP140 in permissive cells did not render them non-permissive. Nonetheless, expression of Vif in cells caused the dispersal of SP140 from nuclear speckles, or its retention in the cytoplasm (Madani et al. 2002). These nuclear speckles are known as PML bodies and have been reported to have functions in transcription, DNA repair, viral defense, cell stress, cell cycle regulation, proteolysis, and apoptosis (Bernardi and Pandolfi 2007).

Other proteins reported to associate with Vif include: SSAT, the spermine/spermidine N1-acetyl-transferase (Lee et al. 1999), a protein involved in polyamine metabolism, the regulation of which could affect viral RNA; cyclophilin A, a member of the peptidyl-prolyl cis-trans isomerase (PPIase) family, which has been shown to interact with many HIV-1 proteins including p55 gag, Vpr, and capsid protein. Cyclophilin A has been shown to be necessary for the formation of infectious HIV virions (Billich et al. 1995); and NVBP, a novel Vif-binding protein (Lee et al. 1999).

Although the interaction of Vif with most of these proteins was reported before Vif was known to degrade A3G, it may be worth re-examining the roles of these proteins in relation to Vif, particularly in light of the recently reported cell cycle disruptions, and the degradation-independent inhibition of A3G induced by Vif.

13 Conclusions

Although the HIV field, and in particular the APOBEC/Vif area, is a rapidly advancing one that aggressively pursues new discoveries, several important questions still remain. It is unclear what (if any) other cellular and antiviral functions APOBEC3 proteins may possess. Similarly, Vif has shown signs of being involved in processes other than APOBEC3 degradation, and these need to be further investigated. The regions and particular amino acids of APOBEC3 and Vif involved in their interaction still need to be fully mapped. In addition, high resolution structures for both Vif and full-length A3G still do not exist. Such structures and further mapping of protein interactions are essential for initiating new drug design studies.

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Interactions of Viral protein U (Vpu) with Cellular Factors

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Abstract Viral protein U (Vpu) is encoded by one of several accessory genes of HIV-1. Like the accessory gene products Vif and Vpr, Vpu targets host proteins such as CD4 for degradation via the recruitment of cellular multi-subunit ubiquitin ligases. Vpu also forms ion channels in cellular membranes. Through one or both of these attributes, Vpu antagonizes host cell factors that restrict the release of progeny virions from infected cells. A key target of Vpu has recently been identified as the interferon-induced transmembrane protein BST-2/CD317 (tetherin), which restricts viral replication by retaining nascent virions on the cell surface. The counteraction of this host defense allows Vpu to be considered an antagonist of the innate immune response to viral infection.

1 Introduction

Viral protein U (Vpu) is encoded by one of several so-called accessory genes of HIV-1 (reviewed in Malim and Emerman 2008). These genes may appear dispensable for viral replication under specific conditions in vitro, but their conservation among diverse primate lentiviral isolates documents their importance during viral replication in vivo. Vpu, like the accessory gene products Vif and Vpr, targets host cell proteins for degradation via the recruitment of ubiquitin ligases (Margottin et al. 1998; Yu et al. 2003; Hrecka et al. 2007). Vpu also forms ion channels in cellular membranes (Schubert et al. 1996b). Through one or both of these attributes, Vpu antagonizes host cell factors that restrict the release of nascent progeny virions from infected cells (Varthakavi et al. 2003). Recent data indicate that one such restriction factor is the interferon-induced transmembrane protein BST-2/CD317, which retains virions on the cell surface (Neil et al. 2008; Van Damme et al. 2008). These observations now allow Vpu to be considered an antagonist of the innate immune response to viral infection.

2 Expression

2.1 Comparative Genetics

Vpu is encoded primarily by HIV-1, although it is also found in a subset of simian immunodeficiency viruses (SIVs), most notably those isolated from chimpanzees and believed to be the precursors of the current pandemic human strains (Gao et al. 1999). HIV-2 strains, like most SIV strains, lack *vpu* genes. However, certain strains of HIV-2 encode envelope glycoproteins that provide a Vpu-like activity in enhancing the release of virions from infected cells (Bour et al. 1996).

2.2 mRNA

The Vpu protein is expressed from bicistronic mRNAs that also express the envelope glycoprotein (Env), suggesting a genetic as well as distant functional relationship

between the two genes (Schwartz et al. 1990). These mRNAs are incompletely spliced and require the viral Rev protein for nuclear export and translation. Consequently, Vpu (and Env) are expressed relatively late during the viral replicative cycle.

2.3 Subcellular Distribution

Vpu is a small, transmembrane protein that accumulates at steady-state within various cellular membrane systems including the endoplasmic reticulum (ER), Golgi, *trans*-Golgi network (TGN), and endosomes including those involved in recycling endocytosed proteins to the plasma membrane (Pacyniak et al. 2005; Varthakavi et al. 2006; Van Damme and Guatelli 2007). Some Vpu proteins, specifically those of HIV-1 group M, clade C, appear to reside at least partly at the plasma membrane (Pacyniak et al. 2005). The trafficking itinerary of Vpu is poorly defined; conceivably, Vpu, like many endosomal proteins, may transit the plasma membrane en route to its apparent steady-state destinations. The trafficking of Vpu is controlled at least in part by sequences within its cytoplasmic domain (Ruiz et al. 2008). Vpu is not believed to be incorporated into virions.

3 Structure

The 16 kD Vpu protein has almost no ecto- (luminal) domain, a single membrane-spanning domain, and a cytoplasmic domain that interacts with several cellular proteins (Strebel et al. 1988; Cohen et al. 1988). In the well-studied and prototypical protein encoded by the group M, clade B sequence found in the molecular clone NL4-3, Vpu comprises 81 amino acid residues: 4–5 residues are luminal; approximately 23 span the lipid bilayer; and the remainder are cytoplasmic. Multimerization of the protein allows the transmembrane domain (TMD) to form ion channels that are selective for monovalent cations (Schubert et al. 1996b). The cytoplasmic domain (CD) contains a canonical sequence that recruits the cellular protein β-TrCP, the substrate adaptor for an SCF-E3 ubiquitin ligase complex, in a serine-phosphorylation-dependent manner (Margottin et al. 1998).

3.1 Transmembrane Domain

The structure of the transmembrane domain of Vpu has been determined using NMR spectroscopy (Fig. 1): it is an α-helix containing a high proportion of hydrophobic residues (Park et al. 2003). Near the base of the helix is a tryptophan that may anchor the TMD by interacting with phospholipid head groups along the cytoplasmic leaflet of the plasma membrane. Molecular simulations suggest that the TMD forms oligomeric structures within the lipid bilayer, enabling activity as an ion channel (Lopez et al. 2002).

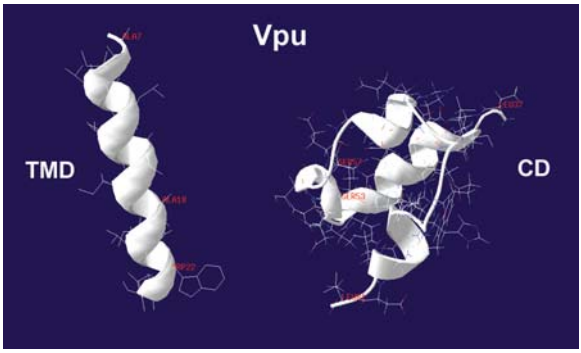

Fig. 1 Structure of Vpu as determined by NMR spectroscopy. The transmembrane domain (TMD: *left*) and cytoplasmic domain (CD: *right*) of Vpu are shown. The TMD is an α-helix that oligomerizes to form cation-selective ion channels; it may also mediate interactions with cellular transmembrane proteins. The cytoplasmic domain contains two conserved serine residues (here numbered 53 and 57 but corresponding to residues 52 and 56 in the prototypical Vpu protein of the HIV-1 clone NL4-3). When phosphoryated, these serines direct interaction with the host protein β-TrCP, a substrate adaptor for a multisubunit ubiquitin ligase complex through which Vpu induces the degradation of host proteins

3.2 Cytoplasmic Domain

The structure of the cytoplasmic domain of Vpu has also been characterized by NMR spectroscopy (Fig. 1): short α-helical domains are bridged by interconnecting loops (Willbold et al. 1997). The membrane-proximal α-helix is amphipathic and appears to lie along the inner leaflet of the plasma membrane. Within the CD is the highly conserved sequence DSGxxS, through which Vpu interacts with β-TrCP, linking cellular substrates including CD4 to degradation pathways via ubiquitination (Margottin et al. 1998). Phosphorylation of the serines within the DSGxxS sequence is required for this interaction. The CD of Vpu also interacts directly with the CD of the viral receptor, CD4 (Margottin et al. 1996; Bour et al. 1995). The ternary interaction of CD4, Vpu, and β-TrCP induces degradation of CD4 by the proteasome, as described below.

4 Function

4.1 Recruitment of an E3-Ubiquitin Ligase Complex to Membrane Proteins

Vpu interacts directly with β-TrCP, the substrate adaptor for an SCF-multi-subunit E3 ubiquitin ligase complex (Margottin et al. 1998). This interaction allows Vpu to recruit β-TrCP to cellular membranes and induce the ubiquitination of target proteins, leading to their destruction via proteasomal or endo-lysosomal degradation.

4.1.1 CD4

Vpu appears to act in the ER to induce the proteasomal degradation of CD4 (Margottin et al. 1998). Serines 52 and 56 are required for this activity, consistent with their essential role in the interaction between Vpu and β-TrCP. Notably, the downregulation of CD4 is also a function of the HIV-1 accessory protein Nef, although unlike Vpu, Nef acts early in the viral replication cycle and directly removes CD4 from the plasma membrane by enhancing its endocytosis and endo-lysosomal degradation (Aiken et al. 1994). The purposes of downregulating CD4 are several-fold and include prevention of "superinfection" (the re-infection of already infected cells, which can induce apoptosis), enhancement of virion-release, and blockade of incorporation of CD4 into virions (Benson et al. 1993; Ross et al. 1999; Lama et al. 1999). (Virion-associated CD4 can inhibit infectivity by interacting with the viral envelope glycoprotein; Lama et al. 1999; Levesque et al. 2003). By targeting CD4 in the ER, Vpu can also free the envelope glycoprotein precursor gp160 from CD4, facilitating its processing by TGN-resident cellular proteases into gp41 and gp120, an event required for viral infectivity (Willey et al. 1992).

4.1.2 BST-2/CD317

The cellular protein BST-2/CD317 has recently been identified as a cellular "restriction factor" that inhibits the release of retroviral particles from infected cells and is counteracted by Vpu (Neil et al. 2008; Van Damme et al. 2008). This relationship is the apparent basis for the long-described ability of Vpu to enhance the release of retroviral particles (virions) from infected cells (Klimkait et al. 1990; Strebel et al. 1988; Terwilliger et al. 1989). Like CD4, BST-2 is a transmembrane protein (Ohtomo et al. 1999), and it is downregulated from the cell surface by Vpu (Bartee et al. 2006; Van Damme et al. 2008). Like CD4, the downregulation of BST-2 by Vpu requires serines 52 and 56 in the Vpu CD, linking this function with the β-TrCP/SCF-ubiquitin ligase pathway (Van Damme et al. 2008). However, inhibition of the proteasome does not appear to inhibit the downregulation of BST-2 by Vpu, suggesting that if ubiquitination is involved, it likely triggers endo-lysosomal rather than proteasomal degradation (Van Damme et al. 2008).

4.2 Interference with Degradation of Host Cell Proteins

Although Vpu recruits the β-TrCP/SCF E3-ligase complex, it is not itself degraded via this complex. Consequently, Vpu is a "pseudosubstrate" for β-TrCP/SCF-ubiquitin ligase complexes, and it can saturate these complexes, competitively inhibiting the degradation of physiologic, cellular substrates (Besnard-Guerin et al. 2004).

This leads to the stabilization of specific proteins and the dysregulation of cellular processes that they control. Two examples of this are the effects of Vpu on Iκβ and β-catenin (Besnard-Guerin et al. 2004; Bour et al. 2001).

4.2.1 Iκβ

Iκβ is a cytosolic inhibitor of the transcription factor NF-κβ, and it is a physiologic substrate of β-TrCP/SCF-ubiquitin ligases. The expression of Vpu stabilizes Iκβ and as a consequence enhances the inhibition of NF-κβ (Bour et al. 2001). This, in turn, leads to a decrease in the expression of NF-κβ-dependent anti-apoptotic proteins such as Bcl-xL and TRAF-1 and consequent induction of caspase-mediated apoptosis (Akari et al. 2001). Vpu may contribute to viral-induced apoptosis by this mechanism.

4.2.2 β-catenin

β-catenin is a cytoplasmic protein that is involved in cell adhesion and, when highly expressed, interacts with nuclear transcription factors to induce the expression of various oncogenes. As in the case of Iκβ, the expression of Vpu stabilizes β-catenin, and it accumulates both in the cytoplasm and the nucleus (Besnard-Guerin et al. 2004). The consequences of this phenomenon for the pathogenesis of HIV-infection remain obscure.

4.3 Ion Channel Activity

Vpu forms cation-selective ion channels in cellular membranes (Schubert et al. 1996b). Consequently, Vpu has been categorized as a "viroporin," along with other virally encoded transmembrane proteins such as the M2 protein of influenza A virus, the p7 protein of hepatitis C virus, and the 6K protein of Sindbis virus (Gonzalez and Carrasco 2003). In conductance studies using artificial lipid bilayers, the individual ion channels formed by Vpu are evanescent, likely reflecting the association and dissociation of Vpu oligomers (Park et al. 2003). Molecular modeling studies suggest that these channels represent pentameric (or possibly hexameric) associations of Vpu TMD monomers (Lopez et al. 2002).

The correct amino acid sequence of the TMD of Vpu is required for the enhancement of virion-release but is dispensable for the degradation of CD4 (Schubert et al. 1996a). This observation has suggested that ion channel activity is mechanistically related to the enhancement of virion-release. In support of this model, ion channel blocking compounds such as hexamethylene amiloride appear to inhibit both transmembrane conductance and enhancement of virion release by Vpu (Ewart et al. 2002). Furthermore, the replacement of the alanine-18 residue in the Vpu

TMD with histidine, which mimics the sequence of the influenza A M2 TMD, renders the release of HIV-1 virions sensitive to the adamantanes, amantadine and rimantadine, anti-influenza drugs that block the ion channel activity of M2 (Hout et al. 2006). Nevertheless, exactly how ion channel activity would induce enhancement of virion release remains obscure, and the correlation of these two activities has not been extensively tested by mutational analysis.

In the case of influenza A virus, M2-mediated neutralization of the pH gradient along the secretory system (which normally increases in acidity from the ER to the plasma membrane) allows the receptor-binding hemagglutinin protein of certain isolates to avoid conformational changes that would render it prematurely fusion-competent (Sakaguchi et al. 1996). This mechanism does not apply to Vpu and HIV-1, because Vpu does not conduct protons and HIV-1 Env does not exhibit pH-dependent fusogenicity.

4.4 Delay in Protein Traffic Along the Biosynthetic Pathway

Both HIV-1 Vpu and influenza A M2 appear to delay secretion along biosynthetic membrane systems (Sakaguchi et al. 1996; Vincent and Abdul Jabbar 1995). This phenomenon may contribute to the ability of Vpu to decrease the levels of class I MHC on the cell surface, as discussed below (Kerkau et al. 1997). Conceivably, such a delay could also affect posttranslational modification of the viral envelope glycoprotein, but Vpu has not been reported to affect the proteolytic cleavage or the glycosylation of Env.

4.5 Enhancement of Virion-Release

Two decades of research on the virology of Vpu have recently culminated in the discovery of a novel component of the innate immune response to infection with enveloped viruses: the identification of BST-2 as an interferon-induced cellular factor that restricts the release of viruses from infected cells (Neil et al. 2008; Van Damme et al. 2008).

Several observations regarding the effect of Vpu on virion release were key to this discovery. In the absence of *vpu*, virions accumulate on the cell surface and within endosomal vesicles, many of which are coated with clathrin and derived from the plasma membrane (Klimkait et al. 1990; Neil et al. 2006; Van Damme and Guatelli 2007). This phenotype of *vpu* is not evident in all cell types, although it is observed using natural host cells such as CD4-positive T cells and macrophages (Schubert et al. 1995; Sakai et al. 1995). Cells that support the Vpu-mediated release of HIV-1 virions also support a similar effect on other retroviruses (Gottlinger et al. 1993), and even on virus-like particles of Ebola virus (Neil et al. 2007); these observations suggest that Vpu acts on a cellular rather than on a viral

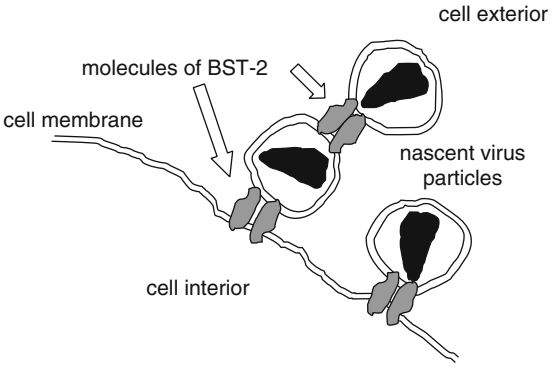

Fig. 2 Model of the interferon-induced transmembrane protein BST-2/CD317 as a host cell restriction factor that prevents lipid-enveloped virions from escaping the cell surface after budding. This activity led to the designation of BST-2 as a "tetherin." Whether BST-2 is actually incorporated into nascent viral particles (virions) is unknown

protein to enhance release. When cells that support the Vpu effect are fused with cells that do not, the resulting heterokaryons support the effect of Vpu; these data suggest the presence of a cellular inhibitor of viral release that Vpu counteracts (Varthakavi et al. 2003). The ability of a specific protease (subtilisin) to release retained virions from intact cells suggests that this inhibitor is a cell surface protein (Neil et al. 2006). Finally, cells that do not express the putative inhibitor can be induced to do so by treatment with type-I interferons (Neil et al. 2007).

Deductive reasoning from the published literature as well as transcriptional profiling of specific cell types treated with interferon allowed this elusive inhibitor to be identified as the interferon-induced gene product BST-2 (also known as HM1.24 and CD317) (Neil et al. 2008; Van Damme et al. 2008). The expression of BST-2 appears both necessary and sufficient for a cellular environment in which Vpu enhances virion release. This protein has hence been referred to as a "tetherin" based on its ability to retain nascent virions on the surface of infected cells (Neil et al. 2008). A hypothetical model of virion-tethering is shown in Fig. 2.

Although the ability of Vpu to enhance virion release appears directly related to its ability to antagonize the tethering activity of BST-2, the basis of this antagonism is not fully elucidated. The simplest explanation, and one supported by the initial data, is that Vpu decreases the expression of BST-2 at the plasma membrane, the presumed site of BST-2's action as a tethering factor (Van Damme et al. 2008). The potential mechanisms and protein–protein interactions that underlie this down-regulation of BST-2 by Vpu are discussed below.

4.6 Evasion of Adaptive Immunity

In addition to its role as an antagonist of the novel innate antiviral mechanism mediated by BST-2, Vpu may contribute to the evasion of adaptive immunity via the modulation of both class I and class II MHC, as well as CD40 (Kerkau et al. 1997; Hussain et al. 2008; Henderson et al. 2004).

4.6.1 Downregulation of MHC-I

Although not known to interact directly with class I MHC, Vpu has been reported to decrease its expression at the cell surface (Kerkau et al. 1997). This effect may involve both inhibition of the biosynthesis of MHC-I and enhanced degradation via recruitment of β-TrCP. Downregulation of MHC-I could decrease the density of viral antigens on infected cells and so provide escape from the control of viral replication by cytotoxic T lymphocytes. However, like the case of CD4, the potent ability of the HIV-1 Nef protein to downregulate MHC-I obscures the significance of Vpu in providing evasion of immune surveillance by this mechanism (Collins et al. 1998). In addition, this observation has not yet been independently verified.

4.6.2 Downregulation of MHC-II

A yeast two-hybrid screen for cellular proteins that interact with Vpu identified the invariant chain of MHC-II (Hussain et al. 2008). This led to the observation that Vpu downregulates the surface levels of class II MHC. The significance of this effect is uncertain, but presumably Vpu could contribute to immune evasion by deceasing antigen presentation to CD4-positive T cells during the adaptive immune response.

4.6.3 Upregulation of CD40

Vpu has been reported to increase the expression of CD40, a member of the TNF receptor superfamily (Henderson et al. 2004). Although this effect was reported in endothelial cells, it could conceivably occur in HIV-infected dendritic cells and macrophages, where CD40 plays a co-stimulatory role. The consequences, if any, of CD40 upregulation by Vpu in such cells are unknown.

5 Interactions with Cellular Proteins

Vpu has been reported to interact directly with several cellular proteins. While the role of Vpu in the recruitment of β-TrCP seems unequivocal, the significance of the interaction of Vpu with other cellular proteins is less clear.

5.1 β-TrCP

Human β-TrCP (β-transducin repeat-containing protein) was discovered in a yeast two-hybrid screen in which the cytoplasmic domain of Vpu was used as bait (Margottin et al. 1998). As noted above, β-TrCP is a substrate adaptor or receptor

for an SCF (Skp1-cullin-F-box) multisubunit E3 ubiquitin ligase complex. β-TrCP is an F-Box and WD repeat-containing protein. β-TrCP interacts with the skp1 subunit of the E3 ligase complex via its N-terminal F-box, and it interacts with Vpu via its C-terminal domain, which contains seven WD repeats that form a series of β-strands in a propeller-like arrangement (Wu et al. 2003). As noted above, Vpu interacts with β-TrCP via a canonical DpSGxxpS motif, in which "pS" indicates a phosphoserine. This motif, or variations of it, is found in cellular substrates of the β-TrCP/SCF E3 ligase such as β-catenin. Indeed, the crystal structure of the WD repeat region of β-TrCP complexed with a β-catenin substrate peptide has been solved (Wu et al. 2003). Although the structure of a similar complex with Vpu is not available, NMR data indicate that serine phosphorylation allows electrostatic interactions and hydrogen bonding between Vpu and a binding pocket on β-TrCP (Evrard-Todeschi et al. 2006). The residues LIER located just upstream of the DpSGxxpS sequence in the Vpu CD provides additional binding to a hydrophobic pocket in the WD domain of β-TrCP.

The consequences of the interaction of Vpu and β-TrCP involve ubiquitination of cellular membrane proteins such as CD4 and possibly BST-2. The result of this modification is degradation of the targeted proteins, either via the proteasome as has been documented in the case of CD4 (Schubert et al. 1998), or within the endo-lysosomal system as may be the case for BST-2.

5.2 CD4

To induce ubiquitination and ultimately degradation of target proteins, Vpu must interact with them. In the case of CD4, this interaction occurs between the cytoplasmic domains of both protein (Margottin et al. 1996; Bour et al. 1995). The structural basis of this interaction is not precisely known, but it has been roughly mapped by functional analyses to the membrane proximal region of CD4 including residues KRLLSEKKT, which appears at least in part α-helical, and the membrane proximal α-helix in Vpu including residues YRK and DRLI (Wittlich et al. 2007).

5.3 BST-2

BST-2 is a type II transmembrane protein (the N-terminus is cytoplasmic and the C-terminus is lumenal) that is found on the plasma membrane and within endosomes (Kupzig et al. 2003). The membrane topology of BST-2 is unusual in that in addition to containing a transmembrane domain, the protein is modified by the addition of a glycosylphosphatidylinositol (GPI) anchor at its C-terminus. Consequently, BST-2 interacts with the lipid bilayer twice. This bifunctional membrane binding activity could allow the protein to tether nascent virions to cells by bridging cellular and virion membranes (Fig. 2). BST-2 associates via its GPI anchor with

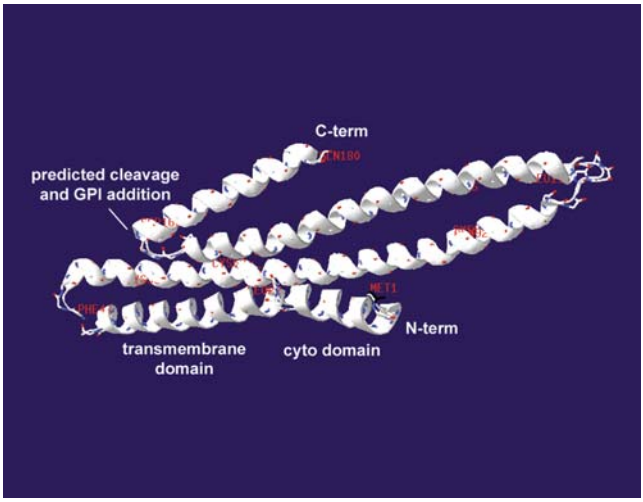

Fig. 3 Predicted structure of human BST-2. Most of the protein is predicted to be α-helical, including the cytoplasmic domain and the transmembrane domain. The ecto- or lumenal domain is predicted to form a coiled-coil hairpin. Removal of the C-terminal α-helical domain is predicted during the addition of the GPI anchor. This structure was predicted using the SAM-T06 server of the University of California-Santa Cruz

cholesterol enriched lipid raft membrane domains (Kupzig et al. 2003). This ideally positions the protein to tether virions of HIV-1, influenza virus, and Ebola virus, all of which bud from raft domains.

The structure of BST-2 is not yet known, but a predicted model is shown in Fig. 3. The protein appears almost entirely α-helical, including an α-helical cytoplasmic domain and an α-helical transmembrane domain. The α-helical ecto- (lumenal) domain is predicted to form a coiled-coil hairpin, bringing the C-terminal site of protein cleavage and GPI-addition back to the lumenal leaflet of the lipid bilayer. The CD of BST-2 contains two lysines that are potential targets for ubiquitination (Ohtomo et al. 1999). BST-2 appears to exist in native form as a cysteine-linked dimer; it contains two conserved predicted N-linked glycosylation sites in its ectodomain; and it appears to be heterogeneously glycosylated (Ohtomo et al. 1999).

Exactly how BST-2 tethers virions is not known, though both its cytoplasmic and C-terminal domains are required (Neil et al. 2008). As noted, BST-2 could tether virions to cells by keeping its transmembrane domain in the plasma membrane while embedding its GPI anchor within the cholesterol-enriched virion membrane. Alternatively, BST-2 could be incorporated into virions, and dimerization between virion- and cell-associated BST-2 could provide the linkage.

Exactly how Vpu counteracts BST-2 is also not understood but likely involves removal of BST-2 from the plasma membrane (Van Damme et al. 2008). The two key domains of the Vpu protein are functionally united by this activity: down-regulation of cell surface BST-2 requires both the Vpu TMD and the serines within

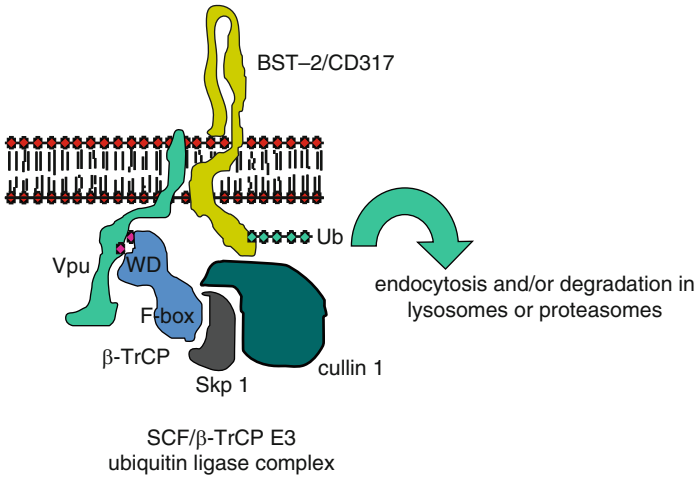

Fig. 4 Hypothetical model of the downregulation of BST-2 from the cell surface by Vpu. Vpu interacts with BST-2, potentially via its transmembrane domain. Vpu also recruits β-TrCP via the interaction of phosphoserines (*red circles* on Vpu) with the WD domain of β-TrCP. β-TrCP is a component of a multi-subunit SCF-ubiquitin ligase complex. Ubiquitination leads to endocytosis and/or targeting of BST-2 to lysosomes for degradation; alternatively, degradation could occur via the proteasome. Presumptive components of this model are the interaction of Vpu with BST-2 and the induction of ubiquitination of BST-2 by Vpu. Vpu-mediated degradation of CD4 proceeds by a related mechanism, in which the interaction between Vpu and CD4 involves the cytoplasmic domains of both proteins, the process takes place in the ER, and the degradation of CD4 is mediated by the proteasome

the DpSGxxpS sequence in the CD (Van Damme et al. 2008). A hypothetical model for the cooperation of the TMD and the CD of Vpu in the downregulation of BST-2 is shown in Fig. 4. In this model, Vpu interacts directly with BST-2, recruits β-TrCP to BST-2, and induces ubiquitin-mediated trafficking events that remove BST-2 from its site of action at the plasma membrane. Several aspects of this model are currently speculative. For example, a direct interaction between Vpu and BST-2 has not yet been shown experimentally, although microscopic data indicate that the two proteins co-localize extensively at steady-state in endosomal vesicles. This co-localization depends on an intact TMD sequence within Vpu, supporting the hypothesis that the two proteins interact via their transmembrane domains (Neil et al. 2008; Van Damme et al. 2008). In this model, the requirement for an intact Vpu TMD for enhancement of virion release relates to the ability to interact with BST-2 within the lipid bilayer, rather than to ion channel activity. Similarly, the role of ubiquitination in the antagonism and downregulation of BST-2 by Vpu has not yet been shown directly, but this hypothesis is supported by the role of serines 52 and 56 in Vpu and by the ability of Vpu to decrease the steady-state levels of endogenous BST-2 within cells (Van Damme et al. 2008; Bartee et al. 2006). Finally, the model explains the contributions of both the TMD and serines 52 and 56 in the enhancement of virion release, but it does not account for the partial activity of a truncated Vpu missing most of the CD including the DpSGxxpS

sequence (Schubert et al. 1996a). One possibility is that the Vpu TMD and the membrane-proximal sequence of the CD are sufficient to partly sequester BST-2 within the endosomal system, diverting it from the site of tethering action at the plasma membrane.

BST-2 is constitutively expressed not only on activated T cells (a major host cell for HIV-replication) but is also found on the surface of B cells and plasmacytoid dendritic cells (Vidal-Laliena et al. 2005; Blasius et al. 2006). The role of BST-2 on these professional antigen-presenting cells (APCs) is unknown. Since BST-2 appears able to bind virions, it could conceivably act as an uptake receptor for extracellular virions in such cells. This line of speculation becomes especially intriguing if BST-2 is incorporated into virions: could BST-2 be induced by interferon to flag progeny virions for uptake into professional APCs? Such a mechanism would place BST-2 at the interface between innate and adaptive immunity, and it would provide a second reason for viral antagonism of this protein independent of restricting virion release from infected cells.

5.4 CAML

Calcium-modulated cyclophilin ligand (CAML) was recently identified as a Vpu-binding partner in a yeast two-hybrid screen using full-length Vpu protein (Varthakavi et al. 2008). CAML is an integral membrane protein with three predicted C-terminal membrane-spanning domains. CAML binds cyclophilin B, regulates the T cell tyrosine kinase p56*lck*, binds a lymphocyte specific TNF-receptor family member, and is required for efficient recycling of the epidermal growth factor receptor (EGFR) to the plasma membrane (Tran et al. 2003, 2005; Bram and Crabtree 1994; von Bulow et al. 2000). The interaction of Vpu with CAML involves the N-terminal region of CAML, but the key domains within Vpu have yet to be mapped (Varthakavi et al. 2008). Remarkably, CAML appears to fulfill functional criteria as a second cellular restriction factor that Vpu antagonizes to enhance virion release. How CAML would directly tether virions is unclear, since the protein is resident in the ER and not detected on the plasma membrane (Holloway and Bram 1998). Similarly, how Vpu antagonizes CAML is unknown. One possible approach to integrate the data on CAML is to consider that the protein might be required for recycling of BST-2 to the plasma membrane. In such a scenario, both cellular factors would be required for the restriction of virion release relieved by Vpu.

5.5 Invariant Chain of MHC-II

As noted above, an interaction between the invariant chain of MHC-II (CD74) and Vpu has been detected in a yeast two-hybrid screen (Hussain et al. 2008). This interaction maps to the cytoplasmic domains of both proteins. The invariant chain is

a chaperone that guides the trafficking of the immature MHC-II complex via the plasma membrane to a late endosomal compartment for loading with foreign antigens derived from the extracellular environment (Hiltbold and Roche 2002; McCormick et al. 2005). Somewhat surprisingly, the expression of Vpu does not appear to affect the levels of the invariant chain (representing the immature MHC-II complex) on the cell surface, but it does decrease the level of mature MHC-II (Hussain et al. 2008).

5.6 UBP

U-binding protein (UBP) is yet another cellular protein found to interact with Vpu in a yeast two-hybrid screen (Geraghty et al. 1994). UBP is a 34-kD ubiquitously expressed protein also known as the small glutamine-rich tetratricopeptide repeat protein (SGT) (Czieplucha et al. 1998). SGT contains four copies of a 34-amino acid tetratricopeptide repeat (TPR). Vpu interacts with the TPR region of SGT via the membrane proximal region of its CD (Dutta and Tan 2008); residues 27–45 are sufficient for the interaction and the KILRQ sequence may be key. UBP/SGT is a member of a family of chaparones that interact with Hsp70 and Hsp90; it also interacts with the structural protein Gag of HIV-1 (Callahan et al. 1998). The functional consequences of the interactions between UBP/SGT and HIV-1 proteins are unclear, but overexpression of UBP has a negative effect on virion release, and Vpu disrupts the association between UBP and Gag (Callahan et al. 1998). Whether UBP/SGT can be integrated into newly emerging models of Vpu as an antagonist of BST-2 and/or CAML remains to be seen.

5.7 TASK-1

TASK-1 is a widely expressed cellular potassium channel with four transmembrane domains. The N-terminal TMD of TASK-1 has homology with the TMD of Vpu, and the proteins interact when co-expressed in cells in a mutually inhibitory manner: Vpu inhibits the conductance of TASK-1, whereas TASK-1 inhibits the enhancement of virion release by Vpu (Hsu et al. 2004). The N-terminal TMD of TASK-1 further appears able to provide a modest degree of Vpu-like activity in enhancing the release of virions. While the actual role, if any, of TASK-1 in Vpu-function remains unclear, the protein provides a precedent for the interaction of Vpu with a cellular protein via their TMDs; as noted above, this mechanism could apply to other cellular targets of Vpu such as BST-2.

6 Conclusion

The study of the accessory protein Vpu of HIV-1, like that of the accessory protein Vif, has revealed a novel aspect of the innate host defense to viral infection. While many questions remain to be answered, the enhancement of the release of virions

from infected cells by Vpu almost certainly represents the antagonism of the interferon-induced restriction factor BST-2/CD317 (tetherin) by a specific viral protein. Like the antagonism of the APOBEC family cytidine deaminases by Vif, the counteraction of BST-2 by Vpu likely involves the recruitment of multisubunit cellular ubiquitin ligases. The ability of Vpu to down-regulate the expression of not only BST-2 but also CD4 and MHC-I highlights the breadth of activity that can be marshaled against innate and adaptive immunity by viral recruitment of these cellular regulators. The integration of the many additional interactions and effects of Vpu within this paradigm remains one of many challenges to the field.

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TRIM5alpha

Byeongwoon Song

Contents

Abstract TRIM5α protein blocks retroviral replication at early postentry stage reducing the accumulation of reverse transcriptase products. TRIM5α proteins of Old World primates restrict HIV-1 infection whereas TRIM5α proteins of most New World monkeys restrict SIV_{mac} infection. TRIM5α protein has a RING domain, B-box 2 domain, coiled-coil domain, and PRYSPRY domain. The PRYSPRY domain of TRIM5α determines viral specificity and restriction potency by mediating recognition of the retroviral capsid. The coiled-coil domain is essential for TRIM5α oligomerization, which contributes to binding avidity for the viral capsid. The RING domain and B-box 2 domain are required for efficient restriction activity of TRIM5α protein but the mechanisms remain to be defined.

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1 Introduction

Following entry into the host cells, retroviruses must execute a series of processes including the uncoating of the viral core, reverse transcription, nuclear import, and integration of the viral DNA into the host genome before they establish a successful infection (Arts and Wainberg 1996; Whitcomb and Hughes 1992). The prevalence of retroviral DNAs in the genomes of all eukaryotes suggests that they have been present throughout the course of evolution, and about 8% of the human genome is made of the relics of past infections in the form of extinct endogenous retroviruses (Bannert and Kurth 2004). The integration of viral DNA and expression of viral proteins are potentially mutagenic or pathogenic. Therefore, the ability of an organism to limit or restrict retrovirus replication should have a selective edge over virus-susceptible counterparts. There are multiple genetic barriers to HIV-1 replication in most nonhuman primates hampering our efforts in developing a robust animal model for HIV-1 infection and pathogenesis. A major cellular factor responsible for blocking HIV-1 infection in Old World monkeys at early postentry step was identified as TRIM5α by Sodroski and colleagues in 2004 (Stremlau et al. 2004). Our efforts in understanding how TRIM5α interferes with the replication cycle of HIV-1 will hopefully allows us to manipulate this system to induce antiviral states in the near future. What follows is a brief review of the postentry restrictions of retroviruses, followed by highlights of these recent new developments on the antiretroviral activity of TRIM5α.

2 Postentry Restrictions

2.1 Fv1

Evidence of a specific, postentry restriction of retroviruses was first provided by studies of murine leukemia virus (MLV) replication in mouse cells of different genetic backgrounds regarding the *Fv1* gene, which was first identified as a locus that controlled susceptibility to Friend leukemia virus disease (Lilly 1970; Pincus Hartley and Rowe 1971). The virus resistance induced by *Fv1* was genetically dominant over susceptibility and was evident in cells cultured in vitro. Among a variety of *Fv1* alleles, two alleles of *Fv1* were shown to provide resistance to infection by particular MLV types. The $Fv1^b$ allele, present in Balb/c mice, blocks infection of N-MLV, whereas the $Fv1^n$ allele, present in NIH/swiss mice, blocks infection of B-MLV. This block occurs after reverse transcription but prior to integration, and targets the MLV capsid (DesGroseillers and Jolicoeur 1983; Jolicoeur and Rassart 1980; Ou et al. 1983; Pryciak and Varmus 1992; Sveda and Soeiro 1976). Indeed, a single amino acid residue at position 110 in the viral capsid can determine the susceptibility of the virus to the blocking effects of different *Fv1*

alleles (Kozak and Chakraborti 1996). Fv1 activity is saturated or titrated at high multiplicity of infection.

Fv1 gene arose from the germ-line integration of an endogenous retrovirus and encodes a Gag-like product (Best et al. 1996). An intact sequence corresponding to the major homology region (MHR), which is conserved in all retroviral capsids and contributes to capsid–capsid interactions, is important for Fv1 function (Bishop et al. 2001). However, the mechanism of the Fv1-mediated block is not understood. It is possible that Fv1 interferes with the trafficking of preintegration complex (PIC) or inhibits the integration of PIC into the host chromosome.

2.2 *Ref1 and Lv1*

In addition to governing the ability of retroviruses to infect particular mouse strains, early postentry restriction can also determine tropism at the species level. N-MLV, for example, inefficiently infects human cells and certain cell lines from African green monkeys (Besnier et al. 2003; Towers et al. 2000). The cellular factor restricting N-MLV in human cells has been referred to as Ref1 (restriction factor 1). As for Fv1 mediated restriction, the major determinant for virus susceptibility to Ref1 was the amino acid 110 of the capsid protein, and Ref1 blocked N-MLV in a saturable manner. However, Ref1 blocked infection at a slightly earlier stage than Fv1, before reverse transcription.

HIV-1 encounters a postentry block in Old World monkeys, whereas simian immunodeficiency virus (SIV_{mac}) is blocked in most New World monkey cells (Himathongkham and Luciw 1996; Hofmann et al. 1999; Shibata et al. 1995). The cellular factor dictating the susceptibility of primate cells to the lentiviruses was referred to as Lv1 (lentiviral susceptibility factor 1) (Cowan et al. 2002). Lv1 restricted HIV-1 in a saturable manner (Hofmann et al. 1999; Towers et al. 2000). Like the restriction mediated by Ref1, the Lv1 restriction occurred early after entry, before reverse transcription, and the resistance was dominant over sensitivity (Cowan et al. 2002; Munk et al. 2002). As for Fv1 and Ref1, the determinant for virus susceptibility was the viral capsid protein (Cowan et al. 2002; Dorfman and Gottlinger 1996; Hatziioannou et al. 2003; Owens et al. 2003) and the block could be abrogated with wild-type HIV-1 (Kootstra et al. 2003) or with replication-defective particles lacking reverse transcriptase activity (Besnier et al. 2003; Cowan et al. 2002).

These species-specific, postentry restrictions mediated by Ref1 and Lv1 share common features: (1) the block occurs prior to reverse transcription (Cowan et al. 2002; Himathongkham and Luciw 1996; Munk et al. 2002; Shibata et al. 1995); (2) the viral determinant of the susceptibility to restriction is the capsid protein (Cowan et al. 2002; Kootstra et al. 2003; Owens et al. 2003; Towers et al. 2000); and (3) the host cell restricting factor can be competed by virus-like particles containing proteolytically-processed capsid proteins of the restricted viruses (Besnier Takeuchi and Towers 2002; Cowan et al. 2002; Hatziioannou et al. 2003; Owens et al. 2004).

3 TRIM5α

3.1 Identification of TRIM5α Restriction

While the search for the genes encoding Ref1 and Lv1 continued, the similarities in the nature and timing of the block imposed by the two loci raised the possibility that Ref1 and Lv1 might be human and monkey versions of the same gene. This possibility was further supported by a cross-abrogation experiment in African green monkey (AGM) cells which show a broad range of restriction against retroviruses including HIV-1, HIV-2, EIAV, and N-MLV. For example, the restriction of HIV-1 in AGM cells could be abrogated by EIAV, and the restriction of N-MLV could be abrogated by the lentiviruses (Besnier et al. 2002; Hatziioannou et al. 2003; Stoye 2002; Towers et al. 2000).

A major breakthrough in the field was accomplished with the identification of the gene responsible for Lv1 activity (Stremlau et al. 2004). TRIM5α was identified during a screen for cDNA clones derived from HIV-resistant, rhesus macaque lung fibroblasts that would protect human HeLa cells from infection by single-cycle GFP-expressing HIV-1 vector pseudotyped with VSV-G envelope when introduced into HIV-susceptible human HeLa cells. The promiscuous envelope protein VSV-G allows entry into most mammalian cell types and thus bypasses blocks related to cell-surface binding, fusion, and entry. This particular screen, therefore, specifically revealed the presence of barriers to the first half of the retroviral life cycle, including reverse transcription, integration, and expression. The expression of the rhesus cDNA was sufficient to restrict incoming HIV-1, whereas the human cDNA was not. Rhesus TRIM5α activity was specific for the HIV-1 capsid, as expected for Lv1 activity. In the cells expressing rhesus TRIM5α, the accumulation of reverse transcripts was significantly reduced indicating that TRIM5α blocks HIV-1 replication before or during early reverse transcription (Fig. 1). Furthermore, siRNA-mediated knockdown of endogenous TRIM5α expression in rhesus cells abrogated the early postentry restriction to HIV-1 infection indicating that TRIM5α was required for Lv1 activity. Work performed by several laboratories soon confirmed that Ref1 and Lv1 were indeed the human and monkey orthologues of TRIM5α (Hatziioannou et al. 2004b; Keckesova Ylinen and Towers 2004; Perron et al. 2004; Song et al. 2005c; Yap et al. 2004).

3.2 TRIM5α

TRIM5α is a member of the large family of tripartite motif proteins (TRIM) (Reymond et al. 2001). TRIM proteins contain RING, B-box 2, and coiled-coil domains and thus have been referred to as RBCC proteins (Reymond et al. 2001) (Fig. 2). Human TRIM5 gene is located in chromosome 11p15 in a cluster with other

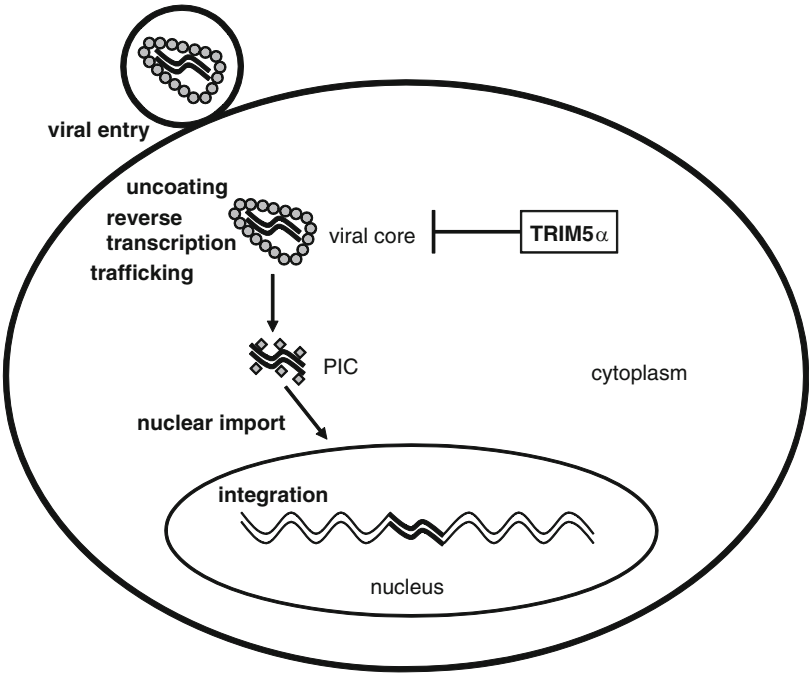

Fig. 1 Postentry restriction of retroviral infection by TRIM5α. The early steps of retrovirus replication cycle and the position of block generated by TRIM5α are shown. *PIC* Preintegration complex

TRIM genes including TRIM3, TRIM6, TRIM21, TRIM22, TRIM34, and TRIM68 (Fig. 2). Among these TRIM genes, TRIM5, TRIM6, TRIM22, and TRIM34 are located at adjacent loci. TRIM5α displays higher identities to adjacent TRIM proteins in RING and B-box domains, but lower identities in coiled-coil and PRYSPRY domains. Consistent with this observation, the carboxy-terminal PRYSPRY domain has recently been shown as a variable region that determines the species specificity of retroviral restriction in primates.

Differential splicing of the TRIM5 primary transcript gives rise to the expression of several isoforms of the protein product (Reymond et al. 2001). The TRIM5α is the largest product (493 amino acid residues in humans) and contains the PRYSPRY domain. The other TRIM5 isoforms lack an intact PRYSPRY domain and are incapable of restricting HIV-1. Two TRIM5 isoforms, TRIM5δ and TRIM5α, are reported to have ubiquitin ligase activity typical of RING-containing proteins (Xu et al. 2003; Yamauchi et al. 2008).

TRIM proteins often self-associate and form nuclear or cytoplasmic bodies of undefined function (Diaz-Griffero et al. 2006; Reymond et al. 2001; Song et al. 2005a). Although TRIM proteins have been implicated in transcriptional regulation,

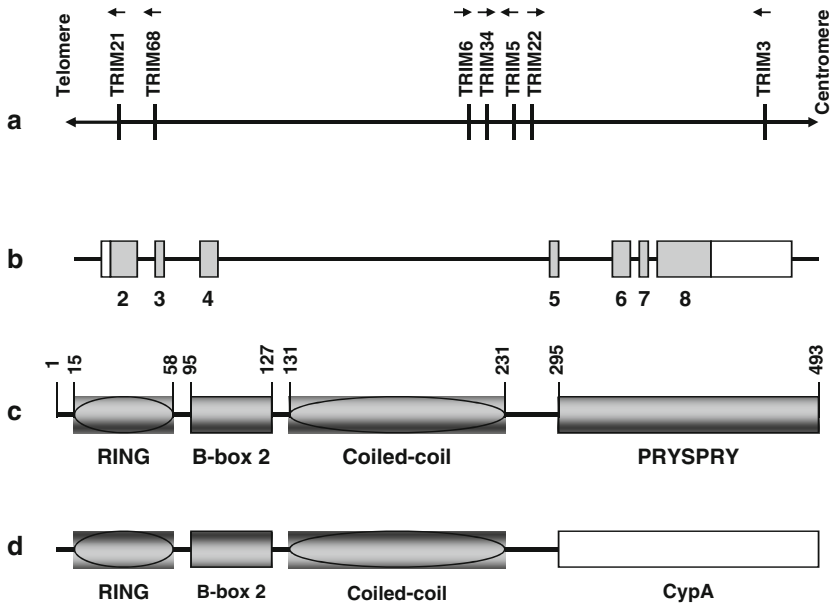

Fig. 2 Chromosomal localization and domain structure of TRIM5α. (**a**) TRIM genes in the segment of 11p15, which is present in the distal region of human chromosome 11. (**b**) Schematic representation of TRIM5 gene. The coding sequences for the alpha isoform is spread across 7 exons, beginning with the RING domain in exon 2 and ending with the PRYSPRY domain in exon 8. (**c**) Schematic representation of the domain structure of human TRIM5α protein. Other isoforms such as TRIM5γ or TRIM5δ do not have the PRYSPRY domain and they do not restrict retroviruses. (**d**) Schematic representation of the domain structure of TRIMCyp fusion protein expressed in owl monkey cells. TRIMCyp fusion consists of the RBCC domain of TRIM5 fused to cyclophilin A due to the insertion of a CypA sequence between exons 7 and 8

cell division, antiviral activity, determination of cell polarity, and differentiation, the precise functions of most TRIM proteins remain to be determined (Meroni and Diez-Roux 2005; Nisole et al. 2005; Towers 2005). TRIM proteins arose with the metazoans and have expanded in number during vertebrate evolution (Reymond et al. 2001). To date, more than 70 TRIM proteins have been identified in the human genome; homologues exist in other species as well. Dysregulation and mutations of some TRIM family members have been linked to a variety of pathological conditions, including genetic diseases and oncogenesis. Many TRIM proteins, including TRIM19, TRIM21, TRIM22, TRIM34, and TRIM5α itself, can be upregulated by interferon, supporting their potential role as effectors in the antiviral cellular response (Asaoka et al. 2005; Chelbi-Alix et al. 1995; Gongora et al. 2000; Orimo et al. 2000; Tissot and Mechti 1995). Indeed, several primate TRIM proteins, including TRIM1, TRIM5α, TRIM19, TRIM22, TRIM32, and TRIM34 have been shown to have antiviral activity against different viruses (Nisole et al. 2005).

3.3 *TRIMCyp*

Cyclophilin A (CypA) is a highly conserved peptidyl prolyl isomerase that binds to HIV-1 capsid (CA) (Franke et al. 1994; Luban et al. 1993; Thali et al. 1994). HIV-1 directly interacts with the CypA activie site by virtue of residues in a loop between the fourth and fifth alpha helices of CA (4–5 loop) (Bukovsky et al. 1997; Gamble et al. 1996). A proline residue P90 in HIV-1 CA is required for CypA binding (Franke et al. 1994). Blocking the CypA-CA interaction by either mutation of the critical proline itself or nearby residues in CA, by mutation of the cyclophilin gene, or by addition of the drug cyclpsporin inhibited virus replication in human cells (Braaten et al. 1996; Braaten and Luban 2001; Dorfman and Gottlinger 1996; Franke et al. 1994; Thali et al. 1994). The affected step was early after entry, before reverse transcription, at the same time as the Ref1 and Lv1 blocks. Analysis of the retroviral replication cycle, using RNA interference to disrupt CypA in the virion producer cell or in the target cell, indicated that target cell CypA alone promotes HIV-1 infectivity (Kootstra et al. 2003; Towers et al. 2003).

Owl monkey cells restricted HIV-1 whereas most other New World monkey cells blocked SIV_{mac} infection (Hofmann et al. 1999). In contrast to the positive effect of CypA-CA interaction on HIV-1 replication in human cells, Towers et al. showed that inhibiting CypA in owl monkey cells rescued HIV-1 restriction (Towers et al. 2003). This was later explained by the identification of TRIM5-cyclophilin A fusion protein (TRIMCyp) (Nisole et al. 2004; Sayah et al. 2004), which arose by retrotransposition of a complete CypA cDNA into TRIM5 intron 7 (Fig. 2). These findings raised a possibility that the CypA domain in the owl monkey TRIMCyp and the PRYSPRY domain in the rhesus TRIM5α could provide a binding domain to target the incoming HIV-1 core and the N-terminal domain(s) of TRIM5α and TRIMCyp could serve an effector function.

It was also shown that inhibiting CypA in Old World monkey cells reduced HIV-1's sensitivity to TRIM5α (Berthoux et al. 2005; Keckesova et al. 2006; Kootstra et al. 2003; Stremlau et al. 2006b) and this was concluded to be due to CypA-mediated prolyl isomerization of CA residue P90 impacting on sensitivity to TRIM5α binding (Berthoux et al. 2005; Keckesova et al. 2006). It was also reported that TRIM5α does not have a role for CypA sensitivity of HIV-1 in human cells (Hatziioannou et al. 2005; Keckesova et al. 2006; Sokolskaja et al. 2006).

Initially, a TRIMCyp fusion gene was thought to exist only in the owl monkey, a New World monkey. However, recent studies reported that the TRIMCyp fusion gene is also found in Old World monkeys including rhesus macaque and pig-tailed macaque (Brennan et al. 2008; Liao et al. 2007; Newman et al. 2008; Virgen et al. 2008; Wilson et al. 2008). Rhesus TRIMCyp restricts infection of HIV-2 and FIV but not HIV-1 (Wilson et al. 2008) and pig-tailed TRIMCyp restricts FIV but not HIV-1 (Brennan et al. 2008; Virgen et al. 2008). TRIMCyp genes of Old World monkeys were proposed to be generated independently from that in owl monkeys as indicated by different position of CypA cDNA sequence, and these events constitute a remarkable example of convergent evolution.

4 Restriction Activity of TRIM5α

4.1 Interspecies Variation of TRIM5α and Retroviral Restriction

Sequence analysis revealed significant interspecies variability in the PRYSPRY domains of TRIM5α proteins of Old World and New World monkeys (Sawyer et al. 2005; Song et al. 2005b). These studies showed substitution patterns indicative of selection in the PRYSPRY domain and revealed lineage-specific expansion and sequential duplication in the PRYSPRY domain (Song et al. 2005b). For the sequences encoding the PRYSPRY domain, the Ka/Ks ratio was very high, indicative of selectively driven diversity. These results suggest that occasional, complex changes were incorporated into the TRIM5α PRYSPRY domain at discrete time points during the evolution of primates. Some of these time points correspond to periods during which primates were exposed to retroviral infections, based on the appearance of particular endogenous retroviruses in primate genomes.

Soon after the identification of TRIM5α as a restriction factor blocking HIV-1 replication at early postentry steps in rhesus monkey cells, several laboratories cloned the TRIM5α cDNAs from diverse primate species and tested their antiretroviral activities (Table 1) (Hatziioannou et al. 2004b; Newman et al. 2006; Ohkura et al. 2006; Perez-Caballero et al. 2005a; Sawyer et al. 2005; Song et al. 2005a, 2005c; Stremlau et al. 2004; Yap et al. 2004). The ability of TRIM5α proteins from different primate species to restrict infection by various retroviruses (Table 1) and the evidence for positive evolutionary selection of TRIM5 genes support the possibility that a major natural function of TRIM5α is its antiviral activity.

Recent studies identified TRIM5 genes with restriction activity against divergent retrovirus in cows (Si et al. 2006; Ylinen et al. 2006) and rabbits (Schaller et al. 2007) and revealed several TRIM5-like genes in rodents (Tareen et al. 2009), and

Table 1 Restriction of retroviruses by primate TRIM5alpha proteins

Group	Species	HIV-1	SIV_{mac}	N-MLV
Hominoids	Human	−/+	−	++
	Chimpanzee	−/+	−	++
	Gorilla	−/+	+	++
	Orangutan	−/+	−/+	++
Old World monkeys	Rhesus macaque	++	+	++
	Pigtailed macaque	++	−	++
	AGM(pyg)	++	−	++
	AGM(tan)	++	++	++
	Sooty mangabey	++	−	++
New World monkeys	Squirrel monkey	−	++	−
	Tamarin (red-chested)	+	++	+
	Tamarin (cotton top)	+	++	++
	Tamarin (emperor)	+	++	++
	Spider monkey	++	++	++

− No restriction, + weak restriction, ++ strong restriction, −/+ no restriction or weak restriction

phylogenetic analysis of these TRIM genes suggest that these factors have evolved from a common ancestor with antiretroviral properties and have undergone independent evolutionary expansions within species.

4.2 Role of TRIM5α Domains

The RING domain, a cycteine-rich zinc binding sequence, found at the N-terminus of TRIM5α is involved in specific protein–protein interactions and often associated with E3 ubiquitin ligase activity (Freemont 2000; Pickart 2001). Indeed, ubiquitination of TRIM5α (Diaz-Griffero et al. 2006; Yamauchi et al. 2008) and TRIM5δ (Xu et al. 2003) has been demonstrated. It was shown that TRIM5 is able to ubiquitinate itself in a RING domain-dependent manner (Xu et al. 2003; Yamauchi et al. 2008). Deletion of the RING domain as well as point mutations affecting residues known to be critical for ubiquitin ligase activity (C15 and C18) significantly reduced the HIV-1 restriction activity of rhesus TRIM5α (Stremlau et al. 2004).

The B-box is a distinct zinc binding sequence present on a number of developmentally important proteins (Torok and Etkin 2001) but the exact function of the B-box is unknown. The deletion or disruption of the B-box domain completely abolished HIV-1 restriction activity of rhesus TRIM5α, suggesting that this domain is essential for restriction activity (Javanbakht et al. 2005; Li et al. 2006a; Perez-Caballero et al. 2005a; Stremlau et al. 2004). Alteration of arginine 119 of human TRIM5α or the corresponding arginine 121 of rhesus TRIM5α diminished the abilities of the proteins to restrict retroviral infection and removal of the positively charged side chain from the B-box 2 arginines 119/121 resulted in diminished proteasome-independent turnover of TRIM5α (Diaz-Griffero et al. 2007b). A recent study by Sodroski's group suggests that the B-box 2 domain of TRIM5α promotes cooperative binding to the retroviral capsid by mediating higher-order self-association (Li and Sodroski 2008). Thus, the B-box domain of TRIM5α modulates capsid binding and retroviral restriction.

The coiled-coil region is composed of multiple alpha-helices involved in protein-protein interactions that may result in homo- or hetero-multimers (Meroni and Diez-Roux 2005; Nisole et al. 2005; Reymond et al. 2001). In vitro cross-linking studies demonstrate that the coiled-coil domain plays a critical role in oligomer formation of TRIM5α protein (Javanbakht et al. 2006b; Mische et al. 2005). It is proposed that oligomer formation allows the B30.2 domain of TRIM5α protein to be better positioned for binding to the target capsid. Consistent with this hypothesis, TRIM5α mutants lacking the coiled-coil domain fail to restrict viral infection (Javanbakht et al. 2006b; Perez-Caballero et al. 2005a).

The PRYSPRY domain, located at the C-terminus of TRIM5α protein, has a core composed of two β-sheets sandwiched together to form a central hydrophobic core and loops of variable length and containing non-conserved residues that protrude out from the core structure, based on comparisons with the molecular structures of

related proteins (Grutter et al. 2006; Masters et al. 2006; Ohkura et al. 2006; Woo et al. 2006). PRYSPRY domain was shown to be the determinant of the specificity of restriction (Nakayama et al. 2005; Ohkura et al. 2006; Perez-Caballero et al. 2005a; Stremlau et al. 2005; Yap et al. 2005). Substitution of R332 of human TRIM5α with a negatively-charged or non-charged amino acid is sufficient to allow restriction of HIV-1 without altering its ability to restrict N-MLV (Li et al. 2006b; Yap, et al. 2005). The regions of variability among TRIM5α PRYSPRY domains for different species are located on the protruding variable loops (Ohkura et al. 2006; Song et al. 2005b; Woo et al. 2006). Initial studies using chemical crosslinking suggested that TRIM5α may function as a trimer, but recent studies using purified recombinant TRIM5-21R, which contains the RING domain of TRIM21 in the backbone of TRIM5, by Sodroski's and Sundquist's groups suggest that TRIM5α forms stable dimers and recognizes retroviral capsids through direct interactions mediated by the PRYSPRY domain (Kar et al. 2008; Langelier et al. 2008).

4.3 How TRIM5α Works?

The viral determinant of susceptibility to TRIM5α-mediated restriction is the capsid protein (Cowan et al. 2002; Hatziioannou et al. 2004a; Owens et al. 2004; Owens et al. 2003; Towers et al. 2000). Restriction of retroviral infection by TRIM5α is saturated at high levels of input virions or virus-like particles and only the properly processed and assembled form of a condensed viral core is able to abrogate the restriction (Besnier et al. 2002; Cowan et al. 2002; Dodding et al. 2005; Forshey et al. 2005; Munk et al. 2002; Owens et al. 2004). Studies from several laboratories suggest that the PRYSPRY domain of TRIM5α is responsible for recognizing a conformational ligand on the viral capsid and that the RBCC domains provide an effector function by unknown mechanism (Javanbakht et al. 2006b; Li et al. 2006b; Owens et al. 2004; Perron et al. 2006, 2007; Sayah et al. 2004; Sebastian and Luban 2005; Stremlau et al. 2006a). Several possibilities can be envisioned for the possible mechanisms for TRIM5α restriction: it may bind and sequester the incoming virion core in a subcellular compartment; it may modify the virion core and target for degradation; it may interfere with normal uncoating; or it may inhibit trafficking of preintegration complex. Multiple mechanisms or pathways might be involved in TRIM5α restriction.

4.3.1 Inhibition of Normal Uncoating

In most instances, TRIM5α proteins impair retroviral infection early after entry into target cells, reducing the efficiency of reverse transcription (Keckesova et al. 2004; Perez-Caballero et al. 2005b; Stremlau et al. 2004), raising the possibility that TRIM5α might interfere with normal uncoating of the incoming viral cores.

Recently, it has been shown that TRIM5α binds to the restriction-sensitive retroviral capsid (Sebastian and Luban 2005; Stremlau et al. 2006a) and causes an accelerated uncoating (Stremlau et al. 2006a). Uncoating is a poorly understood process, and it is still uncertain whether it is an active process requiring energy and/or specific host cell components, or whether it occurs passively (Greber et al. 1994; Narayan and Young 2004). HIV-1 cores are relatively unstable in vitro and, in the infected cell the capsid protein is thought to undergo disassembly soon after virus entry (Forshey et al. 2002; Grewe et al. 1990). Analyses of the viral components of the HIV-1 reverse transcription or preintegration complexes failed to detect significant amounts of the capsid protein (Bukrinsky et al. 1993; Farnet and Haseltine 1991; Fassati and Goff 2001; Karageorgos et al. 1993; Miller et al. 1997). The analysis of HIV-1 Gag mutants suggests that capsid disassembly demonstrates precise requirements; both increases and decreases in capsid stability resulted in decreased HIV-1 replication ability (Forshey et al. 2002). Therefore, an accelerated or premature uncoating could account for one of the modes of TRIM5α-mediated HIV-1 restriction.

4.3.2 Ubiquitin Ligase Activity of TRIM5α

Protein ubiquitination and the subsequent degradation of ubiquitinated proteins by the proteasomal pathway are essential for a wide range of cellular functions (Freemont 2000). Proteasome-independent functions of protein ubiquitination are also involved in regulating a variety of protein functions including transport and processing (Schnell and Hicke 2003). Recently, a RING domain-dependent, auto-ubiquitination activity of TRIM5 protein has been demonstrated in vitro (Xu et al. 2003; Yamauchi et al. 2008). Then, an interesting question arises: what is the substrate of the TRIM5α enzyme other than itself? The identification of interaction partner or cofactor of TRIM5α will hopefully answer the question. Ubiquitination of the incoming HIV-1 cores could potentially lead to the degradation of the modified viral cores by proteasome system or could interfere with the trafficking of the modified viral cores. However, TRIM5α-mediated ubiquitination of HIV-1 cores has not so far been demonstrated.

4.3.3 Proteasome

It has been shown that disrupting proteasome function relieves rhesus TRIM5α restriction of HIV-1 late RT products even though 2-LTR circle production and viral infection remained blocked, suggesting some contribution of proteasome activity to TRIM5α activity (Anderson et al. 2006; Wu et al. 2006). Therefore, a two step process of TRIM5α restriction was proposed: TRIM5α acts prior to complete reverse transcription of viral RNA and may also inhibit trafficking of the preintegration complex. A recent study showed that treatment of cells with proteasome inhibitors prevented TRIM5α-dependent loss of particulate CA

protein (Diaz-Griffero et al. 2007a), indicating the potential involvement of proteasome activity in TRIM5α-induced virus uncoating. How much proteasome contributes to the restriction activity mediated by TRIM5α and its mechanism remain to be defined.

4.3.4 TRIM5α Turnover

Human and rhesus TRIM5α proteins stably expressed in HeLa cells were shown to be rapidly turned over, with half-lives of 50–60 min (Diaz-Griffero et al. 2006). The high rate of TRIM5α turnover creates opportunities for a rapid regulation of the levels of these proteins in response to viral infection or other stimuli. Both proteasome-dependent and proteasome-independent modes for the turnover of TRIM5α protein has been proposed (Diaz-Griffero et al. 2006, 2007b).

Recently, it was shown that TRIM5α is targeted for degradation by a proteasome-dependent mechanism following encounter of a restriction-sensitive retroviral core (Rold and Aiken 2008). This study proposed two potential outcomes of TRIM5α-CA interaction: (1) proteasomal degradation of a TRIM5α-CA complex, resulting in functional decapsidation of the viral core and a premature uncoating, and (2) dissociation of CA from the core followed by its release from TRIM5α, leading to destruction of the restriction factor and decapsidation of the core but not necessarily degradation of CA. It will be interesting to determine whether HIV-1-induced degradation of TRIM5α is dependent on the self-ubiquitination activity of TRIM5α or dependent on other host cell ubiquitin ligases.

4.3.5 TRIM5α dynamics and trafficking

It was shown that TRIM5α cytoplasmic bodies are highly mobile and use the microtubule network to navigate throughout the cytoplasm, and that TRIM5α proteins are dynamically exchanged between the cytoplasmic bodies and the diffuse cytoplasmic population (Campbell et al. 2007), suggesting a more active role of TRIM5α in antiviral activity. Furthermore, it was reported that there is a dynamic interaction between rhesus TRIM5α and HIV-1 viral complexes, including the de novo formation of TRIM5α cytoplasmic body-like structures around viral complexes (Campbell et al. 2008).

A previous study showed that heat shock proteins Hsp70 and Hsp90 colocalize with TRIM5α cytoplasmic bodies (Diaz-Griffero et al. 2006). Hsp70 and Hsp90 proteins are the components of molecular chaperones which play a critical function in protein folding by promoting and maintaining the native conformation of cellular proteins and in some cases in protein sorting (Young et al. 2004). It remains to be determined whether these molecular chaperones directly interact with TRIM5α and contribute to the turnover, trafficking, or the restriction activity of TRIM5α protein. Our understanding of the mechanism of HIV-1-restricting activity of TRIM5α may

depend on the complete understanding of the components and function of the TRIM5α complexes and the dissection of the interaction partners of TRIM5α.

4.3.6 Cyclophilin A

The CypA-CA interaction has been shown to assist HIV-1 replication in some human cells (Franke and Luban 1996; Hatziioannou et al. 2005; Sokolskaja et al. 2004; Thali et al. 1994). Initially, it was thought that CypA in producer cells plays an important role in HIV-1 replication. However, recent findings support that CypA is more important in target cells than in producer cells for HIV-1 replication (Kootstra et al. 2003; Towers et al. 2003). It has been hypothesized that human encode an unknown factor that can negatively affect HIV-1 replication, and CypA binding to HIV-1 CA can protect HIV-1 from this unknown factor (Sokolskaja, et al. 2006; Towers et al. 2003). In contrast to the positive effects of CypA on HIV-1 replication in human cells, CypA exerts negative effects on HIV-1 replication in Old World monkey cells because CypA-CA interactions sensitize HIV-1 to the restriction from Old World monkey TRIM5α proteins (Berthoux et al. 2005; Keckesova et al. 2006; Stremlau et al. 2006b).

CypA interacts with diverse lentiviral capsids including HIV-1, SIV_{cpz}, SIV_{agmTAN}, and FIV (Lin and Emerman 2006). It was proposed that CypA binding to HIV-1 CA induces the conformational change of viral core and renders HIV-1 CA more recognizable by the PRYSPRY domain of TRIM5α (Berthoux et al. 2005; Keckesova et al. 2006). However, HIV-1 variant (e.g., G89V), which does not bind CypA, is still susceptible to the TRIM5α restriction in a CypA-independent manner (Lin and Emerman 2008; Stremlau et al. 2006b). These findings support the idea that TRIM5α restriction of HIV-1 is composed of both CypA-dependent and CypA-independent components (Keckesova et al. 2006; Lin and Emerman 2008; Stremlau et al. 2006b). The ridge formed by helices 3 and 6 on CA has been reported to determine viral susceptibility to the TRIM5α restriction (Owens et al. 2004). A recent study showed that two loops on the HIV-1 capsid, one between the 4th and 5th helices (4–5 loop) and the other between the 6th and 7th helices (6–7 loop), are responsible for the HIV-1 susceptibility to the CypA-dependent TRIM5α restriction (Lin and Emerman 2008).

4.4 Polymorphism

An analysis of sequence data collected from HIV/AIDS cohorts, human genomic DNA diversity collections, and human SNP databases revealed polymorphism in TRIM5 (Goldschmidt et al. 2006; Javanbakht et al. 2006a; Sawyer et al. 2006; Speelmon et al. 2006; van Manen et al. 2008). These include residues in the RING domain (H43Y), in the B-box 2 domain (V112F), in or near the coiled-coil domain (R136Q, R238W, G249D), and in the PRYSPRY domain (H419Y). Two

polymorphisms in the TRIM5 gene (H43Y and R136Q) were shown to affect the antiviral activity of TRIM5α in vitro. For example, human TRIM5α with the H-to-Y change at position 43 showed a reduced ability to restrict N-MLV in tissue culture-based assays (Goldschmidt et al. 2006; Javanbakht et al. 2006a; Sawyer et al. 2006). For the residue at position 136, one study reported that R-to-Q change at position 136 rendered a slightly more effective restriction of HIV-1 (Javanbakht et al. 2006a), although a different study did not detect a difference (Goldschmidt et al. 2006). A recent study reported that an accelerated disease progression was observed for individuals who were homozygous for the 43Y genotype as compared to individuals who were heterozygous or homozygous for the 43H genotype (van Manen et al. 2008), suggesting that polymorphisms in the TRIM5 gene may influence the clinical course of HIV-1 infection.

5 Conclusion

The recent discovery of TRIM5α has revealed a complex interaction between the incoming virion and the host factor, influencing the postentry replication steps in the retroviral life cycle. TRIM5α reduces the accumulation of reverse transcriptase products possibly by interfering with the normal uncoating process. Little is known about the uncoating process, that occurs shortly after a retrovirus fuses with the cell membrane. There are many questions to be addressed to understand the mechanisms of TRIM5α restriction. Defining the capsid uncoating process and the cellular factors involved will be critical to understanding the mechanisms of TRIM5α restriction. The role of ubiquitin and TRIM5α E3 ubiquitin ligase activity needs to be determined. The availability of methods for producing and purifying TRIM5 derivatives should expedite studies of their structure and mechanism of action in restricting retroviral infection. Equally important is identifying TRIM5α-binding proteins or cofactors and understanding the normal function of TRIM5α. The elucidation of TRIM5 mechanism may facilitate pharmacological and genetic intervention to induce currently nonrestrictive human TRIM genes to target and restrict HIV-1.

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Human Immunodeficiency Virus Type-1 Gag and Host Vesicular Trafficking Pathways

Hin Chu, Jaang-Jiun Wang, and Paul Spearman

Contents

Abstract The Gag protein of HIV-1 directs the particle assembly process. Gag recruits components of the cellular vesicular trafficking machinery in order to traverse the cytoplasm of the cell and reach the particle assembly site. The plasma membrane is the primary site of particle assembly in most cell types, while in macrophages an unusual intracellular membrane-bound compartment bearing markers of late endosomes and the plasma membrane is the predominant assembly site. Plasma membrane specificity of assembly may be directed by components of lipid rafts and the cytoplasmic leaflet component PI(4,5)P_2. Recent work has highlighted the role of adaptor protein complexes, protein sorting and recycling pathways, components of the multivesicular body, and cellular motor proteins in facilitating HIV assembly and budding. This review presents an overview of the relevant vesicular trafficking pathways and describes the individual components implicated in interactions with Gag.

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1 Introduction

Retroviral structural proteins actively engage components of cellular vesicular trafficking pathways during the virion assembly process. Retroviral Gag proteins form the core of the viral particle, and are sufficient to direct the formation of virus-like particles when expressed in cells in the absence of all other viral components. HIV $Pr55^{Gag}$ (Gag) is a myristoylated precursor polyprotein that interacts with cellular membranes soon after translation (Spearman et al. 1997; Tritel and Resh 2000). Gag may be translated on membrane-free or membrane-bound ribosomes, and some evidence suggests that Gag is first found deep in the cytoplasm in a perinuclear location (Perlman and Resh 2006; Stanislawski et al. 1980). The assembly of HIV particles occurs at the plasma membrane in infected T lymphocytes and in most commonly used cell lines in which the assembly process has been examined. A notable exception is the human macrophage, in which particles are found primarily within large intracellular tetraspanin-enriched compartments that remain contiguous with the plasma membrane (Deneka et al. 2007; Raposo et al. 2002; Welsch et al. 2007). An important theme for this review is that in order to reach the site of assembly at either the plasma membrane or the intracellular assembly compartment, Gag must traverse the viscous cytoplasm of the cell. A growing body of evidence indicates that specific interactions with vesicular trafficking pathways allow Gag to reach the site of assembly. In order to address the components involved in active trafficking of Gag, we will first discuss current views of assembly at the plasma membrane versus intracellular endosomal compartments. We then discuss specific molecules and pathways implicated in the transit of Gag to the site of assembly.

Gag is cleaved by the HIV protease during and immediately following budding into its component subunits, ordered from N- to C-terminus as matrix (MA), capsid (CA), spacer peptide 1 (SP1), nucleocapsid (NC), spacer peptide 2 (SP2), and p6 (Fig. 1a). Interactions relevant to assembly events occur in the context of the intact precursor protein, but we will refer to relevant regions of the polyprotein involved in host protein interactions by the nomenclature of the cleavage product. Most of the relevant host interactions implicated in trafficking events involve the N-terminal MA region of Gag, while late budding events require interactions with specific motifs in the C-terminal p6 segment. The structural contributions provided by individual segments of Gag to the immature HIV capsid are increasingly understood but are not the focus of this review; readers are referred elsewhere for detailed descriptions of this important aspect of HIV assembly (Ganser-Pornillos et al. 2008; Wright et al. 2007). A schematic diagram of the Gag polyprotein and electron micrographs illustrating plasma membrane assembly in an epithelial cell line and intracellular assembly events in a macrophage are provided in Fig. 1.

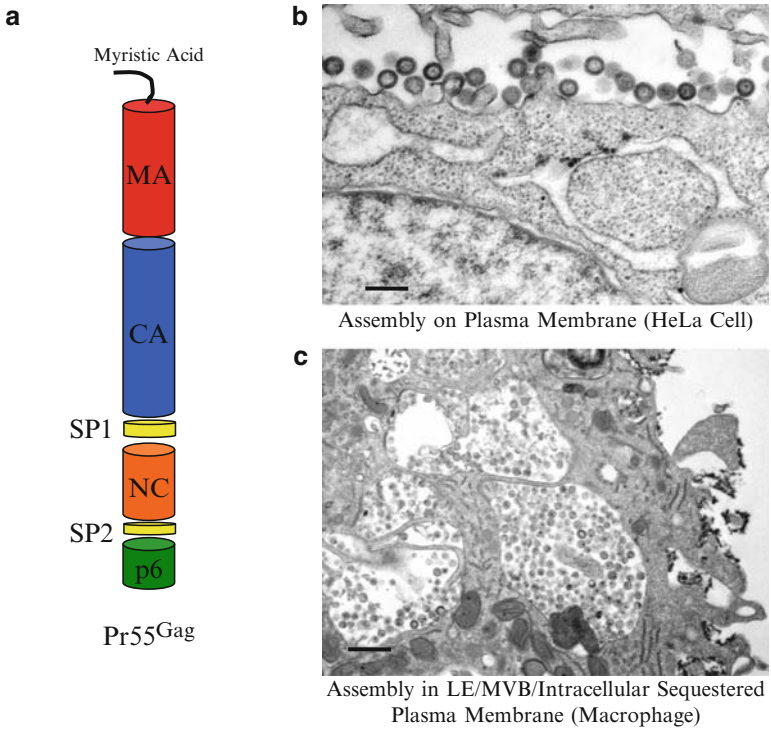

Fig. 1 Schematic of Gag and major sites of assembly. (**a**) Schematic diagram of the $Pr55^{Gag}$ polyprotein and its cleavage products, with the N-terminal MA domain oriented toward the top. (**b**) Electron micrograph of particle assembly at the plasma membrane of HeLa cells in which Gag was expressed by transfection. (**c**) Electron micrograph of HIV particle assembly within internal membrane compartments in macrophages infected with HIV. The site of assembly is acknowledged to be predominantly the plasma membrane as in (**b**); uncertainty regarding the nature of the intracellular compartment in (**c**) is discussed in the text

2 Gag and Late Endosome Compartments/MVBs

Multivesicular endosomes (MVEs) are a subset of endosomes of typically spherical morphology, with a diameter of ~0.4–0.5μm (Gruenberg et al. 1989; Gruenberg and Stenmark 2004). MVEs translocate to the pericentriolar region of the cells along microtubules, where they fuse with other endosomes or mature into late endosomes (Aniento et al. 1993; Rink et al. 2005), which are highly pleiomorphic and composed of tubular, multi-vesicular, and multi-cisternal regions (Piper and Luzio 2001). A subset of highly multi-vesicular late endosomes that are involved in the degradative pathway are referred to as multi-vesicular bodies (MVBs). Late endosomes function not just as degradatory organelles, but they are also important sorting stations. For example, in B cells and dendritic cells, major histocompatibility complex class II (MHCII) molecules are transported to the cell surface from late endosomes through the MVB-like MHC class II compartments (MIICs) (Calafat

et al. 1994; Murk et al. 2002). At the same time, mannose-6-phosphate receptors (MPR) are transported to the trans-golgi network (TGN) from late endosomes for further recycling (Goda and Pfeffer 1988). Cargo proteins that are destined for degradation are eventually sorted to lysosomes, one of the two major cellular sites of protein degradation. The other site is the proteasome (Hershko and Ciechanover 1998). Lysosomes are often thought of as the end point of the endocytic pathway. They contain more than 40 acidic hydrolases and carry out a degradatory role at a pH between ~4 and 5 (Nilsson et al. 2003). However, a subset of specialized lysosomes known as secretory lysosomes are capable of storing secretory products and releasing the products to the plasma membrane under appropriate conditions (Blott and Griffiths 2002; Stinchcombe et al. 2004).

There is a substantial body of evidence associating HIV Gag and the late endosome/MVB compartment in cells. Although the precise nature and nomenclature of the compartment remains debatable, it is clear that Gag associates with intracellular compartments bearing classical late endosome markers in a variety of cell types. The markers implicated include tetraspanins (CD63, CD81, CD82) as well as Lamp-1 and MHC class II (MHC II). The association of Gag with these intracellular compartments is apparent in studies utilizing confocal microscopy or immunoelectron microscopy in fixed cells or by live cell imaging techniques (Nydegger et al. 2003; Pelchen-Matthews et al. 2003; Sherer et al. 2003). Extracellular infectious virions also incorporate LE/MVB markers, suggesting that the LE/MVB is a productive budding site (Nguyen et al. 2003). HIV budding shares many common features with the formation of intraluminal vesicles within the MVB, such as the requirement for recruitment of ESCRT-1 and ESCRT-III complexes (Morita and Sundquist 2004). Thus, an attractive model to explain the presence of both intracellular sites of assembly and those at the plasma membrane has been postulated, stating that in most cells (exemplified by T cells) Gag recruits ESCRT components away from the MVB to the plasma membrane budding site, while in others (such as the macrophage) the abundance of ESCRT components at the MVB facilitates budding at this site. The involvement of AP-3 in the transport of Gag to the LE/MVB further supported this model, as AP-3 is known to be required in the transport of MVB markers (CD63, LAMP-1) to this compartment (Dell'Angelica et al. 1997; Dong et al. 2005). Some groups have even argued that the assembly of retroviruses on intracellular endosomes can be generalized to all cell types, and that this pathway is dominant for transmission of virus from cell-to-cell (Fang et al. 2007; Gould et al. 2003). The exosome hypothesis has not been widely accepted, however, in light of the abundant evidence establishing the importance of the plasma membrane assembly site.

Despite much circumstantial evidence, the apparent LE/MVB site of assembly may not actually be a classical LE/MVB. Although this compartment bears classical LE/MVB markers, the compartment is nonacidic (Jouve et al. 2007). The Bieniasz group has proposed that assembly occurs at the plasma membrane in all cell types, and that the HIV particles found within intracellular compartments of macrophages and other cells are either products of endocytosis or phagocytosis (Finzi et al. 2007; Jouvenet et al. 2006). Indeed, the intracellular sites of budding in macrophages have recently been shown by the Krausslich and Marsh laboratories to

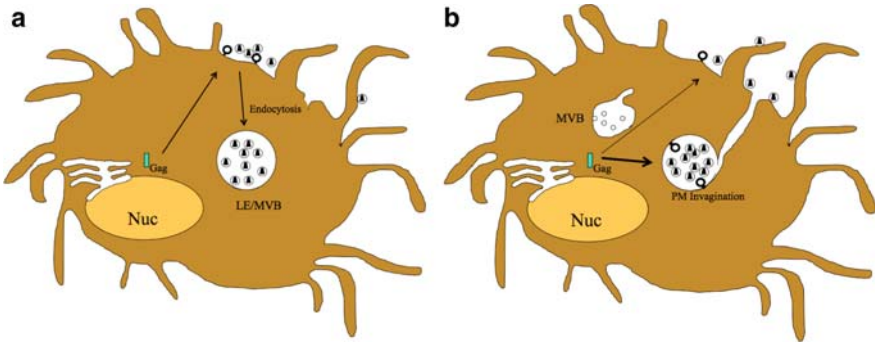

Fig. 2 Models for the internal assembly site in macrophages. (**a**) Particle assembly at the plasma membrane (PM), followed by endocytosis of membrane-tethered particles to the internal LE/MVB compartment. (**b**) Particle assembly within sequestered subcompartments of the plasma membrane. Assembly is depicted here as occurring at the peripheral PM and at the PM invagination

be formed of sequestered plasma membranes that are continuous with the cell surface (Deneka et al. 2007; Welsch et al. 2007). Using ruthenium red as an EM tracer of the plasma membrane, these investigators demonstrated staining of virus-containing intracellular compartments that was separate from endosomes. Endocytosed beads failed to localize to the intracellular, ruthenium red-positive site of particle formation (Welsch et al. 2007). These studies suggest that a novel, very convoluted subcompartment of the plasma membrane is the site of intracellular assembly in macrophages. Within these compartments, HIV is postulated to bud from tetraspanin-enriched membranes, although tetraspanins themselves do not enhance particle budding or infectivity (Ruiz-Mateos et al. 2008). Although both the endocytosis/phagocytosis model and the internal plasma membrane compartment model agree that the plasma membrane is the dominant site of particle assembly, the mechanisms suggested by these models are radically different. As depicted in Fig. 2, endocytosis of tethered particles or of budding portions of the plasma membrane would place the particles within true, closed endosomal compartments (Fig. 2a), while the sequestered, convoluted PM compartment would be entirely distinct from endosomes (Fig. 2b), despite sharing LE/MVB markers. In addition, the implications for the initial trafficking events in the assembly process are distinct, as indicated by the arrows in Fig. 2. Further characterization of this unique assembly compartment in macrophages, and of a similar compartment in dendritic cells, will be of great interest. Carter and Ehrlich have carried out a comprehensive review of HIV-1 trafficking in macrophages (Carter and Ehrlich 2008).

3 Gag and ESCRT

Genetic studies in the budding yeast *Saccharomyces cerevisiae* defined a class of genes that are responsible for vacuolar protein sorting (*vps* genes) (Bankaitis et al. 1986; Rothman and Stevens 1986). The *vps* genes encode products required for

regulating distinct stages of vacuolar protein trafficking and are highly conserved in eukaryotic cells. A subset of these genes, the class E *vps* genes, encode products that are required for protein sorting in the MVB pathway (Odorizzi et al. 1998). In mammals, one or more homologues have been identified for each of the approximately 18 known yeast class E proteins. The majority of the 18 class E *vps* gene products assemble into four distinct heteromeric protein complexes known as the ESCRT (endosomal sorting complex required for transport) -0, -I, -II, and -III complexes (Babst et al. 2002a, b; Katzmann et al. 2001). The ESCRT complexes are responsible for the biogenesis of MVBs by facilitating the sorting and inward budding of mono-ubiquitylated cargoes into intralumenal vesicles (Williams and Urbe 2007). In 2001, the Sundquist and Carter laboratories both reported an association between the PTAP motif within the p6 region of HIV Gag and the UEV domain at the N-terminal of TSG101 (Garrus et al. 2001; VerPlank et al. 2001). The identification of a direct link between the PTAP L domain motif and the cellular/MVB budding machinery (TSG101 and the ESCRT complex) stimulated a burst of studies in this field. A model arising from these studies posits that viruses divert the cellular machinery normally involved in the formation of intralumenal vesicles at the MVB to the site of viral assembly, and utilize this machinery as an exit mechanism from the cell. Specifically, by mimicking Hrs, HIV Gag is able to hijack the TSG101 subunit of ESCRT-I to recruit downstream machinery for its own budding at the plasma membrane or into intracellular membrane-bound compartments. Consistent with this model, disruption of the Gag-TSG101 interaction by overexpression of the dominant negative form of TSG101 or depletion of TSG101 with siRNA inhibits HIV budding (Demirov et al. 2002; Martin-Serrano et al. 2001). More recently, the central role of AIP1/ALIX in connecting Gag to the ESCRT-III complex and the rest of the cellular budding machinery has been defined (Fisher et al. 2007; Popov et al. 2008; Usami et al. 2007). For a comprehensive review of the role of ESCRT complexes in HIV budding, the reader is referred to other references (Hurley and Emr 2006; Morita and Sundquist 2004; Williams and Urbe 2007). One reason to emphasize the role of ESCRT here is that it provides clear evidence of interactions occurring between Gag and the cellular vesicular biogenesis/sorting machinery. This interaction implies but does not establish that Gag may also utilize other vesicular machinery in the cell, such as vesicle-associated adaptor proteins. The strong relationship between Gag and the normal MVB-associated cellular sorting machinery is also striking in light of the assembly process in macrophages, where intracellular assembly occurs in compartments enriched in MVB markers as already discussed.

The subcellular distribution pattern of the ESCRT complex components may play a role in determining the site of HIV budding. Welsch and colleagues reported in 2006 that the majority of ESCRT components were associated with cellular membranes, and that more ESCRT components were present on endosomal membranes in macrophages than in T lymphocytes, which demonstrated higher ESCRT concentrations on the plasma membrane (Welsch et al. 2006). This study suggested that the ESCRT complexes play a role in determining the location of

assembly and budding, arguing against the idea that ESCRT complexes are mainly cytosolic and are recruited to the plasma membrane site of budding by Gag (Martin-Serrano et al. 2001). However, deletion of p6 failed to affect Gag localization in HeLa cells or macrophages, supporting the idea that Gag/ESCRT interactions function primarily at the budding step rather than in affecting Gag trafficking (Ono and Freed 2004).

4 Plasma Membrane-Specific Interactions: Lipid Rafts and PI(4,5)P_2

A plasma membrane Gag "receptor" that explains why assembly occurs predominantly at the plasma membrane has been postulated for many years (Wills and Craven 1991). Two cellular components that may help explain the preference of Gag for particle assembly on the plasma membrane are lipid rafts and the inner leaflet-associated lipid phosphatidylinositol (4,5) bisphosphate [PI(4,5)P_2]. Lipid rafts are postulated microdomains of membranes that are enriched in cholesterol and glycosphingolipids and provide lateral organization to the lipid bilayer. Although controversy remains regarding the size, composition, and even the existence of such domains, a number of important roles have been ascribed to rafts (Hanzal-Bayer and Hancock 2007). The lipid envelope of HIV particles is enriched in sphingolipids and cholesterol, suggesting differential incorporation of typical raft lipids into budding HIV particles (Aloia et al. 1988; Brugger et al. 2006). Gag associates strongly with detergent-resistant membranes (DRMs) at cold temperatures, a characteristic of proteins that associate with lipid raft microdomains (Ding et al. 2003; Lindwasser and Resh 2001; Nguyen and Hildreth 2000; Ono and Freed 2001) and depletion of cholesterol inhibits particle release and infectivity (Ono and Freed 2001). The association of Gag with rafts could explain a preference of Gag for the inner leaflet of the plasma membrane, rather than the ER membrane, for example, as a budding site. The density of Gag-associated DRMs, however, is markedly different than that of classical rafts, and many typical raft markers do not fractionate together with Gag DRMs (Ding et al. 2003; Lindwasser and Resh 2001). An emerging concept in this regard is that Gag multimers may concentrate or recruit raft components into the particle budding site, and in so doing alter the classical biochemical characteristics of rafts. Depletion of cholesterol inhibits the higher-order multimerization of Gag, suggesting that rafts may provide a selective platform favoring the formation of Gag multimers at the plasma membrane (Ono et al. 2007).

PI(4,5)P_2 has emerged as an important determinant of the specificity of Gag's interaction with the plasma membrane. PI(4,5)P_2 is a lipid that localizes preferentially to the inner leaflet of the plasma membrane. When the distribution of PI(4,5)P_2 was altered through overexpression of polyphosphoinositide 5-phosphatase IV or constitutively active Arf6 (Arf6 Q67L), particle production was severely

diminished. Moreover, these interventions resulted in the redistribution of particle formation to late endosomes (Ono et al. 2004). Interactions between MA and PI(4,5)P_2 appear to influence the efficiency of Gag protein membrane binding (Chukkapalli et al. 2008). The structural basis for MA-PI(4,5)P_2 interactions has been determined, and provides a compelling model to explain how the interaction could lead to enhanced Gag interactions with the inner leaflet of the plasma membrane. Membrane binding studies combined with mutagenesis of MA had suggested that a myristoyl switch underlies the interaction of MA with cellular membranes (Spearman et al. 1997; Zhou and Resh 1996). The structure of myristoylated MA was subsequently solved by NMR, and showed that myristic acid was partly sequestered in a pocket within the globular head of the molecule (Tang et al. 2004). Recent work examining the MA-PI(4,5)P_2 interaction demonstrated that PI(4,5)P_2 binds directly to HIV-1 MA and is postulated to trigger myristate exposure (Saad et al. 2006; Shkriabai et al. 2006). The resulting complex provides an effective membrane anchor tying Gag to the inner leaflet of the plasma membrane. The anchor revealed by the MA-PI(4,5)P_2 interaction includes the inositol head group and 2' fatty acid chain of PI(4,5)P_2, which extend into a hydrophobic cleft in MA, and the extended myristate of MA, which contacts the inner leaflet of the membrane. The data supporting the role of PI(4,5)P_2 in assembly are further strengthened by observations from the Rein laboratory indicating a role for inositol phosphates in the formation of virus-like particles of normal size in vitro (Campbell et al. 2001) and the in vivo studies from the Summers laboratory (Saad et al. 2007). Interactions between Gag and inositol hexakisphosphate (IP6) in vitro cause dramatic conformational changes in Gag in solution that regulate Gag-Gag interactions (Datta et al. 2007). While the precise relationship of this effect with IP6 to the interaction of MA with PI(4,5)P_2 is not clear, the potential for the Gag-PI(4,5)P_2 interaction to regulate Gag-Gag multimerization on cellular membranes has considerable support. Recent studies utilizing mass spectrometry revealed that HIV-1 and MLV virions are highly enriched in PI(4,5)P_2 compared to plasma membrane levels (Chan et al. 2008). It should also be noted that the MA-PI(4,5)P_2 interaction is also conserved in HIV-2 and EIAV (Saad et al. 2008; Chen et al. 2008). Given this weight of evidence, continued work on the role of PI(4,5)P_2 in assembly is likely to lead to important new findings.

5 Role of Adaptor Protein Complexes in HIV Assembly

Adaptor protein (AP) complexes are key regulators of protein sorting in the secretory and endocytic pathway. AP complexes recognize the sorting signals on the cytoplasmic tail of cargo proteins and recruit scaffold proteins for vesicle formation. The first two AP complexes identified, designated as AP-1 and AP-2, were identified from purified clathrin-coated vesicles and were originally termed assembly proteins 1 and 2 for their roles in bridging clathrin to vesicles (Keen 1987; Zaremba and Keen 1983). Two more AP complexes, AP-3 and AP-4, were later discovered from

sequence homology searches of AP-1 and AP-2 in mammalian cDNA libraries (Dell'Angelica et al. 1997, 1999; Hirst et al. 1999; Simpson et al. 1997).

All four members of the AP complex family are ubiquitously expressed cytosolic heterotetramers. In addition, AP-1 and AP-3 contain cell-type specific isoforms. AP-1B, which contains the μ1B subunit, is specifically expressed in polarized epithelial cells (Ohno et al. 1999). AP-3B, which contains the β3B and μ3B subunits, is only expressed in neuronal and neuroendocrine tissues (Newman et al. 1995; Pevsner et al. 1994). Each of the AP complexes consists of 4 subunits, including 2 large subunits (γ, α, δ, ε, and β1, β2, β3A/B, β4), a medium subunit (μ1A/B, μ2, μ3A/B, μ4), and a small subunit (σ1, σ2, σ3, σ4). One of the large subunits of each heterotetramer (γ, α, δ, or ε) mediates membrane binding by interacting with membrane phosphatidylinositols such as $PI(4,5)P_2$ or $PI(3,4,5)P_3$. The other large subunits (β1-3) recruit clathrin using their clathrin binding motifs in the hinge region (Shih et al. 1995). It is clear that AP-1 and AP-2 are highly enriched in clathrin-coated vesicles, but the association between AP-3 and clathrin remains ambiguous. Biochemical studies have implied that clathrin is absent in AP-3 containing vesicles (Simpson et al. 1996). However, it has also been shown that the β3 subunit of AP-3 contains the conserved clathrin binding motif and is therefore fully capable of recruiting clathrin (Dell'Angelica et al. 1998). The medium subunits (μ1-4) recognize sorting signals on the cytoplasmic tails of cargo proteins and also provide additional membrane anchor strength by binding to membrane phosphatidylinositols. The sorting signals that facilitate sorting into clathrin-coated vesicle are the tyrosine-based signal NPXY and YXXΦ, and the dileucine-based signals DXXLL and [DE]XXXL[LI] (Barre-Sinoussi et al.), where X can be any residues and Φ is a bulky hydrophobic residue. It should be noted that the NPXY signal, unlike the YXXΦ and the dileucine-based signals, is only known to interact with monomeric clathrin adaptors such as the auto-recessive form of hypercholesterolemia protein (ARH) (Mishra et al. 2002) and Disabled 2 (Dab2) (Morris and Cooper 2001). The smallest subunits, σ1-4, play a predominantly structural role by stabilizing the core of the heterotetrameric complex, as suggested by a structural study based on AP-2 (Collins et al. 2002). In addition to their structural roles, σ1 and σ3 have also been suggested to play a part in the recognition of the dileucine-based sorting signals, [DE]XXXL[LI], from HIV-1 nef and LIMP-II, in the context of γ-σ1 and δ-σ3 hemicomplexes respectively (Janvier et al. 2003).

AP complexes are involved in protein trafficking at different locations of the post-Golgi network. AP-1A mediates bidirectional vesicle transportation between the trans-Golgi network (TGN) and endosomes. AP-1B is responsible for vesicle sorting from the TGN to the basolateral plasma membrane in polarized epithelial cells. AP-2 is well known for its role in sorting cargo proteins into clathrin-mediated endocytic vesicles. AP-3A is essential for sorting of vesicles from early endosomes or the TGN to late endosomes, MVBs, lysosomes, and lysosome-related organelles. AP-3B is believed to play a role in the formation of synaptic vesicles in neuronal cells. AP-4 has been implicated in vesicle sorting from the TGN to lysosome as well as basolateral trafficking from the TGN to the basolateral plasma membrane.

Table 1 Direct interactions between adaptor protein complexes and HIV-1 components that have been implicated in the assembly pathway

Complex	AP Complexes (interaction region)	HIV-1 components (interaction region)	Reference
AP-1	AP-1 (μ subunit)	HIV-1 Gag (MA)	(Camus et al. 2007)
	AP-1 (μ subunit)	HIV-1 Env (gp41 Y_{712}SPL motif)	(Wyss et al. 2001)
AP-2	AP-2 (μ subunit)	HIV-1 Gag (MA-CA junction YPIV motif)	(Batonick et al. 2005)
	AP-2 (μ subunit)	HIV-1 Env (gp41 Y_{712}SPL motif)	(Wyss et al. 2001)
AP-3	AP-3 (δ subunit hinge)	HIV-1 Gag (MA H1 helix residue 11–19)	(Dong et al. 2005)
	AP-3 (μ subunit)	HIV-1 Env (gp41 Y_{712}SPL motif)	(Wyss et al. 2001)
TIP-47	TIP-47 (not known)	HIV-1 Gag (MA residue 5–16)	(Lopez-Verges et al. 2006)
	TIP-47 (not known)	HIV-1 Env (gp41 $Y_{802}W_{803}$ motif)	(Lopez-Verges et al. 2006)

AP complexes have been implicated in the assembly and budding of HIV and other enveloped viruses (Table 1). In 2005, we identified an interaction between AP-3 and HIV Gag (Dong et al. 2005). Specifically, the delta subunit of AP-3 (AP-3δ) binds to helix 1 of the MA region on HIV-1 Gag. This interaction is important in the productive pathway of particle assembly, as disruption of the AP-3-Gag interaction by a dominant-negative form of AP-3δ or depletion of AP-3 complexes with siRNA altered Gag subcellular distribution and severely limited particle release (Dong et al. 2005). When the Gag-AP-3 interaction was disrupted, Gag localized minimally with the LE/MVB compartment, suggesting that AP-3 directs Gag to this compartment. Further support for this model was provided in a recent report by the Piguet laboratory (Garcia et al. 2007). These investigators found that AP-3 was required for particle production in HIV-infected dendritic cells, and that assembly occurred within a tetraspanin- and AP-3-enriched intracellular compartment in these cells. A role for AP-3 in productive trafficking of Gag may not solely require assembly in an intracellular compartment. Arguably, AP-3 may also facilitate the transportation of HIV-1 Gag to the plasma membrane directly, as has been reported for VSV glycoprotein (Nishimura et al. 2002). A unified model for the role of AP-3 in HIV assembly is needed, including further definition of the role of AP-3 in HIV-infected macrophages.

Shortly after the discovery of the AP-3-Gag interaction, the Thali laboratory reported a link between AP-2 and HIV-1 Gag (Batonick et al. 2005). This report demonstrated that the μ subunit of AP-2 was able to bind HIV-1 Gag at the tyrosine-base motif located in the MA-CA junction. In contrast to the AP-3-Gag interaction, disruption of the AP-2-Gag interaction enhanced particle release. Recently, Berlioz-Torrent and Basyuk's group demonstrated that the μ subunit of AP-1 also binds to the MA region of HIV-1 Gag and the MA-p12 portion of MLV Gag (Camus et al. 2007). Disruption of AP-1-Gag interaction of HIV or MLV using

an AP-1 μ subunit knockout cell-line lead to a defect in particle release in both cases. Overexpression of AP-1 μ subunit rescued the release defect in a dose-dependent manner. The potential involvement of multiple adaptor protein complexes in directing the trafficking of Gag creates a complex picture that will require further work to provide clarity.

The HIV Env glycoprotein is another HIV virion component that has been heavily implicated in interactions with AP complexes. A major unanswered question in the field is how Env reaches the site of assembly and associates with Gag. In 1997, the Bonifacino laboratory demonstrated that a membrane proximal tyrosine-based motif (Y_{712}SPL) located in the cytoplasmic domain of the transmembrane protein gp41 (TM) was able to bind specifically to the μ subunits of AP-1, AP-2, and AP-3 (Ohno et al. 1997). The interaction between this tyrosine-based motif and the AP-2 μ subunit mediates the internalization of Env from the plasma membrane via clathrin-mediated endocytosis (Boge et al. 1998). This particular motif is also important for basolateral targeting of viral budding (Deschambeault et al. 1999; Lodge et al. 1997). In addition to the Y_{712}SPL motif, a highly conserved dileucine motif ($L_{855}L_{856}$) located in the C-terminal of Env TM is also critical for the accurate subcellular localization of Env and its association with AP-1 (Wyss et al. 2001). Recently, this dileucine motif has also been shown to be involved in the AP-2 dependent clathrin-mediated endocytosis of HIV-1 Env (Byland et al. 2007). Although it is clear that adaptor protein complexes mediate endocytosis of Env, it has not yet been firmly established how this might play a role in bringing Gag and Env together. The potential for Gag and Env to be co-transported to a common site of assembly is raised by the fact that both structural components of the virion interact directly with separate AP subunits.

TIP-47 (tail-interacting protein of 47kDa) is an adaptor-like protein that was recently described to interact with both HIV Env and Gag. TIP-47 was initially identified as a binding partner of the cytoplasmic domain of mannose-6-phosphate receptors (MPRs) as an essential mediator of the sorting of MPRs from late endosomes to the TGN (Diaz and Pfeffer 1998; Orsel et al. 2000). In 2003, the Berlioz-Torrent's group identified an interaction between TIP-47 and Env (Blot et al. 2003). Env was shown to bind TIP-47 via a $Y_{802}W_{803}$ diaromatic motif in the cytoplasmic tail of gp41, and the interaction was shown to be responsible for the retrograde transport of Env from the plasma membrane to the TGN (Blot et al. 2003). Recently, the same group reported the ability of TIP-47 to bind not only to Env but also to Gag (Lopez-Verges et al. 2006). They demonstrated that TIP-47 interacted with Gag at residue 5-16 of the MA region, largely overlapping the MA region implicated in AP-3-Gag interaction (H1 helix, residues 11–19). Abolishing the TIP-47-MA interaction by mutations or siRNA silencing impaired both virion infectivity and Env incorporation (Lopez-Verges et al. 2006). The potential role of TIP-47 as a Gag-Env adaptor protein that may determine the site of assembly of intermediate complexes is intriguing, and will require further investigation. Independent reports of a role of TIP-47 in assembly would help to establish the validity of this very intriguing finding.

6 GGAs, Arf Proteins, and Assembly

GGA (Golgi-localized γ-ear containing Arf-binding) proteins are a family of clathrin-associated factors engaged in protein sorting. GGA proteins are recruited to membranes through direct interaction with GTP-bound Arf proteins. In a recent study, depletion of GGA2 and GGA3 unexpectedly enhanced virus release, indicating a negative modulatory role in particle production (Joshi et al. 2008). In contrast, GGA overexpression inhibited the release of retroviruses in a late domain-independent manner. The inhibition of particle production required an intact Arf-binding domain, implicating Arf proteins in the assembly process. Consistent with this, depletion of Arf proteins also inhibited assembly. Impaired membrane binding was noted in these experiments as a partial explanation for the defects in particle production contributed by depletion of Arf proteins. Together, the data suggest that Arf proteins may play an important role in the trafficking and interactions of Gag with the plasma membrane.

7 Role of Rab GTPases and Host Motor Proteins in HIV Assembly

Rab proteins are members of the small Ras-like GTPase superfamily. To date, 11 Rab proteins have been identified in yeast and more than 60 in human cells (Zerial and McBride 2001). Together with an array of Rab effectors, Rab proteins play key roles in multiple processes within the vesicular trafficking pathway. Rab proteins are initially synthesized as soluble proteins in the cytosol, followed by prenylation by the Rab geranylgeranyl transferase (RGGTase) for the addition of one or two geranylgeranyl groups (Farnsworth et al. 1994), which allow the attachment of Rab proteins into the membrane of organelles. Among their many functions, Rab proteins have been best characterized for their role in providing specificity during the vesicle tethering process. More recently, Rab proteins have been shown to regulate the movement of vesicles and organelles along the cytoskeleton network via their interactions with both microtubule-dependent (dyneins and kinesins) and actin-dependent (myosins) motor proteins. For instance, Rab7 is able to recruit the dynein motor through one of its effectors known as Rab7-interacting lysosomal protein (RILP). The recruitment of the dynein motor by Rab7 and RILP results in a minus end transportation of Rab7-positive endosomes towards the microtubule organizing center (MTOC) (Johansson et al. 2007). Similarly, Rab27a is capable of recruiting the myosinVa actin motor through its effector melanophilin and promote proper transport of Rab27a-positive melanosomes along the actin network (Jordens et al. 2006). In some cases, the motor protein can act as an effector of a Rab protein. As an example, Rab6 has been reported to bind directly to Rabkinesin-6, which is a kinesin-like protein that localizes to the Golgi. This finding suggests a role of Rab6 in the movement of Golgi-associated vesicles along microtubules through an interaction with Rabkinesin-6 (Echard et al. 1998).

Several lines of evidence suggest a role of Rab GTPases in retrovirus assembly. Rab7 and its effector protein RILP have been shown to influence HIV-1 assembly, as overexpression of RILP leads to perinuclear localization of HIV-1 Gag (Nydegger et al. 2003). Rab7 and RILP are involved in the vesicular transport of late endosomes towards the minus end of MTOC along microtubules, a process that is dependent on the action of dynein motors. This suggests a model in which Rab7 and RILP may direct endosome-associated HIV-1 Gag towards the MTOC. Intriguingly, the "D" type retrovirus Mason-Pfizer monkey virus (M-PMV) Gag forms intracytoplasmic immature capsids that are retained adjacent to perinuclear recycling compartments via a direct interaction with dynein/dynactin motor complex (Sfakianos et al. 2003). The direct interaction is mediated by a short peptide sequence known as the cytoplasmic targeting-retention signal (CTRS) located within the matrix region of Gag. A specific mutation within the CTRS (R55W or R55F) disrupts dynein interaction and converts the perinuclear "D" type assembly of M-PMV to plasma membrane "C" type assembly (Rhee and Hunter 1990). The structural basis for this morphogenetic switch was recently revealed when the NMR structure of the M-PMV MA R55F mutant was solved. R55F MA demonstrated a dramatic conformational change from wild-type MA that buried the CTRS motif within a hydrophobic pocket, preventing interactions with the dynein light chain Tctex-1 (Vlach et al. 2008). The M-PMV Gag interaction with cellular dynein therefore builds a compelling story for a direct role of motor proteins in defining the capsid assembly site. M-PMV Env expression enhances the release of M-PMV capsids from the perinuclear compartment, and requires an intact recycling compartment to do so (Sfakianos and Hunter 2003). Expression of a dominant-negative Rab11a prevented efficient transit of capsids to the plasma membrane for budding. Thus, Rab11a may play a key role in allowing recycling of endocytosed Env to a compartment where Gag-Env interactions occur and facilitate capsid transit from the perinuclear region. Although HIV does not form intracellular immature capsids like M-PMV, it is tempting to speculate that HIV Gag may be transiently transported or retained in the perinuclear region in a dynein-dependent manner, where it may subsequently encounter endocytosed HIV Env via adaptor protein-coated vesicles involved in further trafficking steps. Alternatively, HIV Gag protein or oligomeric intermediates could interact with kinesins such as KIF4 (discussed below).

There may be additional roles for Rab proteins and associated motors in the assembly of HIV particles. Rab9, a Rab GTPase that facilitates cargo binding by TIP47 (Carroll et al. 2001), has also been suggested to play an important role in HIV assembly. Depletion of Rab9 by siRNA was shown to sequester Gag within cells and to cause a reduction in particle output (Murray et al. 2005). No additional studies have yet been performed to establish the specific step disrupted by Rab9 depletion. Because of their central role in recruiting effector molecules involved in vesicular trafficking, it is likely that further roles for Rab proteins engaged in specific steps of virus assembly will be elucidated.

If dynein motors are involved in retaining Gag near the MTOC, then kinesins may potentially play a role in movement of Gag to the periphery of the cell. This remains

a relatively poorly studied aspect of Gag trafficking. KIF4 is a plus-end directed, microtubule-associated motor protein that was found to bind directly to murine leukemia virus Gag (Kim et al. 1998), and subsequently reported to interact with MPMV, SIV, and HIV-1 Gag (Tang et al. 1999). Knockdown of KIF4 was recently shown to reduce intracellular Gag protein levels and inhibit particle production (Martinez et al. 2008), supporting a role for this kinesin in assembly.

8 Conclusions

Significant advances have been achieved in the field of retrovirus assembly over the past decade. Retroviral structural proteins utilize specific vesicular transport pathways to bring essential components together in the cell, and to reach the particle assembly site. Transit of Gag and Env in the cell requires an intimate relationship with endosomal trafficking pathways in ways that are being increasingly worked out. Adaptor protein-directed endosomal trafficking steps are required, and Rab proteins and associated effectors play roles that are just beginning to be defined. A putative model of Gag trafficking and HIV particle assembly is presented in Fig. 3, including a role for adaptor proteins in directing transport, $PI(4,5)P_2$ at the

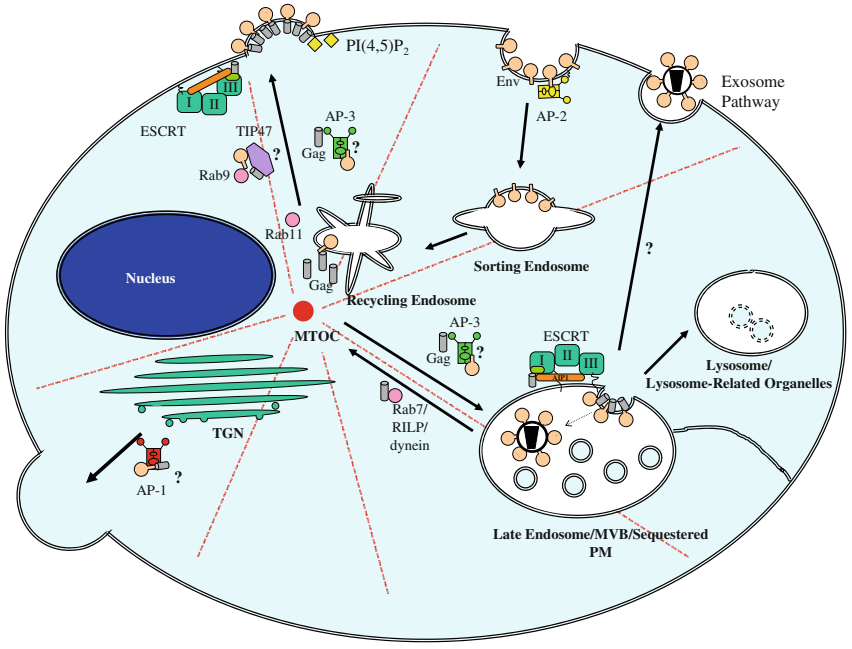

Fig. 3 Schematic diagram illustrating the role of many of the described host factors involved in Gag trafficking. As noted in the text, areas of controversy/uncertainty exist, some of which are indicated by *question marks* in this diagram

plasma membrane, ESCRT complexes for budding at the PM and MVB, and Rab proteins involved in trafficking. The complexity of the model as drawn does not do justice to the large number of other cellular factors that may play a role in the trafficking of Gag. The significance of several of the steps depicted, such as the release of particles from intracellular sequestered sites, remains to be clarified. It is likely that a number of additional host factors involved in HIV assembly remain to be discovered. The interactions of Gag with vesicular trafficking pathways will remain a rewarding area of research for the future, and should provide new targets for antiretroviral drug discovery.

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The Roles of Tetraspanins in HIV-1 Replication

Markus Thali

Contents

Abstract Tetraspanins are small integral membrane proteins that are known to control a variety of cellular processes, including signaling, migration and cell–cell fusion. Research over the past few years established that they are also regulators of various steps in the HIV-1 replication cycle, but the mechanisms through which these proteins either enhance or repress virus spread remain largely unknown.

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1 Introduction

Virus proteomics, i.e., the mapping of interactions that take place between cellular and viral proteins on a global, whole cell scale, or based on analyses of cellular components incorporated into virions, has led to the identification of cellular proteins, and indeed entire cellular pathways, that are critical for the propagation of HIV-1 and other retroviruses (for recent overviews, e.g., Goff 2007, 2008). The most straightforward approach leading to the identification of such proteins/pathways is the analysis of virion content. Fifteen years ago such an analysis hinted at the possibility that tetraspanins play a role in the replication of this virus by revealing that a member of the tetraspanin family is specifically incorporated into HIV-1 particles (Orentas and Hildreth 1993). Over the past 5 years, various investigators have followed up on this early study, primarily by further characterizing the presence of tetraspanins at the viral budding site. However, of the two dozen reports that now link tetraspanins with HIV-1 replication steps (see Table 1), so far only a few document functional roles in virus propagation. Therefore, and as I will discuss in this review, while we now know that tetraspanins have regulatory roles during HIV-1 replication, we are only at the very beginning of understanding exactly how these proteins function during transmission, and thus ultimately propagation and pathogenesis, of HIV-1.

In the following, I will first briefly summarize what we know about cellular tetraspanin functions. Subsequently, I will review the existing literature on the interaction between tetraspanins and HIV-1. Finally, I will discuss potential mechanisms through which tetraspanins exert their functions and, though only very briefly, I will also touch upon the role of tetraspanins in the replication cycles of other viruses.

Table 1 Tetraspanins at different stages of the HIV-1 replication cycle

Assembly	Ruiz-Mateos, Pelchen-Matthews et al. 2008
Orentas and Hildreth 1993	Grigorov, Attuil-Audenis et al. 2009
Gluschankof, Mondor et al. 1997	
Meerloo, Sheikh et al. 1993	**Transmission to target cells & virus-cell and cell-cell fusion**
Raposo, Moore et al. 2002	Gordon-Alonso, Yanez-Mo et al. 2006
Nydegger, Foti et al. 2003	Sato, Aoki et al. 2007
Pelchen-Matthews, Kramer et al. 2003	Singethan, Muller et al. 2008
Nydegger, Khurana et al. 2006	Weng, Krementsov et al. 2009
Booth, Fang et al. 2006	Krementsov, Weng et al. 2009
Grigorov, Arcanger et al. 2006	
Welsch, Keppler et al. 2007	**Susceptibility of potential target cells**
Deneka, Pelchen-Matthews et al. 2007	von Lindern, Rojo et al. 2003
Jolly and Sattentau 2007	Ho, Martin et al. 2006
Garcia, Nikolic et al. 2008	Yoshida, Kawano et al. 2008
Turville, Aravantinou et al. 2008	
	Viral gene expression in newly infected cells
Release	Tardif and Tremblay 2005
Khurana, Krementsov et al. 2007	
Chen, Dziuba et al. 2008	

2 Tetraspanins: Organizers of Membrane-Based Processes

Tetraspanins form a diverse family of small (20–30 kDa, not including mass contributed by glycosylation) membrane proteins that compromises 33 members in mammals (Hemler 2005). Consistent with their involvement in controlling membrane-based processes such as signaling, adhesion and cell–cell fusion, tetraspanins have emerged at the transition from unicellular to multicellular organisms and have since undergone intense evolution (Huang et al. 2005).

2.1 Structure and Subcellular Distribution of Tetraspanins

Tetraspanins contain short cytoplasmic N- and C-termini and one short inner loop that separates a small (SEL) from a large (LEL) extracellular loop. A recent cryoelectron microscopic analysis together with molecular modeling studies revealed that tetraspanins, due to close juxtapositioning of the four transmembrane segments, span the lipid bilayer as compact, rod-shaped structures. Plasma membrane-based tetraspanins are relatively "invisible" from the outside, as they protrude only approximately 5 nms. This probably explains why only very few of them do serve as receptors. However, as described elegantly by others, *"with the lower half of the bundle embedded in the lipid bilayer, the tetraspanins may serve as pilings in the lipid sea, ideal for docking other transmembrane proteins"* (Min et al. 2006). Indeed, the consensus reached by researchers analyzing these proteins is that tetraspanins function primarily as scaffold proteins that laterally organize various membrane-based cellular functions (e.g., Stipp et al. 2003; Hemler 2005; Levy and Shoham 2005a, b). Biochemical analyses over the past 15 years have demonstrated that they form homodimers and that they also tightly associate with other transmembrane proteins, including specific integrins and members of the immunoglobulin superfamily. These associations link molecular events taking place within membranes with membrane-peripheral signaling complexes and the cytoskeleton. Recent high resolution ultrastructural (electron microscopy) and/or fluorescence microscopy studies performed in our lab as well as by others (Nydegger et al. 2006; Unternaehrer et al. 2007), for the first time visualized these up to few hundred nanometer-wide, tetraspanin-mediated assemblages of proteins (TEMs: tetraspanin-enriched microdomains). Importantly, while these initial studies were performed in fixed cells, using bivalent antibody-based detection (which can exaggerate the discreteness of microdomains due to antibody-induced microclustering), two very recent analyses of tetraspanin distribution at the single molecule level in live cells clearly confirmed that these proteins are locally concentrated, thus forming submicron-sized "interaction platforms" (Barreiro et al. 2008; Espenel et al. 2008). One of these latter studies (Espenel et al. 2008) also confirmed what was previously reported by others (e.g., Yang et al. 2004), i.e., that TEMs are clearly distinct from lipid rafts (now also called membrane rafts).

2.2 Cellular Functions of Tetraspanins

Based on analyses of amino acid sequences of the LEL, the segment known to be the primary binding site for tetraspanin-associated proteins, the 33 members of this family can be subdivided into four subgroups (Seigneuret et al. 2001). Members of the same subgroup apparently can partially fulfill each other's role, in case a specific tetraspanin is ablated (e.g., in mouse knockout systems), suggesting a certain degree of redundancy. While such redundancy provides obvious benefits to organisms, it complicates genetic analyses of functions for individual members of a protein family, and thus not surprisingly, only in a few cases has the deletion of a tetraspanin gene resulted in dramatic phenotypes, such as the loss of fertility in CD9 knock-out mice, or retinal degeneration in peripherin (tetraspanin 22) knock-out mice (reviewed, e.g., in Hemler 2005; see also Fradkin et al. 2002). Nevertheless, genetic studies clearly revealed that tetraspanins play regulatory roles in numerous membrane-based processes and several recent reviews provide an overview of the various functions (Boucheix et al. 2001; Wright et al. 2004; Levy and Shoham 2005a, b; Hemler 2008). Here, I will merely summarize what we know so far about the involvement of two members of subgroup 1 (Seigneuret et al. 2001) of the tetraspanin family, CD9 and CD81, in the regulation of membrane fusion, because, as will be discussed later, these two members of the tetraspanin family, and also CD63 (subgroup 2b), are co-regulators of HIV-1-induced virus–cell or cell–cell fusion.

Importantly, like other scaffold proteins, tetraspanins can both enhance or repress the activities of other cellular proteins. For example, the expression of specific cell surface tetraspanins has been shown to either enhance or slow down cell migration, depending on the conditions. Similarly, signaling cascades can either be augmented or dampened by these proteins, and, as will be described in the following, the same tetraspanins can also act as either positive or negative regulators of cellular fusion processes. CD9 and CD81 were documented to promote myotube formation through their enhancement of muscle cell fusion (Tachibana and Hemler 1999). As was already apparent at that time, these two members of the tetraspanin family do not achieve this through binding to partner proteins on adjacent cells and they do not themselves function as fusion proteins. Rather, they regulate myotube formation through the organization *in cis* of associated, so far still unidentified, cellular fusogens. Interestingly, 4 years after having been recognized to be fusion promoters, the same two tetraspanins (CD9 and CD81) were found to negatively regulate the fusion of another type of somatic cell: Mekada and colleagues showed that the formation of multinuclear phagocytes which ingest infectious microbes, cell fragments etc, is enhanced in CD9- and CD81-null mice (Takeda et al. 2003). This was surprising because, by then, these tetraspanins had also been implicated, besides enhancing myotube formation, in promoting the fusion of germ line cells: CD9 knockout mice oocytes are unable to fuse with sperm (Le Naour et al. 2000; Miyado et al. 2000), and overexpression of CD81 in CD9 knockout mice can partially compensate for CD9's fusion promoting

function (Kaji et al. 2002). Importantly, comparable to the situation in muscle cells, the expression of CD9 in oocytes is required not because tetraspanin acts as cellular fusogen, but because this protein laterally organizes (a) cellular fusion protein(s) (Ziyyat et al. 2006) that interacts in trans with sperm-based proteins, such as Izumo (Inoue et al. 2005), possibly through interactions mediated by its LEL (e.g., Zhu et al. 2002; Higginbottom et al. 2003). Finally, and most intriguingly (at least for virologists), a very recent report demonstrates that CD9 fulfills its fusion control function not through its presence at the oocyte surface but rather upon incorporation into (exosome-like) vesicles that are shed from the oocytes (Miyado et al. 2008).

3 Tetraspanins are Regulators of HIV-1 Replication

3.1 Tetraspanins are present at viral exit sites

The analysis of virus lipid content (Aloia et al. 1993) guided subsequent studies revealing that HIV-1 buds through membrane domains enriched in distinct lipids (reviewed, e.g., in Ono and Freed 2005; see also Brugger et al. 2006). Similarly, three early analyses of cellular proteins incorporated into HIV-1 which revealed the incorporation of a tetraspanin, CD63, into viral particles (Meerloo et al. 1992, 1993; Orentas and Hildreth 1993), foreshadowed what is now well established: HIV-1 exits at segments of cellular membrane that are enriched in tetraspanins. Importantly, one of these early studies, using a solid phase virus capture assay to identify cellular proteins incorporated into HIV-1 particles, combined with flow cytometric analysis of the host cell membrane, already documented that CD63 incorporation into virions is a non-random process. As also shown for the major histocompatibility antigen HLA-DR, this tetraspanin is specifically incorporated into HIV-1 particles released from T lymphocytes. However, except for another study of host cell protein incorporation into HIV-1 virions, which confirmed that CD63 is enriched in infectious particles (Gluschankof et al. 1997), to the best of my knowledge, nobody followed up on these early findings until about 5 years ago.

Tetraspanins were "re-identified" as potential players in HIV-1 replication when different investigators started scrutinizing (primarily using fluorescence and electron microscopy) where exactly HIV-1 buds from cells and how this virus recruits the cellular ESCRT machinery that mediates its release from cells (for reviews, e.g., Freed 2004; Morita and Sundquist 2004; Bieniasz 2006). Initially, it was shown that HIV-1 (and also SIV) components, particularly the viral envelope glycoprotein, Env, at least under certain physiological conditions and in certain cell types, can traffic through sections of the cellular endocytic system (Hunter and Swanstrom 1990; Rowell et al. 1995; Sauter et al. 1996; Ohno et al. 1997; Boge et al. 1998; reviewed in Marsh and Pelchen-Matthews 2000), where the tetraspanin CD63 was known to primarily reside. Further support for the idea that HIV-1 exit sites may share certain characteristics, may perhaps even be somehow related to endosomal

membranes, came from the finding that TSG101, a component of the ESCRT I complex which is required for the formation of intralumenal vesicles of late endosomes/multivesicular bodies (LEs/MVBs), is critical for HIV–1 release. Further, an electron microscopy study by Raposo and colleagues suggested that in macrophages HIV-1 buds into LEs/MVBs (Raposo et al. 2002). Promptly, two studies published in 2003 documented that this virus acquires CD63 (and also CD81 and CD82 if produced in macrophages), when it buds through either what appeared at that time to be LE/MVB membranes of macrophages (Pelchen-Matthews et al. 2003) or when it buds through the plasma membrane in HeLa cells (Nydegger et al. 2003). The latter finding was puzzling because in HeLa cells CD63 has extremely low abundance at the plasma membrane. Nevertheless, based on those data, we hypothesized that this tetraspanin, perhaps together with other members of this family, accumulates at relatively discrete plasma membrane microdomains, and in a subsequent study we indeed provided a first visualization of TEMs, as mentioned above (Nydegger et al. 2006). This analysis, together with biochemical, fluorescence microscopy and again electron microscopy analyses by several other groups, unequivocally confirmed and extended the earlier studies by showing that HIV-1 exits through membrane microdomains enriched in the tetraspanins CD9, CD63, CD81 and CD82 in epithelial cells, T lymphocytes, macrophages and dendritic cells (Booth et al. 2006; Grigorov et al. 2006; Nydegger et al. 2006; Deneka et al. 2007; Jolly and Sattentau 2007; Welsch et al. 2007; Garcia et al. 2008; Turville et al. 2008). Last, but certainly not least, a virion proteomics study of cellular proteins incorporated into HIV-1 released from macrophages not only again revealed the presence of these four tetraspanins, it also reported the incorporation of two additional members of the family (CD53 and tetraspanin 14) (Chertova et al. 2006). How TEMs form and exactly when and how HIV-1 components start interacting with tetraspanins remains to be elucidated. We originally speculated that CD63-containing TEMs at the plasma membrane derive from TEMs that originate in LEs/MVBs and that the viral components perhaps even associate with these domains while they are still part of these organelles (Nydegger et al. 2003). Such a scenario would appear plausible, as it has been documented that the limiting membrane of LE/MVB, upon movement of these organelles to the cell surface, can be inserted as patches into the plasma membrane (Jaiswal et al. 2004). This idea received support from the findings that Rab9 and AP3, cellular proteins implicated in trafficking to and from LEs/MVBs, are necessary for efficient HIV-1 release (Dong et al. 2005; Murray et al. 2005). However, the fact that a very considerable fraction of CD63 traffics to the cell surface before reaching its final destination (LEs/MVBs) (Janvier and Bonifacino 2005), together with other evidence, also makes it likely that the HIV-1 components start associating with tetraspanins only at the plasma membrane, and not while these membrane proteins are carried along vesicles. Indeed, data presented in a recent high resolution (TIRF) microscopy analysis (Jouvenet et al. 2008) also support this idea. The investigators of that study reported that, surprisingly, they did not detect any CD63 association with budding virions at the plasma membrane, while they observed large amounts of this tetraspanin in intracellular vesicles (sometimes containing Gag) moving near

the sites of viral morphogenesis and release, but never fusing with the plasma membrane. However, as previously documented (Nydegger et al. 2006), surface CD63 cannot be detected in settings when total cellular CD63 is visualized (either by GFP-tagging or by overall staining). Thus, the failure by Jouvenet et al. to detect CD63 in viral budding structures (Jouvenet et al. 2008) can presumably be explained technically: the strong fluorescence signal for CD63 that emanated from membrane-proximal vesicles densely packed with this tetraspanin probably prevented the detection of much fainter signals that emanate from the relatively low amounts of CD63 associated with HIV-1 budding structures.

Given their presence at HIV-1 exit sites, are tetraspanins gatekeepers, do they facilitate or even promote particle release? Two recently published studies (Sato et al. 2007; Ruiz-Mateos et al. 2008) in which one tetraspanin (CD63) was either ablated or overexpressed, negatively answers this question for HIV-1 release from macrophages and 293 T cells, and our own investigations of release from HeLa cells and T lymphocytes lead to the same conclusion (Krementsov et al., Retrovirology, in press), though one recent study reports that decreased CD63 expression in macrophages results in reduced HIV-1 particle output (Chen et al. 2008), and an even more recent study correlates reduced levels of CD81 with decreased virus release from Molt T cells (Grigorov et al. 2009). Again arguing against the idea that tetraspanins act as general release factors, recent data from our laboratory demonstrate that CD9 expression can be abrogated without consequences for the rate with which HIV-1 is released from these cells (Krementsov et al., Retrovirology, in press). Initially, this came as a surprise to us, because an incubation of cells producing another lentivirus (feline immunodeficiency virus, FIV, see below) with an anti-feline CD9 antibody (de Parseval et al. 1997), as well as the incubation of HIV-1-producing HeLa cells with an anti-human CD9 antibody (K41) can significantly reduce the rate with which HIV-1 is released from these cells (Khurana et al. 2007). However, as we documented in that latter study, the treatment with K41 resulted in the aggregation of CD9 and other members of the tetraspanin family at cell–cell junctions, thus possibly simply sterically blocking virus release. Altogether, currently available evidence suggests that tetraspanins do not generally act as budding co-factors for HIV-1, though further studies will need to address the question if some of them play a supportive role in certain cell types.

3.2 Tetraspanins in HIV-1 Virions Inhibit Env-Induced Membrane Fusion

If tetraspanins do not act as budding co-factors, why did HIV-1 evolve to exit at membrane segments enriched in these proteins? Given what we now know about the crucial role that CD9 plays in the sperm–egg fusion process, it would have appeared reasonable to hypothesize that tetraspanins, upon incorporation into viral particles, enhance their fusogenicity, e.g., by laterally organizing viral Env.

Indeed, to virologists the recent finding that CD9-bearing exosomes mediate fusion of adjacent cells (sperm and egg, see above) is reminiscent of the phenomenon called "fusion from without" (Bratt and Gallaher 1969); documented for HIV-1 in (Clavel and Charneau 1994): virions that are added to cells in large numbers will act as fusion-bridges, thus promoting the formation of syncytia. However, data recently published by the Koyanagi laboratory (Sato et al. 2007) together with our unpublished observations demonstrate that tetraspanins, if acquired by HIV-1 particles, reduce the fusogenicity of the virions. Indeed, the incorporation of tetraspanins CD9, CD63, CD81, CD82, and CD231 considerably diminishes the infectivity of HIV-1 particles, and these tetraspanins thus act as negative regulators of Env-induced membrane fusion, comparable to how CD9 and CD81 negatively regulate the fusion of monocyte-macrophages. Based on these data, it has been speculated (Sato et al. 2007) that such a fusion-suppressing activity of, e.g., CD63 explains why this tetraspanin is specifically downregulated upon reactivation of chronically infected T lymphocytes, once they increase their virus output (Sato et al. 2007). However, the finding that tetraspanins, despite an overall downregulation from the surface of infected cells (Krementsov et al. Retrovirology, in press), still accumulate at virus release sites (e.g., Jolly and Sattentau 2007) and are still incorporated into virions, as described above, suggests that tetraspanins do not merely act as restriction factors for HIV-1. Rather, combined with the observation that some anti-tetraspanin antibodies appear to negatively affect the alignment of HIV-1 producer and target cells (Jolly and Sattentau 2007), this suggests that they can act as both promoters and inhibitors of HIV-1 transmission. Indeed, as I will lay out below, the role played by tetraspanins expressed at the surface of uninfected or of newly infected cells further supports the idea that these proteins have pleiotropic effects on HIV-1 replication and that these effects can be positive or negative.

3.3 Tetraspanins Regulate HIV-1 Entry and the Transcription of the Viral Genome in Newly Infected Cells

While most of the papers on HIV-1 replication and tetraspanins suggest or describe roles of these proteins during the assembly/release phase of the viral replication cycle, recent reports clearly document that tetraspanins also affect virus replication at the entry phase and upon integration of the viral genome into host chromosomes. Data presented in two studies showed that the treatment of macrophages with either an anti-CD63 antibody (von Lindern et al. 2003) or with recombinant LELs of the tetraspanins CD9, CD63, CD81, and CD151 (Ho et al. 2006) can inhibit HIV-1 entry, probably by blocking a post-binding step. While this suggests positive roles for these tetraspanins in the infection process, CD63, but none of the other tetraspanins analyzed (CD9, CD81, and CD151) was also recently shown to divert the co-receptor CXCR4 from its trafficking to the cell surface, thus reducing its presence there and consequently reducing the susceptibility of cells to HIV-1

(Yoshida et al. 2008). This later report, which suggested that tetraspanins, specifically CD63, negatively regulate HIV-1 replication, is counterbalanced by another report, which documented a potential role for CD81 as a co-stimulatory molecule that enhances the transcription of the newly integrated HIV-1 genome (Tardif and Tremblay 2005). Finally, the authors of a fourth paper remain ambiguous about whether the observed result of CD9 and CD81 downregulation from the surface of potential target cells overall has positive or negative consequences for HIV-1 replication: Sanchez-Madrid and colleagues (Gordon-Alonso et al. 2006) demonstrated that either siRNA-mediated reduction of tetraspanin levels, or antibody-induced interference with normal tetraspanin function in T cells and in CD4-positive target cells, leads to increased fusion of infected and uninfected cells. As will be discussed below, such increased fusion could restrict virus spread, and the presence of tetraspanins at the surface of potential target cells could thus be beneficial for the virus. However, it is also possible that tetraspanins prevent HIV-1 infection because, as was also shown in that paper (Gordon-Alonso et al. 2006), the elimination of tetraspanins from the surface of potential target cells makes these cells more susceptible for HIV-1 infection.

3.4 Tetraspanins Regulate Cell-to-Cell Transmission of HIV-1

As reviewed elsewhere (e.g., Johnson and Huber 2002; Sattentau 2008), and indeed as already proposed 15 years ago (Phillips 1994), HIV-1 apparently is most efficiently transmitted from cell-to-cell, if it is released at cell-cell junctions, into the cleft of what is now called the virological synapse (VS) (Igakura et al. 2003; Jolly et al. 2004; for a review see Piguet and Sattentau 2004; see also Hope 2007). Transmission via the VS may be particularly important in secondary lymphoid organs, which are the major sites of virus replication and where cells can be densely packed, (e.g., in the order of 10^9 cells/ml in lymph nodes, as compared to 10^5–10^6 cells in blood) (see also Sourisseau et al. 2007, for further citations). Evidence that such synaptic transmission takes place in vivo comes from data that document clusters of patient-derived spleen cells that have been infected by HIV-1 derived from the same progeny virus (Cheynier et al. 1994; Hosmalin et al. 2001).

The VS shares certain characteristics with the so-called immunological synapse (IS), which forms between antigen presenting cells and T cells (e.g., Friedl et al. 2005; see also Fackler et al. 2007). Like the IS, the VS represents a transient but nevertheless well-organized functional entity. Comparable to the IS, (and also to the neural synapse), the producer/effector cell, i.e., the presynaptic cell, does not fuse with the target cell (the postsynaptic cell) upon synapse formation. While this lack of fusion may seem normal in the case of the IS and the neural synapse, it certainly comes as a surprise in the case of the VS: why do producer cells, which express Env at their surface, typically not fuse with target cells which express CD4 and chemokine receptors? Why do they not form a syncytium, a multinucleated cell? Adherence without fusion may be explained at least partially by the fact that

unprocessed HIV-1 Gag represses Env fusion activity through an interaction with the cytoplasmic tail of Env (EnvCT) (Murakami et al. 2004; Wyma et al. 2004; Davis et al. 2006; Jiang and Aiken 2006, 2007; for a recent review, see Murakami 2008). Hence, if expressed as part of the virus, most Env becomes fusogenic only when it leaves the producer cell as part of the budding virion, but it can already bind to CD4 before that. One could thus envision a scenario in which a fraction of Env located at the presynapse and still associated with precursor Gag, and thus not fusogenic, triggers adherence of the producer to the target cell, thus allowing for the formation of the synaptic cleft into which virions (with fusion-active Env) can be shed. However, Env-mediated cell–cell fusion is also known to be regulated by cellular proteins, e.g., integrins, present at the surface of producer and target cells (e.g., Ohta et al. 1994; Fais et al. 1996) and it thus seems most likely that viral and cellular proteins, including tetraspanins, act in concert to promote efficient particle transfer by regulating Env-induced membrane fusion. As already mentioned above, CD9 and CD81 prevent syncytium formation through their presence at the virological postsynapse, but they also act at the other side of the VS: our own data demonstrate that the same tetraspanins (CD9 and CD81, and also CD63) prevent HIV-1 Env-induced cell–cell fusion through their presence at the virological presynapse (Weng et al. 2009). Quite likely, such repression of Env-induced fusion by tetraspanins, unlike their fusion inhibitory function in virions (see above), is beneficial for the virus but not for the host, because syncytia, while being able to still produce HIV-1 particles (indeed lots of them; see, e.g., Sylwester et al. 1997), have limited life span and thus cannot continue spreading the virus.

If fusion prevention by tetraspanins is positive for the virus, how can this be reconciled with the finding that tetraspanin incorporation into budding HIV-1 particles reduces their infectivity and is thus detrimental to virus replication and spread? It seems reasonable to assume that fusion regulation at the VS, as well as other transmission related processes that take place at that site, depends on proper spatio-temporal organization of the synapse. Spatial organization of the synapse is now well documented for the IS (e.g., Kaizuka et al. 2007) which, at least in its more stable form (Friedl et al. 2005), has a central zone known as the cSMAC (central supramolecular activation complex) that contains the T cell receptor (TCR), co-stimulatory molecules and signaling components, and an outer ring of proteins known as the pSMAC (peripheral SMAC), comprised of adhesion molecules such as the ICAM-1-LFA-1 pair. Interestingly, a similar localization of ICAM-1 was reported for a VS-like structure that formed when CD4-positive T cells adhered to coverslips coated with gp120 and ICAM-1, with ICAM-1 forming a ring around gp120 that accumulated in the center (Vasiliver-Shamis et al. 2008). A scenario that would reconcile the opposing effects of fusion repression by tetraspanins (negative for the virus because it reduces its infectivity – positive because it prevents syncytium formation) would see tetraspanins, like the adhesion molecules, accumulating preferentially at the VS periphery, where their presence would prevent Env molecules from initiating the fusion of pre- and postsynaptic cells, while the center of the VS, where HIV-1 may bud preferentially, would be relatively deserted by these proteins, thus allowing for the formation of particles

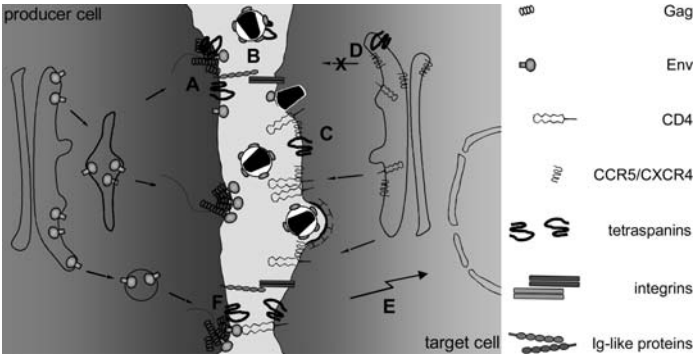

Fig. 1 Tetraspanin functions before, during and after transmission of HIV-1 particles. (A) Formation of viral exit gateways. (B) Incorporation into virions and reduction of infectivity. (C) Repression of Env-mediated virus-cell and cell-cell fusion through interactions with the receptor/coreceptor complex. (D) Reduction of susceptibility to HIV-1 infection through interference with CXCR4 transport to the cell surface. (E) Costimulation of HIV-1 gene expression. (F) Prevention of syncytia formation through interactions with Env in producer cells (see text for details)

with (relatively) few tetraspanins (see Fig. 1 for a scheme of the VS). Examinations of the spatial organization of the VS will allow testing this hypothetical distribution. An alternative, simpler explanation would envision that the virus downregulates tetraspanins to an optimal level that enhances the infectivity of the virions but which still prevents syncytia formation.

3.5 How do Tetraspanins Regulate HIV-1 Entry, Viral Protein Expression, and Env/Receptor-Mediated Fusion Processes?

3.5.1 Tetraspanin Functions in Potential Target Cells and in Newly Infected Cells

While CD63 has been shown to divert CXCR4 from reaching the cell surface, thus preventing infection of cells by HIV-1, it remains to be analyzed if CD63 fulfills this chaperoning function through direct interaction with CXCR4, and if so, where it starts to interact with the coreceptor for HIV-1. Even less is known about the mechanisms with which tetraspanins at the surface of target cells repress fusion of the target cell membrane with the membrane of bound virions and/or bound producer cells. While CD81 is known to associate with CD4 (Imai et al. 1995), downregulation of this HIV-1 receptor apparently is not responsible for the observed fusion repressor function of CD9 and CD81 (Gordon-Alonso et al. 2006). Comparably, receptor/co-receptors in potential target cells are not downregulated upon incubation of these cells with LEL, which inhibits virus entry into macrophages. It seems plausible, however, that tetraspanin knockdowns in T lymphocytes

or incubation of these cells with anti-tetraspanin antibodies, as well as LEL treatment of macrophages, either prevent conformational changes that need to take place for fusion to ensue, or that they alter the microenvironment of the receptors/coreceptors. Such lateral reorganizations may also be at play when CD81 acts as co-stimulator of HIV-1 gene transcription. Since CD81 is known to associate with CD4, one could speculate that an engagement of CD4 by its counter receptor (Env, either on virions or on producer cells) triggers local protein translocations or conformational changes in target cell signaling complexes.

3.5.2 Repression of Fusion Triggered by Virion-Associated or Producer Cell-Associated Env

As described above, we know very little about how tetraspanins regulate infection and post-infection events in lymphocytes. We are even more ignorant about tetraspanin functions in HIV-1 producer cells and in virions. And while these cellular membrane proteins have been established as important players in the replication cycle of other viruses, e.g., in hepatitis C virus (HCV) entry, apparently we cannot extrapolate to HIV-1 from that knowledge. CD81 serves as coreceptor for HCV (e.g., Kapadia et al. 2007; Brazzoli et al. 2008), but the interaction of HCV's envelope glycoprotein E2 with CD81 so far is one of only two cases where a tetraspanin directly interacts with a protein situated in trans, i.e., on the plasma membrane of an adjacent cell (or on the viral membrane). Even the role that the tetraspanin CD82 plays in the replication cycle of another retrovirus (HTLV-1) may be distinct from how tetraspanins regulate HIV-1-induced fusion processes (both virus- or cell-associated fusion processes): while a (probably direct) interaction between HTLV-1 Gag and CD82 was reported to take place (Mazurov et al. 2007), HTLV-1 Env-induced fusion repression by this tetraspanin, unlike what we see in the case of HIV-1 (Weng et al. 2009), does not require coexpression of Gag (Pique et al. 2000). At this point in time, while we do not know the mechanism of fusion regulation by tetraspanins in producer cells, it would appear plausible that these proteins do so by laterally interacting with HIV-1 Env, similar to how they are thought to organize the viral receptors in the target cell.

4 Conclusions – Perspectives

As should be obvious from my remarks above, we are only at the very beginning of understanding the mechanisms that allow tetraspanins to act at various HIV-1 replication steps. Further genetic, biochemical and cell biological analyses are clearly warranted at this point in time. Analyses of how tetraspanins regulate HIV-1 Env-triggered membrane fusion processes at the VS may also benefit from emerging knowledge about the biochemistry and the physics of cellular fusion processes. Clearly, two flat membranes opposed to each other will not

spontaneously fuse. A curved membrane however, as it exists in vesicles or at the tip of a microvillus, can get into closer contact with an opposed flat membrane (because there will be less repulsive force between the two membranes) and this will lower the energy barrier that needs to be overcome in order for membrane fusion to take place. Interestingly, expression of the tetraspanin CD9 has recently been documented to be a key requirement for the formation of proper microvilli (Runge et al. 2007). Considering also the previously mentioned finding that extracellular vesicles enriched in CD9 can trigger sperm-oocyte fusion process (Miyado et al. 2008), one is then tempted to speculate that tetraspanins act as organizers of fusion platforms not only by allowing (or not allowing) access of cellular and viral fusogens to these membrane microsegments (e.g., Singethan et al. 2008), but also by recruiting cellular proteins and lipids that promote curvature of the lipid bilayer.

In conclusion, because of their regulatory functions in fusion platforms that are situated at both sides of the VS, as well as within virions, it will not be easy to dissect exactly how tetraspanins regulate the HIV-1 transmission process. It should also be pointed out that while virus transmission in lymph nodes takes place primarily within a static setting of cells, we know very little about cell-to-cell transmission process in other organs, e.g., in the gut-associate lymphoid system. Quite likely, motile HIV-1-infected cells serve as source for the distribution of the virus at some of those other sites. It will thus eventually become imperative to study tetraspanin functions under conditions that reflect these physiological circumstances, all the more so given that tetraspanins such as CD63 (Mantegazza et al. 2004) or CD9 and CD81 (Takeda et al. 2008) also regulate cell motility, which in turn will probably influence HIV-1 transmission to target cells and thus overall virus dissemination in situ.

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Imaging of HIV/Host Protein Interactions

Cindy M. Danielson and Thomas J. Hope

Contents

Abstract HIV-1 relies on a myriad of interactions with host cell proteins to carry out its life cycle. Traditional biochemical approaches to probe protein–protein interactions are limited in their ability to study the spatial and dynamic interactions that take place in the context of an intact cell. However, issues such as localization and dynamics of interactions between viral and host proteins can be well addressed utilizing fluorescent imaging methods. The past decade has brought about the development of many novel fluorescent imaging techniques which have proved useful to describe the interaction of HIV-1 proteins with the host cell.

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1 Introduction

Encoding only 15 proteins itself, HIV has evolved to usurp many functions of its host cell in order to provide a suitable environment in which it may complete its life cycle. A myriad of interactions exist that redirect host cell functions in order to carry out a successful infection, as do interactions that have in turn evolved to combat HIV infection. In elucidating molecular targets to manipulate therapeutically, one must then consider not only the viral components but also the host cell interactions with these components that may be exploited to derail infection. Understanding this interplay between virus and host may pave the way for novel therapies to disrupt HIV replication.

The traditional manner of both identifying and characterizing protein–protein interactions is the use of biochemical techniques. Much has been uncovered using these techniques, but as the pandemic continues, new strategies for examining these interactions are desperately needed. The biochemical methods employed to probe protein–protein interactions require removing the proteins of interest from the likely relevant milieu around them as well as chemically disrupting the cells from which they are purified, modifications that can limit the physiological relevance of these complex interactions.

Proteins found interacting in biochemical assays are meaningful only if they also interact in vivo. While a protein found in the cytoplasm may be structurally capable of interacting with a nuclear protein, such a theoretical interaction between proteins residing in different subcellular neighborhoods is physiologically irrelevant. Thus, while biochemical studies are useful for characterizing populations of interactions in vitro, novel imaging methods are required to visualize these interactions as they naturally occur in living cells. The use of live cell imaging techniques allows for minimally invasive approaches to more accurately describe the dynamics of such interactions. For not only are we interested in describing the players in these interactions, but also the stage in which they take place.

The past decade has brought forth an abundance of innovative imaging techniques and an increasing pool of knowledge of HIV-1 interactions, and this review will attempt to highlight the most promising of these techniques, how they have impacted our knowledge base, and how they will continue to reveal details unable to be seen by any other means.

2 Techniques

Before understanding how novel imaging techniques have revealed aspects of HIV biology previously unseen, it is necessary to understand the theory of these techniques. The following section will describe the methodology behind a variety of imaging techniques, with an emphasis on ways in which they are useful in the study of HIV-host cell protein interactions. Before considering novel

methodological approaches, an important consideration is the type of fluorescent microscopy to be utilized. Both confocal and deconvolution microscopy allow high resolution fluorescent imaging. Laser scanning confocal microscopy utilizes lasers and pinholes to illuminate a thin layer of the sample, providing a restricted region of illumination that can decrease out of focus fluorescence, generating a clearer image. Deconvolution microscopy uses computer-based image restoration to remove the out of focus light, allowing high resolution imaging. State of the art digital cameras, with highly sensitive and fast image capture, currently allow great flexibility for live cell imaging. The lasers associated with confocal microscopy generate higher levels of phototoxicity, making it less desirable for live cell microscopy. However, the use of a spinning disk confocal addresses this issue to a large degree. In the end, each has its advantage depending on the specific application to be utilized, and for the typical laboratory, access to local equipment often dictates the choice of equipment.

2.1 Colocalization versus Interaction

A common way to demonstrate the interaction of HIV proteins with cellular proteins or compartments is to show that the localization of two or more proteins overlaps within cells. This analysis can be complicated by the fact that the interaction may be transient, and taking a steady state snapshot of the localization of the protein within cells may reveal that only a subset of the populations being visualized in a cell have overlapping expression. Such interactions can be explored further in living cells by utilizing a number of techniques which can provide important insights into the dynamics of such transient interactions. Interpreting image-based analysis can also be confounded if an HIV protein and a cellular protein have overlapping expression, caused not via direct interaction but by both being localized to the same specific cellular compartment. Therefore, the interpretation of observations relating to the interaction of HIV proteins with cellular proteins can be complex. At the same time, these issues can be resolved with controls and appropriate supporting studies.

The most common approach to determining the localization and the potentially overlapping expression of proteins within cells is immunofluorescence microscopy. Typically, the analysis of potential interactions of HIV proteins and cellular proteins begins with the fluorescent imaging of fixed cells. One strength of fixed cells is their higher resolution when compared to live cells, though the method chosen for fixation can be critical to the outcome of this type of analysis because of potential artifacts caused by certain techniques. Simply staining expressing cells with fluorescently labeled antibodies to the proteins can provide initial information. If antibodies to the proteins of interest are not available, then an expression vector for the protein of interest fused to certain small peptides allows immunostaining with antibodies specific to the peptide tag. Alternatively, an expression vector for the

protein of interest fused to a fluorescent protein can be utilized. However, such fluorescent fusion proteins must be carefully validated to ensure that the relatively large tag does not perturb the localization or function of the linked protein of interest. Advantageously, such fluorescent protein fusions can be used in live cell studies to provide insights into the dynamics of potential protein interactions.

One disadvantage of the use of such tagged proteins is that their expression is not likely subject to the regulatory constraints imposed on the endogenous gene. Normal regulation of promoter function or posttranscriptional aspects of regulation are lost in typical expression vectors. Over- or underexpression of the protein under study can alter the normal physiology of virus–host protein interactions. Such issues may be addressed in the future using highly-efficient homologous recombination to introduce the desired tag into the targeted protein in the natural chromosomal location (Lombardo et al. 2007).

The use of fluorophore-tagged viral proteins and interactors is a widely utilized method, and with novel fluorophores continually being added to the repertoire, the investigation of the dynamic nature of interactions can be performed more completely. Though the green fluorescent protein (GFP) from the *Aequorea victoria* jellyfish is still routinely used 14 years after its development (Rizzuto et al. 1995), novel fluorophores with the ability to change colors at the whim of the investigator are making more questions answerable with fluorescence microscopy. Such fluorophores include photoactivatable, photo-convertible, and photo-switchable variants, the use of which allows for pulse-chase type experiments where movement and localization changes can be tracked over time, and thus are useful for studying the dynamics of interactions.

Photoactivatable fluorophores are unresponsive to a given excitation wavelength until they are activated by a brief burst of a specific wavelength of light, after which they behave similarly to their nonphotoactivatable counterparts (Patterson and Lippincott-Schwartz 2002). By selectively activating a very small region within the cell, a specific population of a protein can be tracked over time. As a virion will interact with proteins in a very particular region of the cell, researchers can concentrate on the molecules truly involved in these interactions, no matter how dynamically they move about the cell.

One drawback of photoactivatable fluorescent proteins is the inability to visualize the protein before activation. One solution to this problem is the use of photo-convertible or photo-switchable fluorescent proteins. These fluorophores possess two distinct nonoverlapping fluorescence profiles, enabling the visualization of the entire population of a protein within a cell and selective activation and tracking of a subset of that protein (Gurskaya et al. 2006). Photo-switchable fluorescent proteins have the added benefit of being able to not only switch the fluorescence profile one time, but also to reversibly switch it back to its original state (Ando et al. 2004).

Despite the advancements in fluorescent protein technology, analysis of interactions by visualization alone can be problematic. One problem with interpreting such data is that while colocalization can signify an interaction, it can also simply mean that the proteins occupy adjacent locations; resolving these discrepancies remains a problem with traditional microscopy methods.

2.2 Probing Association by Energy Transfer

Fluorescent imaging analysis of proteins brings to light a wealth of information regarding the subcellular localization and the dynamic movements of proteins, but more objective methods are necessary to definitively label this colocalization as an interaction.

Other techniques utilizing fluorescent proteins can probe the association of viral proteins in more detail. The transfer of energy from a fluorescent protein of one color to a nearby fluorescent protein of another color can reveal information about the distance separating the two proteins.

2.2.1 FRET

Though microscopic studies analyzing colocalization can provide much insight into viral and host cell interactions, distinct fluorescence-based methods are capable of focusing on determining real interactions. Fluorescence resonance energy transfer (FRET), for example, relies of the proximity of two proteins to produce a fluorescence signal. The distance at which a transfer of energy between two proteins can occur ($<$10 nm) is so miniscule that it may be interpreted as an interaction (Stryer 1978). By conjugating putative interacting proteins to a specific pair of fluorophores, an interaction can be determined. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) is a popular pair for FRET due to their partially overlapping fluorescence profiles. The wavelength of light emitted by CFP is the precise wavelength required to excite YFP, a fact conveniently exploited in FRET. If this pair of fluorescent proteins is sequestered in close proximity via some interaction of fusion partners, exciting the sample with the CFP wavelength will produce a YFP emission signal (Fig. 1). In addition to this popular pair, the mCherry and GFP pair is becoming more widely used as this combination is more photostable (van der Krogt et al. 2008).

2.2.2 BiFC

An alternative to FRET is bimolecular fluorescence complementation (BiFC), a technique invaluable for probing interactions. BiFC relies on the production of a fluorescent signal when two interacting proteins attached to complementary halves of GFP come together, complementing the structure of GFP and producing fluorescence (Fig. 2). By separating GFP into halves, neither the N-terminal nor the C-terminal portion is capable of emitting radiation on its own. However, if the proteins to which they are conjugated interact, this will bring the halves close enough to each other to take on the conformation of native GFP and emit fluorescence as usual.

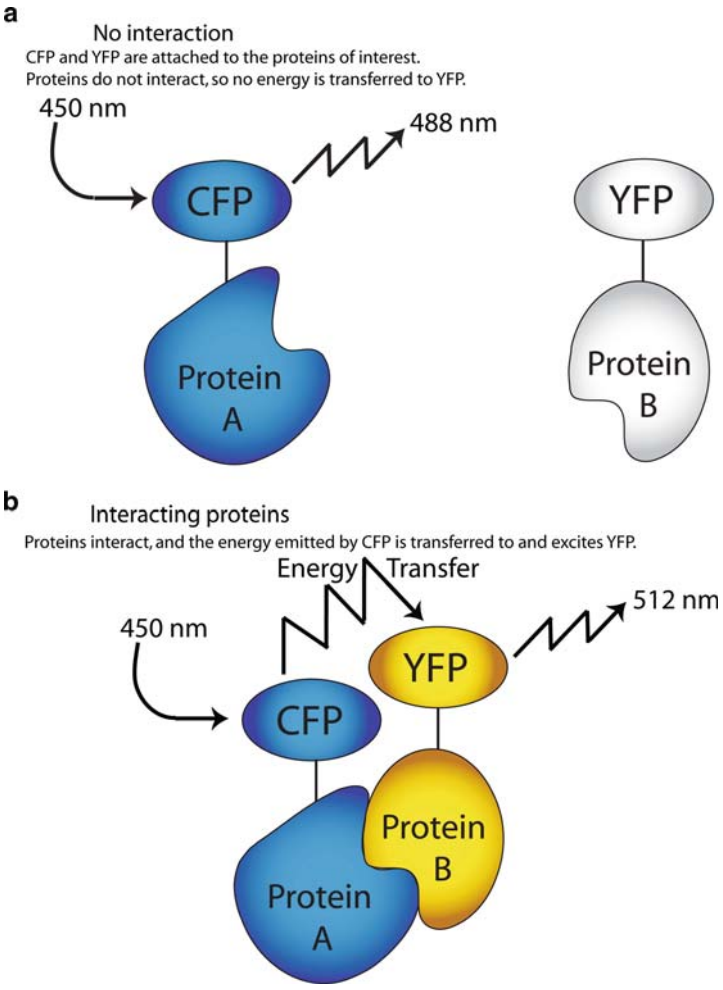

Fig. 1 Fluorescence resonance energy transfer (FRET). (**a**) No interaction. CFP and YFP are attached to the proteins of interest. Proteins do not interact, so no energy is transferred to YFP. (**b**) Interacting proteins. Proteins interact, and the energy emitted by CFP is transferred to and excites YFP

2.3 Improving Imaging Conditions by Increasing Sensitivity and Resolution

Despite the advances in fluorescence light microscopy, there are limitations of fluorescent signal detection and diffraction-limited spatial resolution. Background fluorescence and emission filters make the detection of especially weak signals problematic. Likewise, the highest resolution of light microscopy attainable by traditional optical techniques is approximately 200 nm (Richard 2003), making it

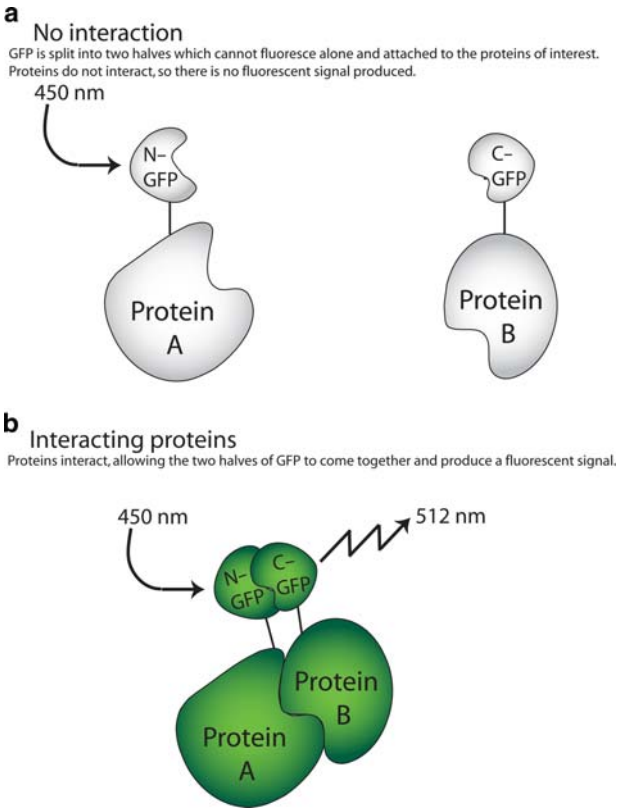

Fig. 2 Bifluorescence complementation (BiFc). (**a**) No interaction. GFP is split into two halves which cannot fluoresce alone and attached to the proteins of interest. Proteins do not interact, so no fluorescent signal is produced. (**b**) Interacting proteins. Proteins interact, allowing the two halves of GFP to come together and produce a fluorescent signal

impossible to determine association from proximity. It is important to define resolution in this case, and this resolution limit means that it is not possible to identify two distinct fluorescent objects unless they are separated by at least 200 nm. New subdiffraction techniques such as stimulated emission depletion (STED) and structured illumination allow imaging at much higher resolution (Klar et al. 2000 and Bailey et al. 1993), but highly specialized equipment is required. Below, we describe alternative methods allowing molecules located at subdiffraction distances to be resolved. Photoactivated localization microscopy (PALM) uses photoactivation and photobleaching to beat the limits, while total internal reflection fluorescence (TIRF) microscopy uses restricted illumination to improve axial resolution and decrease background, allowing the detection of weak signals.

2.3.1 TIRF

One notable advance in fluorescence imaging is total internal reflection fluorescence (TIRF) microscopy. Traditional epifluorescence microscopy illuminates more of the sample than an optical section will capture, creating out of focus fluorescence that causes unnecessary background. By decreasing the portion of the sample in the range of the excitation wavelength, the signal-to-noise ratio is increased, greatly increasing the sensitivity of fluorescent detection and allowing the detection of individual fluors.

TIRF exploits optical principles in order to illuminate only the area of the sample that will be analyzed. The principle works by adjusting the system so that the excitation light passing through the glass coverslip reaches a critical angle, causing all of the light to reflect back into the medium from which it came. On the other side of this medium, an evanescent wave is created that extends up to 100 nm into the sample (Fig. 3) (Axelrod et al. 1984). By focusing the light on such a thin region of the sample, this limits the amount of background fluorescence and thus creates greater sensitivity. This technique is especially useful when interest lies in a comparatively weak signal, which by traditional optical techniques may be masked by stronger signals in a region of the cell that one wishes to ignore. However, the weakness of this method is that the objects of interest must be localized within 100 nm of the coverslip.

The thin region of illumination by the evanescent field is adjustable by altering the critical angle. Modulating the penetration of the evanescent field allows increased resolution in the z-plane, and additional resolution in the z-plane can be obtained by switching between epifluorescence and TIRF. In this way, it is

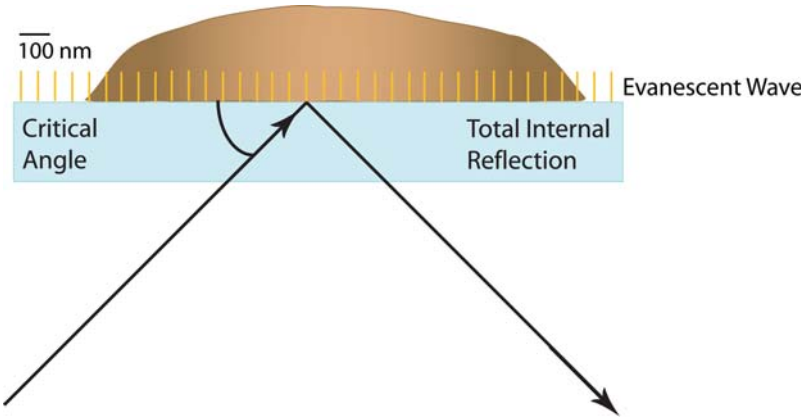

Fig. 3 Total internal reflection fluorescence (TIRF) microscopy. Approaching light at the critical angle undergoes total internal reflection back through the glass coverslip An evanescent wave is created on the other side of the glass, extending approximately 100 nm into the sample. As only this extremely thin region is illuminated, background fluorescence from out-of-focus light is dramatically decreased, creating greater sensitivity

possible to determine when a signal enters or exits the evanescent field, allowing its proximity to the coverslip to be determined. While not a method designed specifically to probe interactions, the increased spatial resolution attainable with TIRF microscopy lends itself to examining colocalization of proteins more accurately.

2.3.2 PALM

A more recent approach for obtaining high-resolution images is photoactivated localization microscopy (PALM). This technique is in theory capable of resolving molecules separated by only a few nanometers, making it an incredibly powerful technique to visualize the spatial and temporal intricacies of certain interactions. In essence, PALM does not increase optical resolution, but instead allows measurement of subdiffraction distances between fluorescent signals.

Though the theory behind PALM has existed for over a decade, the ability to exploit it for high-resolution imaging of fluorescent molecules is still in its infancy. Within a specific diffraction-limited region lie thousands of individual molecules that cannot be visualized separately. PALM is capable of determining the location of a large number ($10^5/\mu m^2$) of these molecules by the use of sequential photoactivation and subsequent photobleaching of subsets of fluorescent molecules within a specific field (Betzig et al. 2006). After this information is obtained, the probability that each molecule resides in a particular location is statistically estimated based on the point spread function of the optical path. Identifying the center of each signal allows the distance between them to be determined. Of more interest to studies of interactions, a dual-color version of PALM has been developed capable of providing superresolution images of two labeled proteins. One notable achievement with this technology is the demonstration that proteins viewed as clearly colocalized by conventional microscopic techniques have been revealed by PALM as very close but distinct clusters of molecules (Shroff et al. 2007). Such a technique should prove valuable for deciphering putative interactions between viral and host cell proteins.

Going even further down the road towards the goal of nanometer resolution is the amalgamation of PALM and single-particle tracking into a method entitled sptPALM (Manley et al. 2008). While single-particle tracking can provide information on the dynamic nature of molecules, it is still subject to diffraction-limited resolution. When using this method to study events occurring on the plasma membrane, the proximity of clusters of molecules renders the interpretation of these paths uncertain. The great spatial resolution of sptPALM, on the other hand, is capable of enabling visualization of the dynamic nature of the plasma membrane by tracking the behavior of thousands of individual molecules. Moreover, the ability to selectively photoactivate populations of molecules allows the determination of multiple trajectories of individual molecules within a very small region of the cell.

3 Examples

Innovative imaging techniques have been used to probe many stages of the HIV life cycle and, as novel methods arise, they will undoubtedly be adapted to visualize interactions that currently seem impossible. A selection of key studies that have utilized these techniques will be highlighted below using examples progressively delving into each stage of the viral life cycle (Fig. 4). As there is an immense wealth of innovative studies of this topic, it is impossible to cite every contribution.

3.1 Entry

HIV enters a target CD4+ T cell after the envelope glycoprotein gp120 binds the CD4 receptor, allowing gp120 to interact with either a CCR5 or CXCR4 coreceptor. After a conformational change exposes the hydrophobic fusion peptide, fusion between the viral and host cell membranes occurs. Upon fusion of the viral envelope to the plasma membrane of the host cell, the viral core is deposited into the host cell cytoplasm. The capsid is lost from the virion in the uncoating process, and reverse transcription converts the viral RNA genome into double-stranded DNA. The preintegration complex containing newly synthesized DNA along with

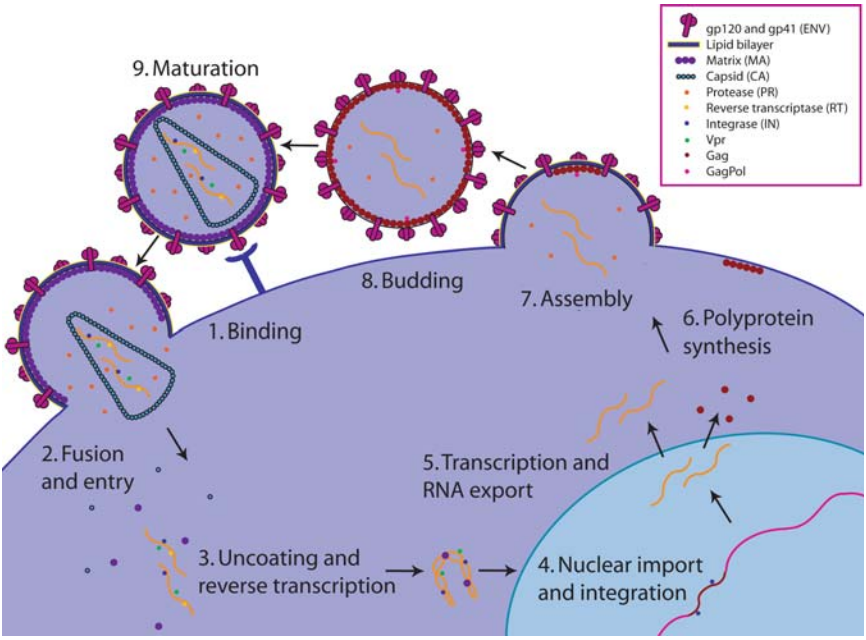

Fig. 4 HIV-1 Life Cycle

several viral and host cell proteins enters the nucleus and integrates into the host cell genome, establishing its everlasting presence (Freed 2001). This process of entry relies on numerous interactions with host cell proteins to achieve the goal of gaining access to the host cell cytoplasm and eventually nuclear DNA. Information regarding the localization of these interactions can be readily gained by exploiting the fluorescence methods described in the previous section.

3.1.1 Mobile Receptors

Normally the HIV entry receptors form locally enriched domains separated by approximately 10 nm, a distance small enough to appear colocalized by simply monitoring their locations by traditional fluorescence microscopy. The envelope gp120 induces interactions between the receptors, but the resolution of light microscopy limits our ability to see any difference. FRET has been used to explore the interaction of receptors due to the presence of virus. Yi et al. (2006) took advantage of the sensitivity of FRET to evaluate the effect of gp120 on interactions between CD4 and CCR5. FRET enables the determination of very closely adjacent or truly interacting molecules, clearing up this ambiguity.

While the receptors normally form separate microdomains on the plasma membrane, complexes including gp120 and the receptors form during HIV entry. Previous studies had inconclusively implicated lipid rafts in entry complex formation, with fluorescence microscopy studies offering conflicting evidence on whether CD4 and CCR5 localization to lipid rafts is important for entry complex formation and infection. A complication in visualizing these interactions is that lipid rafts are dynamic structures less than 70 nm in diameter, well below the resolution of light microscopy. Utilizing FRET to examine interactions between CD4 and CCR5 enabled Yi et al. to assess the importance of lipid rafts on receptor interactions. Using CD4-YFP and CCR5-CFP as a FRET pair, they found that gp120 can bring CD4 and CCR5 together on the plasma membrane of live cells. Because FRET will occur only at miniscule distances, this eliminates complicated interpretations of colocalization and gives a more objective measure of association. Additionally, the authors found that chemically disrupting lipid rafts blocked this gp120-induced FRET signal, and that adding back cholesterol restored it. Studies such as these not only have the potential to visualize an interaction, but to quantify the impact of inhibitory compounds on interactions between virus and host.

3.1.2 Hitching a Ride

The revolution in HIV imaging began with the ability to visualize a virion interacting with living cells. The exploitation of live cell imaging within the HIV field started with the creation of a fluorescently labeled virus whose entry into the host cell and early interactions inside it could be followed under the microscope.

The first observation of HIV in living cells was made possible by attaching a fluorescent protein to the HIV accessory protein Vpr. In 2002, McDonald et al.

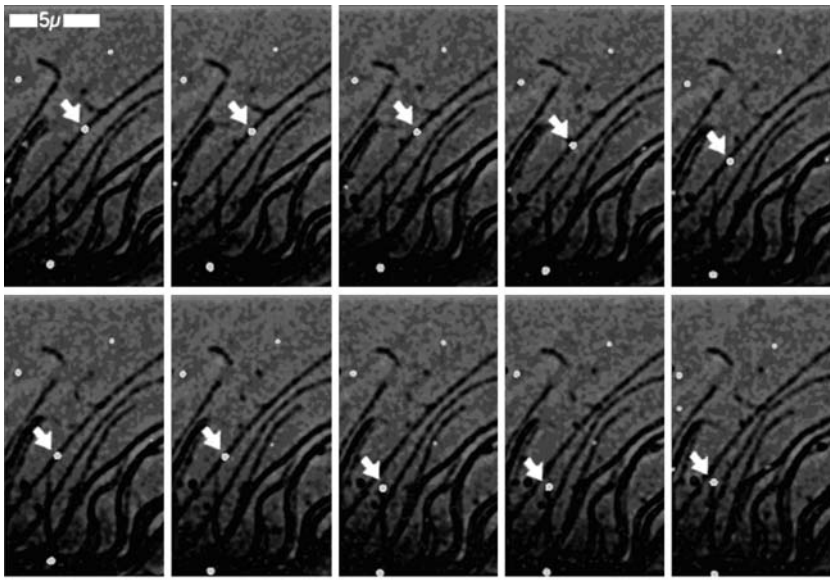

Fig. 5 GFP-Vpr can be used to visualize HIV-1 trafficking within living cells

observed HIV entering cells and hijacking the microtubule network to enable its voyage towards the nucleus (McDonald et al. 2002; Fig. 5). As well as establishing a key method enabling the visualization of HIV entry, this study also highlighted the dependence on the host cell cytoskeleton in order to reach the viral genome's ultimate destination in the nucleus.

Though this remains a valuable method for studying interactions, several limitations are inherent in such an approach. First, Vpr is apparently lost before nuclear entry, limiting the portion of the HIV life cycle that can be visualized with this label. Second, it is not clear whether all virions within the cell have entered via fusion with the cell membrane or have been nonspecifically endocytosed. Finally, the problem of interpreting colocalization cannot be resolved without complementary biochemical techniques.

3.1.3 Discriminating Entry from Endocytosis

One improvement in labeling virions is the use of the 15 N-terminal residues of Src (S15), a signal incorporated into virions that is lost after fusion but not after nonproductive receptor-independent endocytosis. S15 targets the plasma membrane, and this sequence is incorporated into virions (Campbell et al. 2007). Using a fluorescently tagged version of S15 (S15-mCherry) together with GFP-Vpr allows a double label that provides a clear picture of a virion that has entered the host cell via fusion. Being able to visualize a productive entry event allows

interactions within the cell not relevant to productively entered virions to be discriminated and ignored.

This dual-labeled system was used again by Campbell et al. (2008) to visualize the retroviral restriction factor rhTRIM5α interacting with cytoplasmic HIV complexes. The virions observed associating with TRIM5α complexes were specific to GFP-Vpr-labeled particles that had lost the S15 membrane label, indicating that they had entered the host cell cytoplasm by fusion. By being able to identify viral complexes that had entered the cytoplasm after fusion, it was possible to validate that the interaction with TRIM5α had the appropriate specificity.

3.1.4 Journey to the Center of the Nucleus

Despite the utility of GFP-Vpr labeling, one severe limitation of this approach is that it is only possible to visualize early postentry events, leaving any nuclear movements and interactions of the intracellular HIV complex to remain a mystery. One step in the direction of looking further into the nuclear aspects of the HIV life cycle has been achieved with the labeling of integrase. Arhel et al. (2006) reported a FlAsH-tagged integrase that enables the visualization of intranuclear movements. FlAsH (fluorescein arsenical hairpin) is a reagent that specifically binds to a small tetracysteine sequence that may be inserted into a target protein. One virtue of the FlAsH system compared to traditional fluorescent protein labeling is that the sequence added is much smaller than a fluorescent protein like GFP, and as such may be less prone to perturbing the structure and function of the protein to which it is added. The use of this tag on the HIV integrase protein allowed the observation of the kinetics of movement towards the nucleus, supporting both microtubule- and actin-dependent movement. With this system, HIV complexes apparently located in the nucleus exhibited restrained diffuse movement.

Potentially improving on the FlAsH integrase tag to visualize virions trafficking within the nucleus, a subsequent study by Albanese et al. (2008) utilized a fluorophore-tagged integrase (IN) in trans in order to achieve this goal. Because inserting sequences within the proviral sequence often causes processing problems, they exploited the fact that Vpr is incorporated into virions by attaching Vpr to a fluorescently tagged IN. This was done in such a way that, in the presence of HIV protease, cleavage separating Vpr from IN-EGFP will occur. Thus, the Vpr-IN-EGFP was expressed in trans with a proviral construct containing an IN deletion, allowing the production of a virion with fluorescently labeled IN.

Rather than investigate interactions of HIV with host cell proteins, Albanese et al. examined the relationship with host cell DNA. The study of integration sites has revealed that HIV has a preference to integrate into actively transcribed genes. One open question is what determines this preference. Their findings reveal that virions tend not to travel far after entering the nucleus, and that a preference is seen for areas of decondensed chromatin. This work not only begins to reveal what areas of the genome HIV associates with, but opens many doors to further investigate the interactions occurring within the nucleus.

3.2 Exit

After integrating its reverse transcribed DNA into the genome of its host cell, this provirus acts as the template from which RNA is transcribed. Viral proteins are synthesized and virions are assembled at the plasma membrane, where these immature virions bud from the infected cell (Freed 2001) with the help of the host cell's endosomal sorting complex required for transport (ESCRT) machinery. The nascent virions undergo maturation and then go on to infect other target cells. Imaging techniques have provided important insights into the interactions and dynamics leading to new virion assembly.

3.2.1 Correlative Imaging: The Best of Both Worlds

As fluorescently tagged virions enable the study of entry, other methods must be employed to visualize egress of budding virions. One innovative technique used to resolve this difficulty is correlating high-resolution electron microscopy with fluorescent imaging capable of visualizing molecules within the interior of a cell. This approach was used by Larson et al. (2005) in order to visualize retroviral budding. The authors utilized live cell multiphoton laser scanning microscopy (MPM) of transfected Gag and subsequent scanning electron microscopy (SEM) in order to combine the strengths of both methods. Namely, SEM can resolve single budding structures but can only be used for visualizing bulges on the surface, and fluorescence imaging can look within the cell but can only infer budding from the disappearance of a fluorescent signal. As both imaging methods are powerful techniques with their own strengths and weaknesses, the information gained by the combination is invaluable.

Studies of single particles entering the cell have been carried out, but this had not yet been accomplished for budding. Larson et al. visualized real-time budding of single HIV-1 or Rous Sarcoma Virus (RSV) virus-like particles (VLPs) from live cells and correlated their fluorescent results to those seen by SEM. A fraction of punctate Gag spots seen by fluorescent microscopy was found to correspond to budding structures on the plasma membrane as visualized by SEM, confirming the interpretation as sites of budding. Such a method of correlating the same budding structures as seen by two different methods should prove useful in future studies.

3.2.2 ESCRTing Through Microdomains

The use of basic fluorescence microscopy has aided in evaluating the role of previously noted biochemical interactions. For example, while characterization of highly purified HIV particles had already suggested a potential interaction between the tetraspanin CD63 and HIV Gag, the functional importance of this interaction was not understood. Simply staining for CD63 showed that only a small portion of CD63 was present on the plasma membrane, while the majority was located on

intracellular membranes (Nydegger et al. 2003). The next step in investigating this interaction was to selectively stain surface CD63, which revealed that it was present in clustered microdomains rather than being uniformly distributed throughout the plasma membrane (Nydegger et al. 2006).

Aside from traditional microscopy, Nydegger et al. also used electron microscopy (EM) to complement their light microscopy findings. Moreover, by combining the real-time fluorescent imaging of live cells with high-resolution immuno-EM, the size of these microdomains could be appraised. Immuno-EM also enabled the authors to see that tetraspanin-enriched microdomains (TEMs) formed near clathrin-coated areas and cytoskeletal elements. Furthermore, they showed that ESCRT1 components Tsg101 and Vps28, already shown to be required for budding, are recruited to TEMs colocalizing with Gag.

3.2.3 Shared Machinery

Though the budding of HIV-1 virions has been overwhelmingly examined structurally, there is certainly a role for fluorescence microscopy. One demonstration of the strength of traditional colocalization studies is the discovery of shared cellular machinery for viral budding and cytokinesis. Carlton and Martin-Serrano (2007), spurred on by yeast two-hybrid studies revealing interactions of components of the ESCRT and cytokinesis machinery, used fluorescence microscopy to study the localization of these putative interactions.

ESCRT proteins Tsg101 and Alix both bind the centrosomal protein Cep55 involved in cytokinesis. When Cep55 expression is disrupted with siRNA, Tsg101 and Alix no longer exhibit their usual localization. They went on to map the residues of Tsg101 responsible for binding Cep55 and found that, upon deletion, the same aberrant localization occurred. After finding this striking example of a virus usurping the cellular machinery of a function as basic as cell division, they discovered that the Tsg101-Cep55 interaction is required for cytokinesis but not for viral budding. By studying the differential requirements of components involved in requisite cellular functions and exploited for viral replication, it may be possible to separate out specific interactions that may be disrupted for antiviral therapy while preserving necessary cellular functions.

3.2.4 Dynamics of Budding VLPs

Another method for the visualization of assembly and egress is TIRF. Jouvenet et al. (2008) combined TIRF imaging with FRET and fluorescence recovery after photobleaching (FRAP), more objective methods of determining interactions, in order to explore viral budding. TIRF allowed the authors to visualize budding of Gag VLPs in real-time, a difficult task with epifluorescence as the more prominent signal from cytoplasmic Gag overpowers the weak signal from budding VLPs. As

TIRF images regions of the sample within approximately 100 nm of the coverslip, a study of budding from the plasma membrane can benefit greatly from this approach.

In an attempt to characterize the kinetics of budding, the authors first noticed two populations of Gag: slowly appearing and rapidly appearing/disappearing. In order to explore the relevance of these two populations, the authors looked at CD63 as an endosomal marker and correlated these results to the speed at which Gag puncta appeared in order to focus only on real assembly events taking place at the plasma membrane. They found that the majority of the rapidly appearing/disappearing population stained positively for both CD63 and clathrin, implying that this population is associated with endosomes. Thus, they chose to focus on the slowly appearing population representative of budding at the plasma membrane. This use of TIRF allowed the authors to characterize kinetically variant populations based on previously characterized interactions, a feat unthinkable by traditional fluorescence. They also used FRET to interpret the population of slowly emerging particles as Gag molecules moving closer together, and thus undergoing assembly into VLPs. A similar FRET-based assay has been previously utilized to study oligomerization of Gag during the process of assembly, both within living cells and VLPs (Derdowski et al. 2004). Though the Jouvenet study focused primarily on the dynamics of budding, it should prove to be a promising approach to characterize interactions occurring during this process, such as the incorporation of cellular proteins and viral RNA into budding virions.

3.2.5 Superresolution Budding

Betzig et al. (2006) reported a use of PALM in which localization on the scale of nanometers was achieved in high-resolution imaging of Gag at the plasma membrane. PALM combined with TIRF is well suited for the study of proteins at the plasma membrane, such as budding Gag VLPs. This study combined PALM with TIRF microscopy in order to decrease autofluorescence and thus increase the resolution. Though this method requires up to 12 h to obtain a superresolution image, the detail obtained may outweigh the lengthy process for certain questions. As a dual-color version of PALM has been shown to provide resolution on the scale of 20–30 nm, the utilization of such a technique in the future seems to be quite valuable for probing interactions between virion and host cell.

The combination of PALM with single-particle tracking provides the means to investigate the dynamics of molecules in high resolution, opening up many doors to visualize interactions and determine how they are affected in real time at the single molecule level. Manley et al. (2008) use this combined sptPALM technique to track Gag molecules at the plasma membrane at an incredibly detailed resolution, as well as to follow the motion of many subsets of particles by selectively photoactivating them. One interesting finding from this study is that an immobile portion of Gag appeared stuck on the plasma membrane, an observation that the authors postulate may be due to interactions with tetraspanin-enriched microdomains (TEMs). Thus,

differences in the dynamics of Gag may be further explored by the use of dual-colored PALM in order to track interactions with host cell proteins.

3.2.6 Connecting Exit and Entry

Dendritic cells have the ability to bind HIV without becoming productively infected, a strange phenomenon that both contributes to an immune response and at the same time is yet another host cell function that has been pirated by HIV. Aside from binding and degrading HIV virions and producing an antibody response, dendritic cells can also sequester virions and transfer them to T cells in a process called *trans*-infection, and thus contribute to establishing a systemic infection. One unresolved question has been where exactly the dendritic cell stores the virus before passing it off to a T cell. The exosome model proposes storage of virions in a protected compartment within the cell, with *trans*-infection occurring only when fusion with the plasma membrane brings the virions back to the surface. Recent results demonstrated an opposing model in which internalized virions are degraded by the lysosome and only surface-accessible virions are able to cause *trans*-infection (Cavrois et al. 2007).

While evidence existed supporting both models, Yu et al. (2008) used live cell microscopy to resolve the discrepancies between these seemingly contradictory models. First the authors characterized the subcellular components localized within the internal HIV compartment, finding that the compartment colocalizes with actin, and subsequently that formation of it requires the actin cytoskeleton. In order to characterize this compartment, they stained for various cell surface and intracellular markers, finding that tetraspanin CD81 was recruited from its uniform distribution on the cell surface to form a concentration in the compartment. Aside from determining subcellular localization, they used fluorescence microscopy to investigate the time span of HIV sequestration within the compartment, finding that it correlates with the length of time in which *trans*-infection can be detected. The use of the combined Vpr-S15 virion label was used in order to determine which virions had entered the cytoplasm and thus to visualize *trans*-infection. The virion compartment was accessible to both a surface-applied inhibitor and a fluid phase marker, indicating that this compartment was intracellular but nevertheless connected to the surface. Their surprising conclusion was that virions are stored in an internal compartment, but that this compartment is a pocket-like structure contiguous with the plasma membrane that remains surface-accessible. The use of simple antibody staining, combined with the power of live cell microscopy to study the dynamics of a subcellular compartment, allowed a more complete view of opposing models of *trans*-infection.

A similar mechanism has been observed for the transfer of HIV from an infected cell to a target cell. This configuration is called the virological synapse (reviewed in Jolly and Sattentau 2004). Imaging techniques have played an essential role in revealing the function of the virological synapse and addressing the long standing mystery of why infected cells are much more efficient at transmitting HIV relative

to cell-free particles. Such interactions can be sites of direct contact of the plasma membranes of the two cells, or mediated by membrane projections such as cytonemes and nanotubes (Sherer et al. 2007; Sowinski et al. 2008). In all cases, the increase in infectivity is stimulated by concentrating the virus from the infected cell in close proximity with the receptors required for fusion with the target cell. It is difficult to imagine how this important aspect of HIV biology could be studied without the assistance of the investigative power of imaging techniques.

3.3 Regulatory and Accessory Proteins

As well as the structural components of HIV that interact with host cell molecules to exert an effect on infection, the regulatory and accessory proteins of HIV play their own roles in creating a more hospitable environment. Several examples describing the visualization of these interactions are mentioned below.

3.3.1 Tat

HIV depends on regulation of transcription to maintain the viral reservoir, a population of cells with integrated provirus that do not actively produce HIV. The transactivator of transcription (Tat) protein of HIV boosts production of viral RNA synthesis through its interaction with the trans-activation-responsive region (TAR) of the viral RNA, as well as interactions with cellular components. Tat carries out this role through its interaction with positive transcription elongation factor P-TEFb. Tat has been shown to interact with P-TEFb kinase component cyclin T1 in a complex with TAR at the HIV promoter. While the interaction between Tat and cyclin T1 was first recognized biochemically, little was known of its specifics and consequences. A method such as FRET is ideal for visualizing putative biochemical interactions and determining their physiological relevance, the use of which enabled Marcello et al. (2001) to find that Tat influences the subcellular localization of cyclin T1.

Cyclin T1 normally localizes to nuclear foci, while Tat typically resides diffusely throughout both the nucleoplasm and the nucleolus. By simply expressing fluorescently tagged versions of both proteins, they found that a reorganization of both proteins occurs when coexpressed. The authors found that Tat induced cyclin T1 to relocalize from its characteristic accumulations in the nucleus to the sites of transcription and, reciprocally, Tat lost its diffuse localization to accumulate at these same locations. The combination of analyzing changing subcellular location along with FRET to ascertain whether proteins are interacting gives insight into the function of this interaction. Not only do they note that two proteins are located in proximal locations, but this localization is only seen when they are interacting.

Though the interaction of Tat with cyclin T1 and its requisite role in activating transcription were already known, a model had not yet been described to resolve current data. The question was whether P-TEFb remained in association with the

transcription complex after initiation of transcription, or if it dissociated prior to elongation.

Delving into the dynamics of this interaction with an interesting method of visualizing specific RNAs within the cell, Molle et al. (2007) explore the kintetics of the association of Tat and cyclin T1. In order to visualize HIV-1 transcription sites in live cells, they tagged the HIV-1 proviral construct with binding sites for phage MS2, and expressed this along with GFP-tagged MS2. Only when the HIV-1 proviral construct associates with GFP-tagged MS2 will any fluorescence be seen. This system allowed them to visualize the site of transcription as well as to the monitor the localization of Tat and cyclin T1 at this site. They found that Tat must interact with cyclin T1 in order to accumulate at transcription sites. Additionally, the use of FRAP allowed them to see that Tat recovers slowly at transcription sites, bringing up the possibility that Tat may dissociate before complete transcription. The strength of this study is that it used dynamic data from living cells in order to resolve conflicting models.

3.3.2 Nef Oligomerization

The HIV regulatory protein Nef is required for HIV infection to progress into AIDS. Nef is involved in downregulation of CD4 and MHC-I from the cell surface and enhanced infectivity (Freed 2001), roles it carries out through its interactions with various cellular proteins. Its interaction with Hck, a protein-tyrosine kinase, appears to be involved in pathogenesis of HIV. Ye et al. (2004) use BiFC to show that Nef oligomerizes in living cells, a prerequisite for in vivo activation of Hck. Just as BiFC has been used in this study to investigate interactions between Nef molecules that contribute to interactions with cellular molecules, it should be a promising method to reveal interactions between Nef with host cell proteins. Other studies have used BiFC to explore the potential interaction between retroviral Gag and actin (Chen et al. 2007).

4 Conclusions

There is currently a revolution taking place in the identification of cellular factors involved with HIV replication. Using libraries of siRNAs and shRNAs to knock-down cellular factors, multiple groups have identified hundreds of new factors that may interact with HIV proteins and play an essential role in HIV biology. This list will only get longer in the near future. The variety of techniques currently used to visualize interactions between HIV-1 and cellular proteins can provide an extraordinary wealth of information with regard to the functionality of such interactions. Imaging has the potential to reveal subtleties not visible by other techniques, such as the localization and dynamics of interactions. Fluorescent imaging provides a subset of techniques complementary to the standard biochemical methods, and

when combined they present a powerful means to unmask complex questions. With high resolution imaging improving quickly and with different techniques suitable to probe different stages of the viral life cycle, today's scientists are well equipped to answer questions of how HIV-1 proteins relate to the host cell environment, setting up the basis to utilize this knowledge therapeutically.

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Virological and Cellular Roles of the Transcriptional Coactivator LEDGF/p75

Manuel Llano, James Morrison, and Eric M. Poeschla

Contents

Abstract The chromatin-associated cellular proteins LEDGF/p75 and LEDGF/p52 have been implicated in transcriptional regulation, cell survival and autoimmunity. LEDGF/p75 also appears to act as a chromatin-docking factor or receptor for HIV-1 and other lentiviruses and to play a role in leukemogenesis. For both the viral and cellular roles of this protein, a key feature is its ability to act as a molecular adaptor and tether proteins to the chromatin fiber. This chapter reviews the emerging roles of LEDGF/p75 and LEDGF/p52 in diverse cellular processes and disease states.

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1 History and Nomenclature: Transcriptional Coactivator p75, LEDGF/p75, DFS70

Between 1998 and 2003, research in four apparently unrelated fields – transcriptional regulation, cell survival, autoimmunity, and virology – independently identified a polypeptide that migrated in denaturing SDS gels with an approximate molecular weight of 75 kDa (Cherepanov et al. 2003; Ge et al. 1998a; Ochs et al. 2000; Singh et al. 1999). The initial discovery came from micro-sequencing of a protein that co-purified with the general transcriptional coactivator positive cofactor 4 (PC4) (Ge et al. 1998a). Two splice variant cDNAs were identified, one encoding the 75 kDa species (p75) and the other a smaller polypeptide, p52. Like PC4, both proteins enhanced activity of the general transcription machinery in vitro and so were designated transcriptional coactivators p75 and p52. A year later, p75 was isolated from a mouse lens epithelium library and was reported to protect these cells from oxidative damage (Singh et al. 1999). These workers named the protein lens epithelium-derived growth factor p75 (LEDGF/p75), in part because it is one of seven members of the hepatoma-derived growth factor (HDGF) family (Dietz et al. 2002). This term has entered common usage. The implied possibilities that LEDGF/p75 might be secreted or act as a signal-transducing growth factor have been discarded on the basis that the protein is constitutively nuclear (Cherepanov et al. 2003; Llano et al. 2004b; Maertens et al. 2003) and is not detectable in culture supernatants or serum (our and others unpublished data). In addition, LEDGF/p75 knockout mice show no defects in lens development (Sutherland et al. 2006). The general view at present is that LEDGF/p75 is a ubiquitously expressed nuclear protein with a role in transcriptional regulation (Fatma et al. 2001, 2004, 2005 ; Ge et al. 1998a; Kubo et al. 2002; Sharma et al. 2003; Shin et al. 2008; Shinohara et al. 2002; Yokoyama and Cleary 2008). In 2000, p75 was again identified by screening of a cDNA library with human serum reactive against the nuclear autoantigen dense fine speckled protein of 70 kDa (DFS70) (Ochs et al. 2000). This line of work has suggested a role for LEDGF/p75 in autoimmunity (Ganapathy and Casiano 2004; Ochs et al. 2000), and additional studies have suggested roles in cell survival and prevention of apoptosis (Ganapathy et al. 2003; Shinohara et al. 2002). More recently, the protein was isolated by Cherepanov et al. as a polypeptide that co-immunoprecipitated with human immunodeficiency virus type 1 (HIV-1) integrase (IN) when the latter was overexpressed outside the viral context (Cherepanov et al. 2003).

LEDGF/p75 and LEDGF/p52 bind chromatin tightly (Llano et al. 2006b; Nishizawa et al. 2001; Turlure et al. 2006) and interact with other nuclear proteins (Bartholomeeusen et al. 2007, 2009; Ge et al. 1998a; Ge et al. 1998b; Maertens et al. 2006; Yokoyama and Cleary 2008). Several of these proteins are tethered to the chromatin fiber by LEDGF/p75. These properties underlie roles for them in transcriptional regulation, which in turn appears to influence the processes of cellular transformation, differentiation, and survival. We now also know that lentiviruses, the genus of retroviruses to which HIV-1 belongs, have exploited the chromatin-tethering capacity of LEDGF/p75 during their obligate life cycle step of

integration into a host chromosome (Llano et al. 2006a; Shun et al. 2007; Vandekerckhove et al. 2006). LEDGF/p75 dependence is found not only in the primate group of lentiviruses, but also in the two other main groups (ungulate and feline lentiviruses) (Busschots et al. 2005; Cherepanov 2007; Llano et al. 2004b, 2006a; Marshall et al. 2007). Here, we will discuss evidence for the cellular and the viral cofactor activities of LEDGF/p75, concentrating on recent findings. For additional reviews that focus on the HIV-1 cofactor role comprehensively, see Ciuffi and Bushman (2006), Engelman and Cherepanov (2008), Poeschla (2008), and Van Maele et al. (2006).

1.1 Gene Organization and Splice Variants

LEDGF/p52 and LEDGF/p75 are illustrated in Fig. 1. Both are encoded by the gene *PSIP1* (PC4- and SFRS-interacting protein 1) which is 35.7 kb in length and located on human chromosome 9p22.3 (Singh et al. 2000a). There are 15 exons and 14 introns. Gene to protein relationships are presented in Table 1. The two encoded splice variants share the same N-terminal 325 amino acids (encoded by exons 1–9), but have different C-termini, 8 amino acids in the case of p52 and 205 in the case of p75. This C-terminal portion of LEDGF/p75 contains the IN binding domain (IBD, residues 340–417) which is central to the protein's virological and cellular significance (Cherepanov et al. 2004; Vanegas et al. 2005).

Additional LEDGF/p52 splice variants were identified recently by mRNA analyses in promyelocytic leukemia cells (Huang et al. 2007). They elaborate the

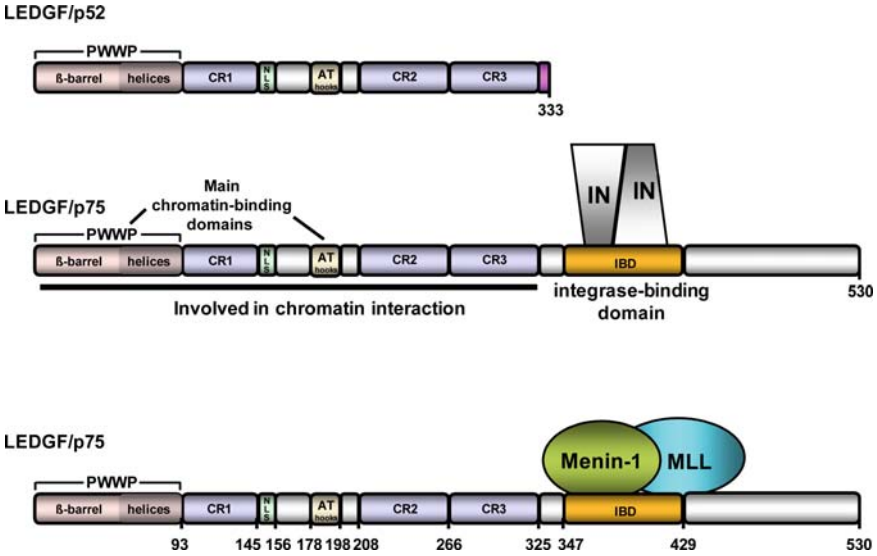

Fig. 1 Domain structure and binding partners of LEDGF/p75 and LEDGF/p52

Table 1 Gene-to-protein relationship in LEDGF proteins

Exon	Amino acids	Protein domain	p75 mRNA segment
1	1–23	PWWP	1–382
2	24–50	PWWP	383–459
3	51–96	PWWP, CR1	460–598
4	97–131	CR1	599–703
5	136–152	CR1, NLS	704–766
6	153–184	NLS, AT	767–863
7	185–210	AT, CR2	864–939
8	211–286	CR2, CR3	940–1168
9	287–326	CR3	1169–1287
10	327–344		1288–1343
11	345–368	IBD	1344–1414
12	369–402	IBD	1415–1516
13	403–472	IBD	1517–1731
14	473–511		1732–1843
15	512–530		1844–poly(A) signal
9-A	[a]287–326	CR3	
10-A	[a]327–z333	C-terminal region	

[a]Exons 9-A and 10-A encode CR3 and the C-terminal region of LEDGF/p52. Exon 9 and exon 9-A have identical nucleotide sequences, except different terminal amino acids are encoded (Q for 9, and H for 9-A)

theme of variant C-terminal domains determining diverse bi-molecular adaptor roles in tandem with the chromatin-binding properties of the N-terminal portion. One, p52b, was highly expressed at the mRNA level. In the putative encoded protein, the last eight amino acids of p52 are substituted by 25 extra amino acids also derived from intron 9 of the gene. The other three LEDGF/p52 variants are expressed at lower levels and they lack exon 6 and exhibit other sequence modifications (Huang et al. 2007). However, endogenous protein expression has not been documented for these p52 splice variants.

Northern blots have identified a 1.8-kb species of mRNA corresponding to LEDGF/p52 and two additional species of 3.4 and 2.8 kb that correspond to p75 (Ge et al. 1998a). These mRNAs and proteins are ubiquitously expressed, with LEDGF/p75 being considerably more abundant in the majority of tissues, although greater relative amounts of p52 mRNA were detected in the brain, testis, and thymus (Ge et al. 1998a), suggesting that splicing of *PSIP1*-derived mRNAs can be regulated in a tissue-specific fashion.

2 Chromatin Binding

LEDGF/p75 and p52 are nuclear proteins that attach to chromatin avidly throughout the cell cycle, though as determined by studying GFP fusions, p52 may have a more restricted intra-nuclear distribution (Nishizawa et al. 2001). The major determinants of sub-cellular distribution are located in the shared N-terminal region (Fig. 1) (Llano et al. 2006b; Maertens et al. 2004; Turlure et al. 2006; Vanegas et al.

2005). Nuclear import is determined by a nuclear localization signal (NLS) at residues 148–156 (Maertens et al. 2004; Singh et al. 2006; Vanegas et al. 2005). This NLS belongs to the classical basic (SV40 large T antigen) family, and nuclear import requires functional Ran, the adaptor protein importin-alpha, and the nuclear import receptor importin-beta (Maertens et al. 2004; Vanegas et al. 2005). NLS deletion leads to cytoplasmic localization of newly synthesized LEDGF/p75. However, in dividing cells, the NLS is actually dispensable for nuclear localization. The protein is chromatin-trapped with such efficiency during mitotic mingling of nuclear and cytoplasmic contents that stably expressed NLS-mutants are seen only in tight association with chromosomes (Vanegas et al. 2005). Chromatin binding is mediated in part through the N-terminal PWWP domain (residues 1–93) (Llano et al. 2006b; Shun et al. 2008; Turlure et al. 2006). Importantly, although there is extensive homology between the PWWP domain of LEDGF/p75 and the same domain in a related IBD-containing HDGF family member, HDGF-related protein 2 (HRP-2), this latter protein (and any associated IN) dissociates from chromatin in mitosis and does not display the tight Triton-resistant chromatin binding of LEDGF/p75 in any part of the cell cycle (Vanegas et al. 2005). It is thus clear that the chromatin binding of PWWP-containing proteins is greatly influenced by other protein regions.

LEDGF/p75 resists extraction from chromatin when cells are lysed in isotonic buffers containing the detergent Triton X-100 (Llano et al. 2006b). Triton-resistance is determined primarily by the cooperative interaction of the PWWP domain with two downstream AT hook motifs (residues 178–198; Fig. 1). Flanking relatively charged regions (CR1, CR2, and CR3) contribute to a lesser extent (Llano et al. 2006b). Turlure et al. also implicated the NLS region in chromatin binding (Turlure et al. 2006). Mutations that disrupt the PWWP domain partially impair Triton-resistant chromatin binding, while simultaneous mutational inactivation of the PWWP and the AT hook domains completely abolishes it (Llano et al. 2006b). Although such mutants localize to the nuclear compartment during interphase, they are excluded from chromatin during mitosis (Llano et al. 2006b; Shun et al. 2008). Shun et al. have further identified specific amino acid mutations within the domain that disrupt chromatin binding, e.g., W21A (Shun et al. 2008).

Sub-nuclear distribution analyzes of LEDGF/p75 and p52 were performed with GFP fusion proteins (Nishizawa et al. 2001). Although LEDGF/p52 and p75 might be predicted to display quite similar sub-nuclear distributions, it turns out that these are distinct with respect to the cell cycle (Nishizawa et al. 2001). In G1, LEDGF/p52 was found in the nuclear periphery, while p75 was distributed diffusely throughout the nucleus, recapitulating the characteristic dense fine speckled pattern observed with autoantibodies. During metaphase, LEDGF/p52 was observed to form a cylindrical pattern surrounding chromosomes whereas LEDGF/p75 associated with chromosomes in a striated pattern. At cytokinesis, LEDGF/p75 nuclear localization was diffuse while LEDGF/p52 retained the cylindrical pattern (Nishizawa et al. 2001). These observations imply that the respective C-terminal regions govern the finer aspects of intra-nuclear segregation. The localization of LEDGF/p75 is likely to be modulated by interactions with other chromatin bound

proteins, some of which are discussed below. Alternatively, the presence of the long C-terminal region of LEDGF/p75 could modify interactions of the shared N-terminal region with chromatin.

2.1 Interactions with Cellular and Viral Proteins

In in vitro assays with recombinant LEDGF proteins and purified general transcription factors, LEDGF/p52 interacted functionally with a number of viral transcriptional activation domains, e.g., VP16, the pseudorabies immediate early protein and adenovirus E1A, as well as with the cellular transcription factor Sp1 and PC4 (Ge et al. 1998a, b). In these in vitro systems p52 fulfilled general transcriptional coactivator criteria; however, with the exception of the VP16 activation domain, LEDGF/p75 did so only marginally (Ge et al. 1998a). In addition, LEDGF/p52, but not p75, has been reported to interact with the essential splicing factor ASF/SF2, thus modulating pre-mRNA splicing (Ge et al. 1998b). Therefore, LEDGF/p52 has been proposed to be a coordinator between transcription and pre-mRNA splicing (Ge et al. 1998b).

The differential interaction of LEDGF proteins with cellular proteins is informative considering that both proteins share the same N-terminal chromatin-interacting region which corresponds to 97.6% of p52. These examples provide further evidence for the long C-terminal region determining diverse molecular partner interactions, and we speculate that they may explain the differences in sub-nuclear localization discussed above (Nishizawa et al. 2001). In support of this hypothesis, a polyclonal antibody raised against LEDGF/p52 recognized both p52 and p75 in immunoblots but immunoprecipitated only LEDGF/p52 from cellular extracts, suggesting that native p75 is not recognized, perhaps because amino acids 326–530 or bound cellular interactors mask N-terminal epitopes (Ge et al. 1998a).

The IBD interacts with lentiviral IN proteins, the c-Myc interactor JPO2 (Bartholomeeusen et al. 2007; Maertens et al. 2006), the menin/MLL histone methyl transferase complex (Fig. 1) (Yokoyama and Cleary 2008), and the pogo transposable element with ZNF domain (pogZ) (Bartholomeeusen et al. 2009). The IBD surfaces involved are not completely overlapping. For example, mutation of D366, which is located in an interhelical loop that extends into the IN dimer interface, abrogates interaction with lentiviral IN proteins but does not affect interaction with JPO2 or pogZ (Bartholomeeusen et al. 2007, 2009; Maertens et al. 2006). However, an F406A mutation in an adjacent loop impairs the binding of these three proteins. Site-directed mutagenesis of LEDGF/p75 IBD residues implicated in IN binding show more overlap with residues required for pogZ than for JPO2 binding. Interestingly, lentiviral INs and pogZ belong to the DDE domain family (Bartholomeeusen et al. 2009). The significance of the JPO2 and pogZ interactions is not clear at present. Analogously to its effects on overexpressed HIV-1 IN (Llano et al. 2004a), LEDGF/p75 protects JPO2 from proteolysis and tethers this protein to chromatin during all phases of the cell cycle (Bartholomeeusen et al. 2007; Maertens et al. 2006). By contrast, interaction with pogZ yields a tightly

chromatin-bound complex in cells in interphase, but no apparent chromosome tethering during mitosis (Bartholomeeusen et al. 2009).

LEDGF/p75 interaction determinants are well understood for two protein complexes, the lentiviral IN dimer and the menin/MLL complex. LEDGF/p75 tethers both of them to chromatin (Llano et al. 2004b; Maertens et al. 2003). Tethering of IN appears to direct integration of lentiviruses into active transcription units (Ciuffi and Bushman 2006; Ciuffi et al. 2005; Marshall et al. 2007; Shun et al. 2007). For Menin/MLL, LEDGF/p75 forms a trimolecular complex that serves to target menin/MLL to genes such as Hoxa9 (Yokoyama and Cleary 2008; reviewed in Roudaia and Speck 2008).

Detailed structural information is now available for the interaction of the IBD and HIV-1 IN (Cherepanov et al 2005a, b: Hare et al. 2009). In addition to the catalytic core domain (CCD) (Busschots et al. 2007; Maertens et al. 2003; Rahman et al. 2007), the N-terminal domain (NTD) of IN also interacts with the IBD (Maertens et al. 2003). As shown in Fig. 2, the IBD is a compact structure comprised of a pair of alpha-helical hairpins (Cherepanov et al. 2005b). The IBD-CCD interface occurs in a pocket that forms between helices α1 and α3 of one IN monomer and helices α4 and α5 in the other IN monomer. In particular, residues connecting IN helices α4 and α5 (the alpha 4-5 connector) and hydrophobic residues in the other monomer engage two inter-helical loops of the IBD, respectively (Fig. 2) (Cherepanov et al. 2005a). Alanine-scanning of this region of the IBD indicated that residues I365, D366, F406, and V408 play essential roles in the LEDGF/p75-IN interaction. Mutation of I365, D366, or F406 disrupts LEDGF/p75-IN interaction (Cherepanov et al. 2005b) and the mutants lack HIV-1 cofactor activity (Llano et al. 2006a; Shun et al. 2007; Vandekerckhove et al. 2006). Differences in these regions of other retroviral IN proteins explain their failure to interact with LEDGF/p75 (Busschots et al. 2005; Cherepanov 2007; Llano et al. 2004b). Within the lentiviral genus of retroviruses, the consistency of IN-LEDGF/p75 interaction despite limited direct sequence homology in key IN residues (Fig. 3) suggests a significant selective advantage during viral evolution. The relatively small, deep cleft occupied by the IBD (Cherepanov et al. 2005a) suggests as well that small molecules could interfere with the LEDGF/p75-IN interaction. Recent progress has been made toward this end (Du et al. 2008; Hou et al. 2008). The IBD-IN NTD interface involves charged interactions that differ from the more lock-and-key IBD-CCD interaction, with acidic residues in the NTD (E6, E10, and E13) interacting with basic amino acids in the IBD (K401, K402, R404, and R405) (Hare et al. 2009).

3 A Role in Transcriptional Regulation

As discussed above, p52 and p75 were initially identified by co-purification with PC4 in HeLa cell nuclear extracts and both displayed transcriptional coactivator activity in vitro (Ge et al. 1998a). Overexpression of LEDGF/p75 was also found to

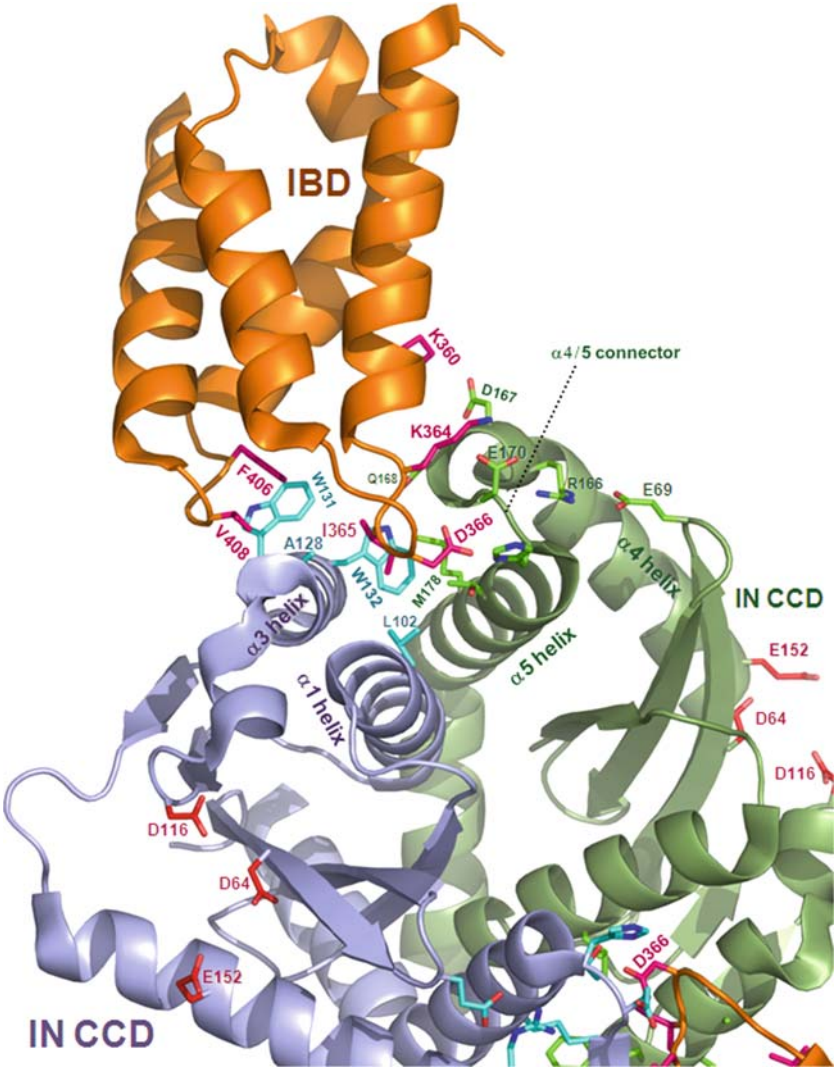

Fig. 2 Co-crystal structure of the HIV-1 IN CCD-IBD interface. The figure was constructed with MacPyMOL from Protein Data Bank file 2BJ4 (www.pdb.org; Cherepanov et al. 2005a). LEDGF/p75 contributes most of the amino acid side chains that make direct contact with IN. D366 engages in a pair of essential hydrogen bonds with the main chain amides of E170 and H171 in the alpha-4/5 connector of one IN monomer. Hydrophobic interactions predominate in interactions with the other IN monomer: IN residues W132, W131, A128, L102 form a pocket in the vicinity of IBD residues F406 and V408; this pocket buries IBD residue I365). IN catalytic center resides D64, D116 and E152, the mutation of which produce purely catalytic defects (reviewed in Engelman 1999) are shown in the lower half of the figure for each IN monomer. Interaction between the HIV-1 IN NTD and the IBD was previously established by biochemical evidence (Maertens et al. 2003), and Hare et al. have recently solved a co-crystal structure for the LEDGF/p75 IBD complexed with a two-domain fragment of HIV-2 IN (NTD+CCD) (Hare et al. 2009). Extensive structural contacts

augment transcription of a set of stress-related genes (Fatma et al. 2001, 2004, 2005; Kubo et al. 2002; Matsui et al. 2001; Sharma et al. 2000, 2003; Shin et al. 2008; Shinohara et al. 2002; Singh et al. 1999, 2001; Takamura et al. 2006) that include heat shock protein 27 (Singh et al. 1999), αB-crystallin, antioxidant protein 2A (Fatma et al. 2001), and involucrin (Kubo et al. 2002). In all these cases, LEDGF/p75 induced the expression of the endogenous proteins as well as reporter genes introduced under the control of the homonymous promoters (Fatma et al. 2001, 2005; Kubo et al. 2002; Shin et al. 2008; Singh et al. 2001). The transcriptional regulation was mediated by LEDGF/p75 binding to heat shock and stress-related elements in the promoters. Binding to these DNA sequences was identified by electrophoretic mobility shift assays (EMSA) and binding specificity was confirmed using competitor oligonucleotides, supershift induction with antibodies to LEDGF, and ablation of interaction following mutagenesis of LEDGF-binding sites in the target oligonucleotides. In addition, the interaction of LEDGF/p75 with some of these promoters was detected by DNase I footprinting. Moreover, mutation of binding sequences in promoters of the LEDGF/p75-responsive genes abrogated the transcriptional coactivator role (Fatma et al. 2001, 2005; Kubo et al. 2002; Shin et al. 2008; Singh et al. 2001). In contrast, however, specific binding of LEDGF/p75 to oligonucleotides containing the reported LEDGF/p75-binding sequences was not seen in independent surface plasmon resonance and electrophoretic mobility shift assays. Instead, LEDGF/p75 was reported to bind nonspecifically to DNA through the two AT-hook motifs and the NLS domain (Turlure et al. 2006). Further studies to clarify the specificity of interaction of LEDGF/p75 with these sequences are necessary.

More recently, LEDGF/p75, but not p52, was implicated in transcriptional regulation of genes involved in the pathogenesis of myeloid leukemias and in the establishment of the embryonic body plan during development (Yokoyama and Cleary 2008). Cytogenetic evidence has identified a translocation that generates a fusion protein between the nucleoporin Nup98 and LEDGF/p75 in patients with acute or chronic myeloid leukemia (Fig. 4) (Ahuja et al. 2000; Grand et al. 2005; Hussey et al. 2001; Morerio et al. 2005). Nup98 translocation occurs commonly in these types of cancers and more than 15 different partners are known to fuse to it. In all these fusion proteins, the N-terminal GLFG repeats of Nup98 are linked to the C-terminal region of the partner genes (Argiropoulos and Humphries 2007; Nakamura 2005; Slape and Aplan 2004). Expression of the mRNA of Nup98-LEDGF fusions was observed only during active disease. One patient exhibited a

Fig. 2 (Continued) between the IBD and the NTD were identified and characterized (Hare et al. 2009). Charged interactions are dominant, with conserved acidic residues in the HIV-2 IN NTD (E6, E10, E13) engaging complementary basic residues in the IBD (K401, R404, R405). Moreover, the NTDs of other lentiviral INs contain the same or closely adjacent glutamic acid residues. Enhancement by LEDGF/p75 of concerted strand transfer activity in vitro was also shown to be impaired by charge-reversing mutation of the basic IBD residues to glutamates in the equine lentivirus (EIAV) IN (Hare et al. 2009). This activity could then be partially restored by reciprocal mutation of the acidic IN residues to lysines, verifying that these charged interactions are the main structural feature. See Chiu and Davies (2007) for a recent review of HIV-1 IN structural biology

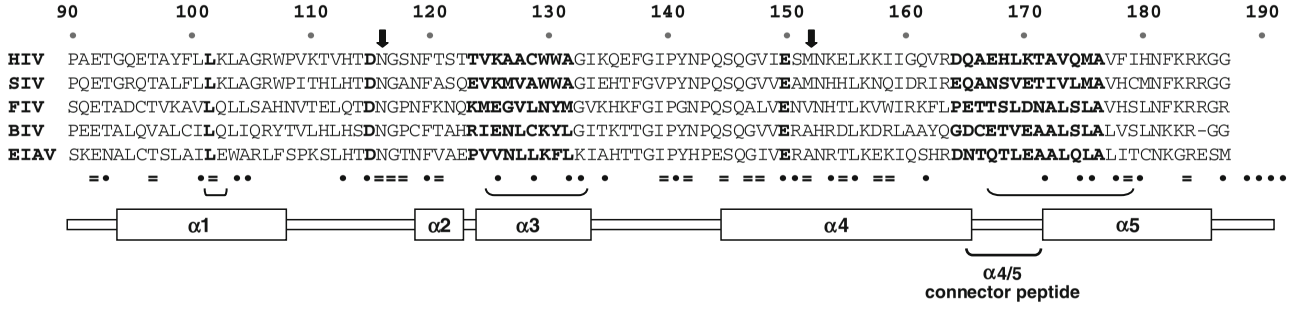

Fig. 3 Alignment of the core catalytic domains of different lentiviral IN proteins. Alignment of the central part of the CCD for IN proteins from the three lentiviral sub-genera (primate, feline, ungulate). Identity is indicated by = and residues with conserved biochemical features by dots. IN alpha helices are indicated below and the segments primarily involved in forming the IBD binding pocket are indicated by bold-face font and brackets. The relative lack of sequence conservation in these regions, e.g., the alpha 4/5 connector, is evident. Figure 2 shows the placement of these protein elements for the interface of HIV-1 IN with LEDGF/p75; catalytic center residues (heavy black arrows here) do not contact the IBD. Although its exact oligomerization state in the PIC is not conclusively established, the weight of evidence is in favor of the enzyme acting as a multimer, with a tetramer likely (Bao et al. 2003; Cherepanov et al. 2003; Faure et al. 2005; Fletcher et al. 1997; Guiot et al. 2006; Heuer and Brown 1998; Hindmarsh et al. 1999; Li et al. 2006; Sinha et al. 2002; Wu et al. 1997). In vitro, an IN dimer enables 3' end processing but a tetramer appears needed for DNA strand transfer activity (Faure et al. 2005; Guiot et al. 2006; Li et al. 2006). Of note, higher order multimers were defined in the Hare et al. co-crystal structure of the IBD with HIV-2 $IN_{NTD+CCD}$ (Hare et al. 2009). Their relevance to the oligmeric state in the virus remains to be determined

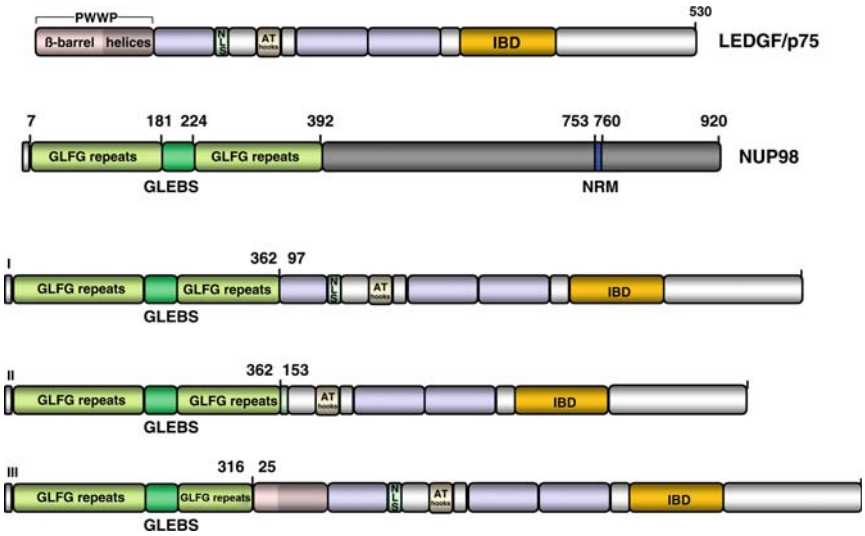

Fig. 4 Structure of NUP 98-LEDGF/p75 fusion proteins. NUP98 contains eight N-terminal GLFG repeats that govern localization of the protein to nuclear GLFG bodies. GLEBS is a sequence within the GLFG repeats that serves as docking site to GLE_{2p}, the yeast ortholog of human RAE_1 a protein involved in mRNA nuclear export. Nucleoporin RNA binding motif (NRM) is an octapeptide with partial homology to the ribonucleoprotein motif. In the NUP98-LEDGF/p75 fusion proteins (I-III) described in leukemic patients, the NUP98 GLFG repeats and the GLEBS element fuse to LEDGF/p75 segments that contain the IBD

fusion of the first 362 residues of Nup98 with a LEDGF/p75 or p52 lacking their first 152 amino acids (Ahuja et al. 2000; Grand et al. 2005), whereas another chimera was generated by the fusion of exon 8 of Nup98 with exon 4 of PSIP1 such that the first 96 amino acids of LEDGF are missing (Morerio et al. 2005). The third reported fusion protein contained the first 316 amino acids of Nup98 and all of LEDGF/p75 distal to amino acid 25 (Hussey et al. 2001). Importantly, each of these fusion proteins lack a functional PWWP domain of LEDGF/p75. The presumed loss of the tethering component may lead to alteration of LEDGF/p75-dependent transcriptional regulation.

Nup98-LEDGF/p75 fusions retain the ability of LEDGF/p75 to interact with the menin/MLL histone methylase complex, which may be implicated in leukemogenesis (Roudaia and Speck 2008; Yokoyama and Cleary 2008). MLL is targeted by numerous translocations that lead to oncogenic MLL fusion proteins, and LEDGF/p75 normally tethers unrearranged MLL to target genes with Menin essentially serving as a connector between LEDGF/p75 and MLL (Fig. 1) (Yokoyama and Cleary 2008). MLL is a nuclear protein containing a SET domain that functions to methylate lysine 4 on histone 3, a genetic modification associated with active transcription. Moreover, LEDGF/p75-dependent recruitment of the menin/MLL complex is mediated by the IBD. The recruitment of menin/

MLL leads to transcriptional regulation of several genes including Hox genes (Yokoyama and Cleary 2008). Properly regulated expression of the latter genes is necessary for the establishment of the vertebrate body plan during development, and altered Hox gene expression during embryogenesis leads to homeotic skeletal transformations similar to those observed in LEDGF/p75 knockout mice (Sutherland et al. 2006). Dysregulation of Hox gene expression has also been observed in LEDGF/p75-deficient cells (Ciuffi et al. 2005). Overexpression of Hox genes is also a salient feature of MLL-associated leukomogenesis. Chromosomal translocations that generate MLL fusion proteins have leukomogenic activity, and LEDGF/p75-mediated tethering is required during leukemic transformation induced by them (Roudaia and Speck 2008).

3.1 Effects on Cell Survival

Overexpression of LEDGF/p75 or treatment of cells with recombinant LEDGF/p75 has been reported to rescue a variety of cell types from death induced by diverse environmental insults, including serum starvation, oxidative damage, heat shock, and UVB irradiation (Ahuja et al. 2001; Huang et al. 2007; Machida et al. 2001; Matsui et al. 2001; Nakamura et al. 2000; Singh et al. 1999, 2000b; Wu et al. 2002). Protection has in general been ascribed to regulation of stress-responsive gene transcription.

A role for LEDGF/p75 in maintaining lysosomal integrity was also recently proposed (Daugaard et al. 2007). LEDGF/p75 knockdown with siRNAs induced lysosomal-dependent cell death in malignant cell lines but not in immortalized or primary cells. These observations contrast with the normal viability observed in CD4+ T cell leukemic lines in which a 97% reduction of LEDGF/p75 mRNA was achieved by stable expression of specific shRNAs (Llano et al. 2006a). Proliferative capacity, morphology, and other aspects, e.g., susceptibility to infection by gammaretroviral vectors, were indistinguishable from control cells expressing higher levels of LEDGF/p75 (Llano et al. 2006a). Effects of LEDGF proteins on cell morphology have been reported in other systems, however. For example, intracellular over-expression of LEDGF/p52 has recently been shown to stimulate dendritic arborization and axonal elongation in neural cells (Zhao et al. 2008a, b)

A role in cell survival processes was further suggested by evidence that these proteins are targeted by caspases (Brown-Bryan et al. 2008; Wu et al. 2002). LEDGF/p75 has been reported to be a substrate for caspase-3 and -7 during apoptosis, which results in protein fragments that lack the pro-survival activity of the full-length protein. Three caspase-cleavage sites were identified at residues 30 and 85 within the PWWP domain, and at amino acid 486 in the C-terminal region.

In contrast to the pro-survival role of LEDGF/p75, p52 overexpression has been reported to promote apoptosis in tumor cells, a phenomenon dependent on the 8 amino acids-long C-terminal region (Brown-Bryan et al. 2008). However, as noted above, in rat retinal ganglion cells, LEDGF/p52 overexpression did not affect viability of these cells and induced neurite growth (Zhao et al. 2008b).

3.2 LEDGF/p75 and Autoimmunity

The alternative name of DFS70 (nuclear autoantigen dense fine speckled protein of 70 kDa) arose when LEDGF/p75 was found to be recognized by autoantibodies present in patients with several chronic inflammatory diseases and cancer (Ganapathy and Casiano 2004; Ganapathy et al. 2003; Ochs et al. 2000). A high prevalence (11–22%) of LEDGF/p75-autoantibodies has also been reported in healthy individuals (Muro et al. 2008; Watanabe et al. 2004). Their pathogenic significance is not clear but it has been proposed that they are natural autoantibodies that are overproduced in some disease states (Ganapathy and Casiano 2004). Epitope mapping conducted with LEDGF/p75 autoantibody-positive serum from 93 patients and 38 healthy controls indicated that 94% of the samples in both groups reacted to a recombinant LEDGF/p75 protein containing residues 349–435, a region that contains the IBD (Ogawa et al. 2004). Reactivity against other regions was considerably underrepresented, leading to a conclusion that the IBD is the major autoimmunity determinant.

4 An Integration Cofactor for HIV-1 and Other Lentiviruses

Following the discovery that LEDGF/p75 co-immunoprecipitated with overexpressed HIV-1 IN (Cherepanov et al. 2003), and a series of studies that characterized domain properties and confirmed the necessary and sufficient role for LEDGF/p75 in mediating IN nuclear and chromatin location (Busschots et al. 2005; Cherepanov et al. 2004, 2005a, b; Emiliani et al. 2005; Llano et al. 2004a, b, 2006b; Maertens et al. 2003, 2004, 2005; Turlure et al. 2006; Vanegas et al. 2005), a fundamental role in integration of HIV-1 and other lentiviruses was demonstrated in several systems (Emiliani et al. 2005; Hombrouck et al. 2007; Llano et al. 2006a; Shun et al. 2007, 2008; Vandekerckhove et al. 2006). Among the seven genera of retroviruses, this cofactor role has turned out to be strictly lentiviral-specific. Accordingly, LEDGF/p75-deficient cells are resistant to infection by HIV-1, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV), but are permissive to infection by the gammaretrovirus murine leukemia virus (MLV) (Llano et al. 2006a). Protein interaction studies are consistent with this, showing that LEDGF/p75 interacts directly with all lentiviral INs tested so far, but not those of alpha-, beta-, delta- and gamma- or spuma-retroviruses (Busschots et al. 2005; Cherepanov 2007; Llano et al. 2004b). This lentiviral selectivity is notable since this cellular protein, and in particular the IBD, is widely conserved – species-specific positive selection is not evident – suggesting it has been available to diverse retroviruses since at least the emergence of bony fishes. LEDGF/p75 mutants that lack either chromatin or IN interaction capacity cannot rescue HIV-1 infection in LEDGF/p75-deficient cells, suggesting that the tethering function is central (Llano et al. 2006a; Shun et al. 2007; Vandekerckhove et al. 2006). This mechanism is also

supported by the change in viral integration site distribution observed in LEDGF/p75-deficient cells (Ciuffi and Bushman 2006; Ciuffi et al. 2005; Marshall et al. 2007; Shun et al. 2007).

HIV-1 IN has two enzymatic activities that are deployed in sequence during viral integration (Brown et al. 1989). After reverse transcription, IN removes a dinucleotide from the 3' end of the viral DNA, a process that appears to occur in the cytoplasm. This 3' processing step is preserved in LEDGF/p75-null cells (Shun et al. 2007). The viral genome (the preintegration complex or PIC) is then imported into the nucleus where IN carries out the second step, strand transfer. This involves the generation of single strand breaks in the opposite strands of the host DNA 5 bases apart, and concerted ligation of both of the viral 3' ends to chromosome 5' ends. Interestingly, PICs isolated from LEDGF/p75 -/- cells were competent for in vitro integration although in vivo integration was markedly impaired.

In cells, LEDGF/p75 associates with tetrameric IN (Cherepanov et al. 2003). The IBD has been demonstrated in vitro to stabilize IN subunit–subunit interactions, promoting the formation of a tetramer (McKee et al. 2008). Importantly, tetramers of IN are implicated in the bona fide strand transfer reaction (Li et al. 2006). Therefore, LEDGF/p75 appears to influence higher order structures of IN and this effect may influence IN enzymatic activity. Effects of LEDGF/p75 on IN catalysis have been studied using in vitro integration assays that mix designed unproteinated DNA fragments with purified recombinant LEDGF/p75 and IN proteins (Cherepanov 2003, 2004, 2007; McKee et al. 2008; Raghavendra and Engelman 2007; Vandegraaff et al. 2006). LEDGF/p75 or the IBD significantly increased IN-mediated 3'-processing of relatively short, 21-mer, double-stranded synthetic oligonucleotides (McKee et al. 2008). In addition, LEDGF/p75 and to a lesser extent the IBD enhanced IN strand transfer activity (Cherepanov 2004, 2007). The effect of p75 on concerted integration in in vitro assays has differed between different lentiviral IN proteins, enhancing full-site integration (properly concerted insertion of both ends) for EIAV IN but promoting uncoupled (half-site) integration with HIV-1 IN (Cherepanov 2007). Promotion of the half-site reaction was corroborated in other studies (McKee et al. 2008; Raghavendra and Engelman 2007) although the balance of half- and full-site integration has also appeared to vary with IN protein concentration and other assay parameters (Pandey et al. 2007). PIC nuclear import is not defective in the absence of LEDGF/p75 nor is nuclear stability of the imported viral genome apparently altered in LEDGF/p75-deficient cells, since abundant non-integrated genomes are detected in the form of two-LTR circles (Llano et al. 2006a; Shun et al. 2007; Vandekerckhove et al. 2006). However, in these cells, the amount of integrated viral genomes is reduced (Llano et al. 2006a; Shun et al. 2007) and the characteristic favoring of active transcription units by HIV-1 (Schroder et al. 2002) is also diminished (Ciuffi et al. 2005; Marshall et al. 2007; Shun et al. 2007). These data suggest the possibilities that intra-nuclear trafficking, chromatin attachment, strand transfer or repair of the semi-ligated integration intermediate by host enzymes could be defective in LEDGF/p75-deficient cells. Direct and definitive evaluation of these variables remains to be done.

The effects of IBD overexpression (as GFP-IBD or GFP fused to somewhat longer fragments of the C-terminal region) are interesting and incompletely understood (De Rijck et al. 2006; Llano et al. 2006a). For example, overexpression of such proteins inhibits HIV-1 integration to roughly the same extent as LEDGF/p75 knockdown. When knockdown is sufficiently stringent to strip LEDGF/p75 from the Triton X-100-resistant chromatin fraction, inhibition of single round HIV-1 reporter virus infection is about 10- to 30-fold. Roughly the same effect occurs with GFP-IBD overexpression, but combination of the two modalities leads to synergistic (> 500-fold) inhibition (Llano et al. 2006a), and up to approximately 10^4-fold inhibition has since been observed (our unpublished data). Moreover, passage of HIV-1 in the presence of such dominant interfering IBD proteins leads to selection of adaptive escape mutations in the IN dimer interface, namely A128T and E170G (Hombrouck et al. 2007).

It remains unclear where these dominant-interfering proteins act in the postentry series of events that culminate in integration or, more generally, where endogenous LEDGF/p75 first engages the viral complex. HIV-1 and FIV PICs isolated from the cytoplasm of cells could be immunoprecipitated with antibodies to LEDGF/p75 (Llano et al. 2004b). While no significant pull-down was observed in a similar experiment (Yan et al. 2009), LEDGF/p75 was noted to restore integration activity to salt-stripped cytoplasmic HIV-1 PICs (Vandegraaff et al. 2006). Whether or not it interacts with IN in the incoming PIC prior to nuclear entry, LEDGF/p75 has not been detected in free virions and it has not been implicated convincingly in PIC nuclear import.

When HIV-1 IN is expressed as a free protein in the absence of other viral components (a situation without a correlate in the viral life cycle), it is readily detected in the nuclei of cells. However, in addition to shifting to the cytoplasm in the absence of LEDGF/p75, IN is also markedly destabilized, such that it is difficult to detect by confocal microscopy or immunoblotting (Llano et al. 2004a). IN mRNA levels are unaffected, however, indicating a posttranslational effect. IN was also found to be ubiquitinated and pharmacological inhibition of the proteasome restored wild-type levels of the protein (Llano et al. 2004a). While LEDGF/p75 mutants lacking IN-binding activity fail to shield IN from the proteasome, mutants that selectively impair chromatin binding or nuclear localization of the LEDGF/p75-IN complex still prevent degradation (Llano et al. 2004a). Recently, it has been reported that IN is targeted for proteasome degradation by binding to the von Hippel-Lindau (VHL) binding protein 1(VBP1), which in turn recruits the Cullin2-based VHL E3 ubiquitin ligase, leading to IN ubiquitination and proteosome degradation (Mousnier et al. 2007). LEDGF/p75 and VBP1 binding sites on IN overlap. While direct evidence is lacking, this suggests that LEDGF/p75 could prevent protesome-mediated degradation of IN by competing with VBP1 binding. The role, if any, of this proteasome-shielding mechanism for IN within the HIV-1 PIC is not clear, and it is certainly not sufficient, because chromatin-binding mutants of LEDGF/p75 that stabilize overexpressed IN do not rescue infection (Llano et al. 2006a).

LEDGF/p75 dependency is observed in murine cells although there is no evidence for a lentivirus that naturally infects rodents (Marshall et al. 2007;

Shun et al. 2007). Lentiviral vectors with minimized viral sequences are also dependent (Llano et al. 2006a; Shun et al. 2007) which suggests that viral genome complexity, size or nucleotide composition (e.g., A-richness) are not involved. Although the chromatin tethering activity of LEDGF/p75 has been established to play a key role, the molecular mechanism is not completely understood (reviewed in (Engelman and Cherepanov 2008; Poeschla 2008)).

4.1 LEDGF/p75 as a Therapeutic Target

Current HIV therapies utilize combinations of drugs that target viral proteins involved in different steps of the viral life cycle. Toxicity, resistance development and expense remain limitations. The now clearly demonstrated efficacy of strand transfer inhibitors such as raltegravir verifies that integration can be targeted with success (Steigbigel et al. 2008). The appropriateness of the LEDGF/p75-IN interaction for therapeutic targeting is supported by the multiple lines of evidence for its role as an HIV-1 dependency factor, the limited physiological effects observed when cells are rendered deficient in this protein, and also by the possibility that resistance potential may be less because one half of the interface is a cellular protein. The interaction appears amenable to small molecule inhibition because of the excellent structural definition that exists, the relatively small size and depth of the IN binding pocket, and the susceptibility of binding to abrogation by single amino acid changes (Cherepanov et al. 2005a). Cell-based and recombinant protein interaction-based screening systems have been used for such screening (Busschots et al. 2009; Du et al. 2008; Hou et al. 2008). Compounds identified have been reported to have anti HIV-1 activity, providing additional support for the concept that LEDGF/p75 plays a fundamental role in HIV-1 infection (Du et al. 2008; Hou et al. 2008).

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Implications of Nef: Host Cell Interactions in Viral Persistence and Progression to AIDS

Nathalie J. Arhel and Frank Kirchhoff

Contents

Abstract The HIV and SIV Nef accessory proteins are potent enhancers of viral persistence and accelerate progression to AIDS in HIV-1-infected patients and non-human primate models. Although relatively small (27–35 kD), Nef can interact with a multitude of cellular factors and induce complex changes in trafficking, signal transduction, and gene expression that together converge to promote viral replication and immune evasion. In particular, Nef recruits several immunologically relevant cellular receptors to the endocytic machinery to reduce the recognition and elimination of virally infected cells by the host immune system, while simultaneously interacting with various kinases to promote T cell activation and viral replication. This review provides an overview on selected Nef interactions with host cell proteins, and discusses their possible relevance for viral spread and pathogenicity.

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1 Introduction

The year 2008 has witnessed a sober reassessment of the state-of-the-art in AIDS (acquired immune deficiency syndrome) research since the discovery of the human immunodeficiency virus (HIV) 25 years ago. Despite many seminal advances in the field, HIV remains an elusive target for eradicating treatment or effective vaccination. Key to the elusive nature of the virus is its ability to evade host or treatment pressures through genetic hypervariability, its integration into the host cell genome, and its persistence in latent reservoirs (Stevenson 2003). In addition, the virus benefits from its ability to interact with components of the infected cell and subvert the cell trafficking, signal transduction, and transcriptional machineries to its advantage: to facilitate virus infection, increase the production of fully infectious progeny viruses, avoid recognition by the immune system, and establish latency.

The HIV and SIV Nef accessory proteins are particularly adept at interacting with their host cell and inducing complex changes that promote efficient virus spread and persistence. Although originally named on the mistaken understanding that it negatively regulates virus transcription (Nef is an acronym for *ne*gative *f*actor), Nef acts as a potent viral enhancer of primate lentiviral persistence.

Particular interest in Nef lies in early observations that correlate its expression with progression to AIDS. Rhesus monkeys infected with simian immunodeficiency virus (SIV) carrying a large deletion in the *nef* gene showed low viral loads and did not progress to simian AIDS (Kestler et al. 1991). Similarly, defective *nef* genes have been detected in several long-term survivors of HIV-1 infection with normal CD4+ T cell counts and very low viral loads (Kirchhoff et al. 1995; Deacon et al. 1995; Mariani et al. 1996; Salvi et al. 1998). As a result, much effort has been undertaken to identify the mechanisms by which Nef promotes viral persistence and accelerates progression to AIDS and to define the molecular interactions with the host cell that are involved.

Nef is unique to primate lentiviruses and present in all HIV and SIV strains characterized to date. Nef proteins have a molecular weight ranging from 27 to about 35 kD and are encoded by sequences extending from the 3' end of the viral envelope (*env*) into part of the 3' long-terminal repeat (LTR). Although the amino acid sequence and length of HIV-1 Nef is variable, several distinct conserved functional domains have been identified (Geyer et al. 2001). HIV-1, HIV-2 and SIV Nef proteins frequently share only about 30% amino acid identity, but most structural properties and the majority of functions are well preserved (reviewed in Kirchhoff et al. 2008).

The best conserved feature of Nef is its N-terminal myristoylation signal, required for membrane binding and critical for most Nef activities. The flexible myristoylated N-terminal anchor is followed by a flexible loop containing a conserved acidic cluster involved in Nef effects on trafficking and a proline-rich region, conserved in HIV-1 but not in HIV-2 nor some SIVs. The latter is involved in SH3-domain binding of tyrosine kinases and Nef effects on signaling (Saksela et al. 1995; Renkema and Saksela 2000; Collette et al. 2000). Other important interfaces for interaction with

cellular proteins are a highly ordered and well-conserved globular core domain and a flexible loop near the C-terminus of Nef, which contains a dileucine-based sorting motif that functions as an endocytosis signal (Craig et al. 1998). The remarkable ability of Nef to interact efficiently with multiple cellular partners may be due to the high degree of flexibility within its folded structure (Geyer and Peterlin 2001).

According to the current literature, Nef may interact with as many as 60 cellular factors and affect the function of more than 180 proteins. These are listed in the web site of the National Library of Medicine: http://www.ncbi.nlm.nih.gov/RefSeq/HIVInteractions/nef.html. All these interactions and effects could potentially be advantageous for the virus. However, most of them remain to be confirmed in virally infected primary T cells or macrophages. Moreover, the underlying mechanisms and the role in viral persistence and progression to AIDS have only been examined for a small proportion of these Nef–host cell interactions.

The aim of this review is to expose some of the reported interactions of Nef with its host cell, and to discuss their possible implications for viral persistence and progression to AIDS. Although an attempt has been made to categorize Nef–host cell interactions into those that facilitate immune evasion and those that directly enhance viral spread, it should be noted that most Nef interactions with its host cell cooperate to ensure the production of progeny virions and to generate an environment that facilitates viral spread.

2 Interactions Facilitating Viral Immune Evasion

2.1 CD8+ T Cell Evasion: MHC-I Down-Modulation

Like many other invading pathogens, HIV and SIV have developed multiple strategies to avoid elimination by the host immune system. One mechanism to reduce immune recognition of infected cells involves down-modulation of the major histocompatibility complex I (MHC-I) (Kerkau et al. 1989; Scheppler et al. 1989). The Nef protein is critical for this ability of HIV and SIV (Schwartz et al. 1996) and sufficient to protect infected primary T cells from killing by CD8+ cytotoxic T lymphocytes (CTL) (Collins et al. 1998). Although the exact mechanisms are still under investigation, consensus evidence indicates that Nef expression in infected cells leads to accumulation of MHC-I in the trans-golgi network (TGN) where it is redirected to TGN-associated endosomal compartments and to lysosomes for degradation (Roeth and Collins 2006). The literature supports two pathways for Nef-mediated down-modulation of MHC-I involving accelerated endocytosis of MHC-I from the plasma membrane (Greenberg et al. 1998a; Le Gall et al. 2000; Piguet et al. 2000) and disruption of normal anterograde transport of MHC-I from the Golgi to the cell surface (Le Gall et al. 2000; Swann et al. 2001; Kasper and Collins 2003; Roeth et al. 2004) (Fig. 1). The prevalence of one mechanism over the other may differ between T cell and non-T cell lines (Kasper and Collins 2003).

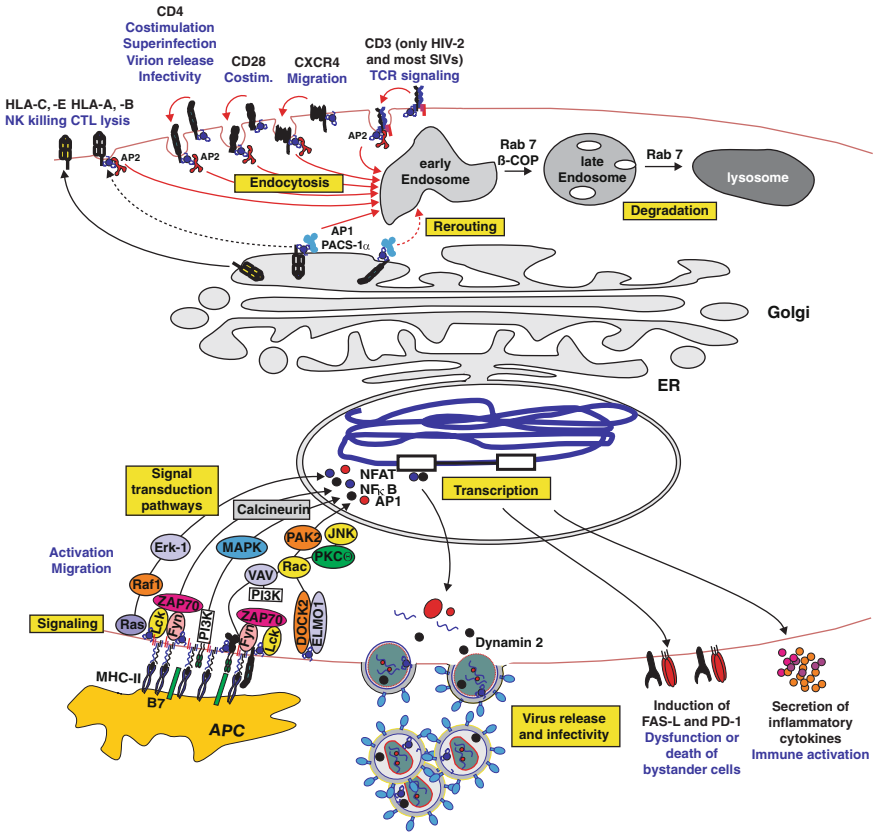

Fig. 1 Schematic presentation of selected Nef functions and interactions in infected T cells. Nef interacts with HLA molecules, CD4, CD28, CXCR4, and CD3 to reduce their surface expression on infected CD4+ T cells, thereby reducing CTL lysis, suppressing cell migration, facilitating virus release and modulating signal transduction by the immunological synapse. Furthermore, Nef interacts with a variety of cellular kinases and other factors to modulate downstream signaling events. HIV-1 Nef promotes the activation of transcription factors, such as NF-AT, NF-κB, and AP-1, to induce the efficient transcription of the viral LTR promoter and of various cellular genes, e.g., those encoding for inflammatory cytokines, activation markers and death receptors. Furthermore, Nef down-modulates CD4 to promote virus release and to prevent superinfection and enhances virus replication and virion infectivity to directly promote virus spread. Please note

Nef interacts directly with the cytoplasmic tail of MHC-I, although this interaction is weak and may be transient or stabilized by other factors (Williams et al. 2002). An N-terminal α helical region, the polyproline repeat, and the acidic domain in HIV-1 Nef are involved in MHC-I down-modulation (Greenberg et al. 1998a; Mangasarian et al. 1999). Notably, however, some HIV-1 and SIV *nef* alleles lead to efficient MHC-I down-modulation although they contain alterations in these residues (Specht et al. 2008).

For rerouting of MHC-I to the lysosomes for degradation, Nef recruits the clathrin adaptor complex AP1 via its μ1 subunit and subsequently ß-COP, which are both implicated in endosomal trafficking and transport through the early secretory pathway, to the cytoplasmic tail of MHC-I (Roeth et al. 2004; Noviello et al. 2008; Schaefer et al. 2008). It is a matter of debate whether accelerated endocytosis of MHC-I from the cell surface requires an interaction between Nef and a coat protein called PACS-1 (phosphofurin acidic cluster sorting protein-1) (Piguet et al. 2000; Lubben et al. 2007; Blagoveshchenskaya et al. 2002). It has also been suggested that Nef-mediated endocytosis of MHC-I involves the ARF-6 (ADP-ribosylation factor 6) endocytic pathway (Blagoveshchenskaya et al. 2002), which is normally involved in the clathrin-independent trafficking of MHC-I between the plasma membrane and endosomal compartments, and PI3K (phosphoinositide 3-kinase) (Swann et al. 2001; Hung et al. 2007). However, the significance of these mechanisms requires further study (Larsen et al. 2004), and the exact mechanism of MHC-I down-modulation is still under investigation (Atkins et al. 2008; Schaefer et al. 2008).

It has been clearly shown that down-modulation of MHC-I by Nef contributes to the ability of SIV to avoid CTL responses in vivo. A point mutation of Y223F near the C-terminus of SIVmac Nef that selectively disrupts the effect on MHC-I reverted within 4 weeks after infection, shortly after the peak of the CTL response (Münch et al. 2001). Furthermore, macaques infected with SIVmac mutants containing difficult-to-revert Nef mutations specifically eliminating MHC-I down-modulation exhibited higher levels of CD8+ T cell responses and showed compensatory mutations in Nef that restored MHC-I down-modulation (Swigut et al. 2004). In support of a relevant role of Nef-mediated down-modulation of MHC-I for viral immune evasion and effective persistence in HIV-1-infected individuals, it has been shown that *nef* alleles from non-progressor perinatally infected children were less efficient in MHC-I down-modulation than those from rapid progressors (Casartelli et al. 2003). The efficiency of CTL responses in infected patients seems to exert a selective pressure on the ability of Nef to down-modulate MHC-I (Carl et al. 2001), and the ability of Nef to down-modulate MHC-I correlated positively with the breadth of the HIV-1-specific CTL response (Lewis et al. 2008). Finally, unusually strong CTL responses have been detected in individuals infected with Nef defective HIV-1 strains (Dyer et al. 1999). Thus, together with the high variability of HIV and SIV leading to the emergence of CTL escape variants and other factors, the ability of Nef to down-modulate MHC-I provides an explanation why CTL responses are usually unable to effectively control viral replication.

2.2 NK Cell Evasion: Selective Down-Modulation of HLA-A and –B

Regulation of cell surface expression of MHC-I is a mechanism used by a number of viruses to evade recognition by CTL (for example herpes viruses, papillomaviruses, HIV). As a counteractive measure, natural killer (NK) cells can gage the

level of MHC-I expressed on cell surfaces and preferentially lyse cells that lack MHC-I. Under normal conditions, NK cell cytotoxicity is blocked by specific recognition of MHC-I molecules by inhibitory NK cells receptors (iNKRs). Reduced expression of MHC-I molecules on the surface of infected cells results in reduced engagement of iNKRs and triggers NK-cell-mediated cytolysis. Thus, down-modulation of MHC-I by HIV-1 Nef (among other viral proteins) should expose infected cells to lysis by NK cells. However, evidence shows that HIV-1 Nef down-modulates class I MHC proteins selectively, reducing surface expression of HLA-A and -B, which are recognized by the majority of CTL, but not of HLA-C and -E, which are recognized by iNKRs (LeGall et al. 1998; Cohen et al. 1999). Selective down-modulation of class I proteins by Nef is accounted for by differences in the cytoplasmic tail of MHC-I molecules, and residues Y320, A323, and/or D327 important for Nef-dependent down-modulation of HLA-A and -B are mutated in HLA-C and -E (LeGall et al. 1998; Cohen et al. 1999). The ability of Nef to selectively down-modulate HLA-A and -B reduces the ability of NK cells to kill HIV-infected cells despite reduced MHC-I surface molecules (Bonaparte and Barker 2003) and is a well conserved property among primate lentiviruses (Specht et al. 2008).

Nevertheless, studies have demonstrated that 30% or more of NK cells present in the peripheral blood do not express any receptors able to bind to HLA-C or -E (Bonaparte and Barker 2004; Mavilio et al. 2003), and that these NK cells have a greater ability to kill CD4+ T cells infected with HIV in which HLA-A and -B are decreased (Bonaparte and Barker 2004). It is therefore very likely that HIV-mediated perturbation of NK cell function (through aberrant expression and function of inhibitory receptors, defective cytokine production, and preferential expansion of NK cell subsets) also plays a role in the decreased ability of NK cells to kill HIV-infected cells. A recent work showed that Nef-pulsed dendritic cells (DCs) modulate NK cell effector function, inhibiting cytotoxic NK cell function while stimulating the pro-inflammatory cytokine-producing NK fraction (Quaranta et al. 2007).

Interestingly, although HLA-C presents self-peptides to NK cells to inhibit cell killing, it also has the ability to present viral peptides to CTL and thus restrict HIV-1 infection (Goulder et al. 1997; Adnan et al. 2006). Moreover, a single nucleotide polymorphism (SNP) upstream of the HLA-C locus associates with increased HLA-C expression and lower viral load at set-point (Fellay et al. 2007). Therefore, the resistance of HLA-C to Nef-mediated down-modulation could offer a promising opportunity for vaccine developments targeting HLA-C-restricted CTL responses.

2.3 Restricting MHC-II Antigen Presentation: Ii up-Modulation

MHC class II molecules, expressed chiefly on B cells, macrophages, and DCs, are specialized in exogenous antigen presentation to CD4+ T cells and are synthesized in the endoplasmic reticulum (ER) together with the invariant chain (Ii or CD74). Ii caps the MHC-II peptide binding site during its transport to endosomal compartments, where acidic pH leads to proteolytic cleavage of Ii thus allowing loading of

appropriate peptides on the MHC-II groove and subsequent transport of mature MHC-II-antigen complexes to the cell surface (reviewed by Rocha and Neefjes 2008). Interestingly, a fraction of MHC II-Ii complexes reaches endosomes not directly from the ER but rather by rapid internalization from the cell surface (Roche et al. 1993). It seems that this MHC-II-Ii fraction shuttles between the endosomes and plasma membrane before peptide loading in endosomes, undergoing repeated cycles of surface delivery and rapid internalization (Lindner 2002) via adaptor protein 2 (AP-2)-dependent endocytosis (Dugast et al. 2005).

Expression of HIV-1 Nef may perturb MHC-II-restricted antigen presentation by up-modulation of Ii cell surface expression (Stumptner et al. 2001; Stumptner et al. 2003). Indeed, stable expression of Ii hampers peptide presentation (Roche et al. 1992; Bertolino and Rabourdin 1996), and Nef-mediated up-modulation of surface Ii might therefore contribute to impaired helper T cell responses observed in AIDS patients (Norris and Rosenberg 2002). Nef interacts directly with both AP-2 and Ii in a dileucine-dependent manner (Toussaint et al. 2008), and it has been suggested that Nef may up-modulate Ii because both compete for AP-2 binding (Mitchell et al. 2008). More recently, however, it has been suggested that Ii up-modulation by Nef is due to impaired AP-2-mediated endocytosis rather than direct competition for AP-2 (Toussaint et al. 2008). Efficient Nef-mediated up-modulation of surface Ii is a well conserved property of primate lentiviruses (Schindler et al. 2003) and can be observed at very low levels of Nef expression (Stumptner et al. 2001). Significant Nef-mediated up-regulation of Ii was also observed in HIV-1-infected macrophages (Schindler et al. 2007a). Further studies are required to obtain definitive proof but our current knowledge suggests that the effect of Nef on Ii represents an important viral immune evasion mechanism in vivo in HIV-1-infected individuals.

2.4 Modulation of Signaling from the Cell Surface

2.4.1 Down-Modulation of TCR-CD3

The T cell receptor (TCR) is a heterodimeric protein consisting of α and β chains. Its function as a receptor is entirely dependent on its association with the CD3 complex, comprised of four transmembrane protein chains: γ, δ, ε, and ζ (zeta), which mediates signal transduction after antigen recognition. All four CD3 chains have intracellular immunoreceptor tyrosine-based activation motifs (ITAM) that become phosphorylated upon receptor ligation, enabling their interaction with cytoplasmic signaling proteins.

Early work showed that the central region of SIVmac239, SIVsmm, and HIV-2 Nef can directly associate with the TCR zeta chain and that this interaction correlates with their ability to down-modulate CD3 from the cell surface (Bell et al. 1998; Howe et al. 1998; Schaefer et al. 2002). Although Nef's endocytic motifs do not appear to be involved, TCR-CD3 endocytosis occurs via the AP-2 clathrin adaptor pathway (Schaefer et al. 2002; Swigut et al. 2003). HIV-1 Nef, on

the other hand, is unable to induce CD3 down-modulation. One study reported that HIV-1 Nef maintains some ability to interact with TCRζ (Xu et al. 1999), whereas several others found that only SIV and HIV-2, but not HIV-1, Nef proteins show this association (Bell et al. 1998; Howe et al. 1998; Schaefer et al. 2002).

To date, SIVs have been detected in about 40 African non-human primate species (reviewed in Pandrea et al. 2008), and phylogenetic studies indicate that SIVs from two of these species, chimpanzees and sooty mangabeys, have been transmitted to humans and gave rise to HIV-1 and HIV-2, respectively (Hahn et al. 2000). A recent study of a wide range of primate lentiviral Nef proteins revealed that the ability of SIVmac239, SIVsmm, and HIV-2 Nefs to down-modulate CD3 is shared by the great majority of primate lentiviruses, and blocks the responsiveness of infected T cells to activation (Schindler et al. 2006). Only Nef proteins from HIV-1 and its SIV counter parts failed to down-modulate TCR-CD3 and to suppress T cell activation and programmed cell death. The structural basis for this fundamental difference in the ability of primate lentiviral Nef proteins to modulate CD3 remains elusive since the core region of Nef that is critical for the interaction with the TCR zeta chain (Schaefer et al. 2002; Swigut et al. 2003) is generally well conserved.

While HIV-1 infection in humans is typically associated with high levels of immune activation and progressive CD4+ T cell depletion, natural SIV infections, such as by SIVsmm in sooty mangabeys and by SIVagm in African green monkeys, do not bring about generalized chronic immune activation or disease. The effect of HIV-1 Nef versus SIVsmm/SIVagm Nef on CD3 surface expression and T cell activation suggests a protective role for SIV Nef against immune activation (Schindler et al. 2006), and TCR-CD3 down-modulation may help the natural hosts of SIV to maintain stable numbers of CD4+ T cells (Schindler et al. 2008). It is interesting, however, that even profound depletion of CD4+ T cells is not associated with disease in naturally infected sooty mangabeys, and it has been proposed that attenuated immune activation following acute viral infection protects these animals from progressing to AIDS (Gordon et al. 2007; Milush et al. 2007).

2.4.2 Down-Modulation of the CD4 Receptor

CD4 assists signal transduction and T cell activation after TCR ligation. It is involved in clustering at the immunological synapse and binding to MHC-II on antigen presenting cells (APCs). CD4 is also the primary receptor for HIV and SIV entry. In support of a critical role for virus replication, three HIV-1 encoded proteins, Vpu, Env, and Nef, ensure that CD4 molecules are kept away from the cell surface upon HIV-1 infection.

It has long been known that Nef down-modulates CD4 (Garcia and Miller 1991) and the underlying mechanisms have been extensively investigated (reviewed by Lama 2003; Roeth and Collins 2006). Notably, Nef uses distinct surfaces to down-regulate MHC-I, CD3 and CD4 from the cell surface. Nef-mediated down-modulation of CD4 involves a dileucine motif in the membrane-proximal cytoplasmic domain of CD4 (Aiken et al. 1994) and an intact dileucine motif, two diacidic

motifs, and a hydrophobic pocket in Nef (Mangasarian et al. 1999; Bresnahan et al. 1998; Craig et al. 1998; Piguet et al. 1998; Lindwasser et al. 2008).

Expression of Nef leads to the endocytosis of surface CD4 by recruitment of the AP-2 clathrin adaptor complex, which directs the receptor to lysosomes for degradation (Aiken et al. 1994; Piguet et al. 1998; Greenberg et al. 1998b; Bresnahan et al. 1998; Craig et al. 1998). Recent findings suggest that ubiquitination of Nef may play a role in CD4 down-regulation (Jin et al. 2008). In contrast to Nef, Vpu and Env are expressed during the late stage of the viral life cycle and interfere with the transport of newly synthesized CD4 to the cell membrane rather than down-modulating CD4 molecules already present at the cell surface (Malim and Emerman 2008). However, intracellular retention mechanisms may also contribute to Nef-mediated down-modulation of CD4 (Rose et al. 2005). In either case, down-modulation of CD4 by Nef is highly effective and most likely important for efficient viral persistence in vivo (Brenner et al. 2006).

The role of CD4 in mediating T cell activation following receptor ligation suggests that down-modulation of CD4 by Nef may be advantageous for the virus because it limits T cell activation. However, since essentially all primate lentiviral Nef proteins down-modulate CD4 with high efficiency, while having very different effects on T cell responsiveness to activation (Schindler et al. 2006), it is likely that other consequences of low CD4 cell surface levels discussed Sect. 3.2.3 are of higher physiological relevance.

2.4.3 Down-Modulation of Co-Stimulatory Molecules

The complete activation of a T cell following antigen-MHC recognition requires a second co-stimulatory signal, provided for predominantly by the CD28 receptor on T cells and the B7 (CD80/CD86) ligand on APCs. Absence of this co-stimulatory signal results in the suppression of the immune response, and induces antigen-specific tolerance and T cell anergy.

In T cells, the Nef proteins of SIVmac239, SIVsmm and HIV-2, and to a lesser extent of HIV-1, down-modulate surface CD28 expression by binding to the cytoplasmic domain of CD28 and accelerating its endocytosis via the AP2 clathrin adaptor (Swigut et al. 2001; Bell et al. 2001; Münch et al. 2005). Thus, the mechanism is similar to CD4 and CD3 down-modulation, although the ability of Nef to down-regulate CD28 can be genetically separated from these functions (Swigut et al. 2001).

In APCs, the HIV-1 Nef protein has been shown to redirect the co-stimulatory molecules CD80 and CD86 away from the surface by binding to their cytoplasmic tails and rerouting them to the Golgi apparatus by a clathrin- and dynamin-independent actin-based endocytic pathway that seems to involve the activation of c-src and Rac (Chaudhry et al. 2005, 2007, 2008). By down-modulating co-stimulatory molecules, as well as other surface receptors, HIV-1 Nef manipulates the functional interaction between T cells and APCs (discussed further in Sect. 2.4.5) to impede the mounting of an effective immune response against the virus.

2.4.4 CXCR4 Down-Modulation

In addition to CD4, chemokine receptors are also essential as co-receptors for HIV-1 entry into target cells (Deng et al. 1996; Feng et al. 1996). Transmitted HIV-1 strains, and those that persist during chronic infection, generally use the CCR5 co-receptor, while HIV-1 variants that utilize CXCR4 or are dual tropic are observed in about 50% of all AIDS patients. HIV-2, on the other hand, is more promiscuous in its use of chemokine co-receptors, and most SIVs use only CCR5, but not CXCR4, as entry co-factors (Marx and Chen 1998).

Some HIV-2 and SIV Nef proteins effectively down-modulate CXCR4 to inhibit T cell migration to the CXCR4 natural ligand, the chemokine stromal-derived factor 1 (SDF-1), whereas HIV-1 Nefs display only weak activity (Choe et al. 2002; Hrecka et al. 2005; Venzke et al. 2006; Wildum et al. 2006). Similar to its effects on CD4, CD28, and CD3, Nef seems to down-modulate CXCR4 by recruiting it to sites of the AP-2 clathrin-adaptor-dependent endocytosis (Hrecka et al. 2005). Notably, HIV-1 and SIVmac239 Nefs also inhibit chemotaxis by binding to the guanine exchange factor DOCK2-ELMO1, a key activator of the Rho GTPase Rac in antigen- and chemokine-initiated signaling pathways (Janardhan et al. 2004; Hrecka et al. 2005).

The chemotaxis of T cells mediated by SDF-1 through CXCR4 is essential for trafficking of T cells during development and the initiation of immune responses, with recruitment of lymphocytes to lymphoid tissues. CXCR4 down-modulation also reduces superinfection of infected cells (Venzke et al. 2006; Wildum et al. 2006). However, this effect is obviously of limited relevance since the primate lentiviruses that down-modulate CXCR4 with the highest efficiency do not utilize it as an entry cofactor. Nef-mediated impairment of T cell chemotaxis, with or without affecting CXCR4 surface expression levels, likely reduces contact with APCs and contributes to immune evasion.

2.4.5 Effects on the Immunological Synapse

As outlined above, Nef most likely forms trimeric complexes with AP-2 and various receptors, i.e., CD3, CD4, CD28, and CXCR4, to target them for endocytosis via AP-2 clathrin adaptors and subsequent degradation in lysosomes. Notably, Nef can use distinct surfaces to interact with AP-2 and to recruit different receptors. As a consequence, all these Nef activities are genetically separable (Akari et al. 2000; Craig et al. 1998; Iafrate et al. 1997; Swigut et al. 2000, 2001), and can (at least in part) be independently adapted to the specific host environment to optimize viral spread (Carl et al. 2001; Patel et al. 2002). Another interesting aspect is that some conserved functions are mediated by different domains in Nef proteins derived from various lineages of HIV and SIV (Swigut et al. 2000; Bresnahan et al. 1999; Hua and Cullen 1997; Lock et al. 1999) suggesting that they evolved independently during primate lentiviral evolution.

Many of the receptors modulated by Nef are involved in the formation of the immunological synapse (Fig. 1), which requires the interaction of the TCR-CD3 complex on T cells with the Ag/MHC-II complex expressed by APCs and of co-stimulatory and adhesion molecules on both cells. As described above, HIV-1 Nefs interfere with this process by modulating MHC-II, Ii, CD4, and, to some extent, CD28. It has been suggested that the ability of HIV-1 Nef to deregulate the function of the immunological synapse may reduce T cell activation and help to prevent damaging high levels of immune activation (Fackler et al. 2007). It is obvious, however, that HIV-1 Nef does a poor job in protecting the infected host because HIV-1 infection is almost invariably associated with high levels of chronic immune activation and progression to AIDS in infected humans. In comparison, HIV-2 and most SIV Nefs impair synapse formation more severely because they also down-regulate TCR-CD3 and are usually more effective in down-regulating CD28 and CXCR4 (Hrecka et al. 2005; Schindler et al. 2006). Cell cultures infected with viruses expressing HIV-1 Nefs are characterized by high levels of activation and apoptosis, whereas PBMCs expressing HIV-2, SIVsmm, or SIVagm Nefs show low levels of activation and cell death. The distinct characteristics of these in vitro cultures are similar to the documented different characteristics of HIV-1, HIV-2, and SIVsmm or SIVagm infection in vivo (Pandrea et al. 2008). Altogether, these findings suggest that HIV-1 Nefs dysregulate the functional interaction of infected T cells with APCs, whereas those of most other primate lentiviruses may disrupt it entirely. The possible importance of this differential ability of primate lentiviruses to disrupt the immunological synapse has been addressed in a recent review (Kirchhoff et al. 2008).

3 Interactions Supporting SIV and HIV Replication

Besides performing multiple functions that facilitate viral immune evasion, Nef also modulates the activation status of the infected T cells and enhances the infectivity of progeny virions to promote viral replication (Fig. 1). As described in the previous chapters, Nef down-modulates several receptors to dysregulate or disrupt antigen-specific signaling by the TCR-CD3 complex. However, HIV-1 Nef proteins also interact with numerous cellular factors to increase the responsiveness of virally-infected T cells to stimulation. The induction of downstream signaling pathways leads to the activation of transcription factors that increase the expression of the HIV-1 provirus as well as of many cellular genes. In addition, HIV and SIV Nefs also act at the latest stage of the virus life cycle by enhancing the infectivity of progeny virions. The effect of Nef on viral replication is most pronounced in primary T cell cultures, particularly if these are infected prior to stimulation (Miller et al. 1994; Chowers et al. 1994; Schwartz et al. 1995) and is most likely dependent on a variety of Nef effects, such as modulation of signal transduction pathway, induction of transcription factors and cellular activation, CD4 down-modulation, and enhancement of virion infectivity.

3.1 Enhancement of Virus Production

3.1.1 Subversion of T Cell Signaling Pathways

Although HIV-1 Nef down-modulates CD4 and (to some extent) CD28, it enhances the responsiveness of T cells to stimulation (Skowronski et al. 1993; Schrager and Marsh 1999; Fenard et al. 2005; Fortin et al. 2004; Wang et al. 2000) and interacts with a large number of cellular signaling proteins (reviewed in Greenway et al. 2003; Renkema and Saksela 2000). While the physiological relevance of these interactions is often poorly understood, they may lead to the induction of various signaling pathways and the activation of transcription factors (Maninnen et al. 2000; Wang et al. 2000; Fortin et al. 2004) that enhance viral and cellular gene expression (Arendt and Littman 2001; Simmons et al. 2001). Thus, HIV-1 Nef may partly uncouple T cell activation from the "normal" antigen-dependent interaction with APCs to increase virus production (Fig. 1). Notably, Nef can manipulate the infected cells very rapidly, as it is abundantly produced early during the viral life cycle. Moreover, it has been shown that selective transcription of *nef* and *tat* in quiescent cells can increase T cell activation and viral replication even prior to viral integration into the host cell genome (Wu and Marsh 2001).

It is well established that the PxxP motif in HIV-1 Nef interacts with the SH3 domains of a number of tyrosine kinases, including Lck, Fyn, Hck, Lyn, and c-Src itself (Saksela et al. 1995; Greenway et al. 1996; Collette et al. 1996; Baur et al. 1997). Lck and Fyn are the first recruited kinases upon T cell receptor ligation and Lck also mediates signaling from CD4, CD8, and IL-2 receptors. In yeast, Nef activated the kinase activities of Hck, Lyn, and c-Src but not of Fyn, Lck, or Yes (Trible et al. 2006). It is conceivable that the well-conserved albeit frequently low-affinity interactions of HIV-1 Nef with various Src kinases contribute to its effect on signal transduction and T cell activation (Renkema and Saksela 2000). It has also been reported that Nef interacts with the SH3 domain of VAV, a Rac1 guanine nucleotide exchange factor (GEF), possibly to trigger the JNK/SAPK signaling cascade and to induce rearrangements of the cytoskeleton (Fackler et al. 1999). However, another study using a comprehensive proteomics approach to directly identify Nef interacting proteins did not detect several of the proteins mentioned above including VAV, but suggests that binding of Nef to the DOCK2–ELMO1 complex, a key activator of Rac, plays a major role in its ability to inhibit chemotaxis and to promote T cell activation (Janardhan et al. 2004).

Nef can be phosphorylated on serine residues and associates with a number of serine and threonine kinases (Renkema and Saksela 2000). Particularly, the interaction of Nef with the p21-activated serine–threonine kinase 2 (PAK-2) has been extensively investigated (Sawai et al. 1994; Lu et al. 1996). PAK-2 is usually activated by Rac1 and Cdc42 and involved in the regulation of several cellular processes, such as cytoskeleton rearrangement, cell morphology, motility, apoptosis, and gene transcription (Daniels and Bokoch 1999; Chu et al. 2004).

It has been proposed that the interaction of PAK-2 with Nef plays a role in T cell activation, viral replication, apoptosis, and progression to AIDS (Lu et al. 1996; Wiskerchen and Cheng-Mayer 1996; Fackler et al. 2000; Linneman et al. 2002; Chu et al. 2004). However, data obtained using Nef mutants selectively impaired in this interaction (Agopian et al. 2006) failed to detect definitive effects on T cell activation, viral replication, and apoptosis (Schindler et al. 2007b). Further effects of HIV-1 Nef involve the modulation of additional effectors and signaling pathways such as protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) families (Lu et al. 1996; Smith et al. 1996; Greenway et al. 1996; Yang and Gabuzda 1999; Wolf et al. 2008), interactions with the Ras-Raf-MAP kinase pathway (Maninnen et al. 2000; Hodge et al. 1998), and modulation of calcium signaling (Skowronski et al. 1993; Baur et al. 1994; Maninnen and Saksela 2002).

The biological consequences of most of these Nef interactions are still poorly understood. Taken together, however, our current knowledge suggests that HIV-1 Nef recruits various signaling proteins to the inner cell membrane and lipid rafts and, by modulating their catalytic activity and bringing them into close proximity, primes T cells for activation. In fact, it has been shown Nef alone can trigger T cell activation signaling pathways, inducing a transcriptional program that is highly similar to that of anti-CD3 T cell activation at least in Jurkat T cells (Simmons et al. 2001). In primary HIV-1 infected T cells, however, expression of Nef is usually not sufficient to induce T cell activation and viral replication but rather increases their responsiveness to stimulation (Djordjevic et al. 2004; Fenard et al. 2005; Fortin et al. 2004; Wang et al. 2000). The exceptions are SIV *nef* alleles that contain an additional SH2 domain and are highly active in enhancing T cell activation and associated with an acute lethal disease in infected rhesus monkeys (Du et al. 1995). Typically, Nef expression in HIV-1-infected T cells increases the induction of various transcription factors, such as NFAT, NFκB, and AP1 Activation Protein 1 upon stimulation with various agents (Wang et al. 2000; Manninen et al. 2000; Manninen et al. 2001; Fortin et al. 2004). Since HIV-1 requires T cell activation for efficient replication, Nef's subversion of T cell signaling pathways increases the transcriptional activity of the viral LTR promoter to promote efficient viral replication (Fig. 1).

Although the polyproline motif mediating interactions between Nef and Src kinases is highly conserved between HIV-1 isolates, three amino acid substitutions in the HIV-2 and SIVmac Nef proteins result in the targeting of different Src kinases (Collette et al. 2000; Karn et al. 1998). To date, relatively little is known about the interaction of SIV and HIV-2 Nefs with cellular kinases and their effects on signal transduction pathways, T cell activation, and virus replication. Further studies seem warranted since differences in the ability of Nef to modulate cellular signaling pathways may affect the levels of immune activation and hence the development of immunodeficiency. The observation that an additional SH2 domain in Nef is associated with acute disease in SIV-infected rhesus macaques (Du et al. 1995) represents a particular striking example for the importance of cellular activation in the clinical outcome of primate lentiviral infections.

3.1.2 Activation of Viral and Cellular Transcription

By modulating the signal transduction machinery Nef augments the expression of its own genome and of a large number of cellular genes (reviewed by Arendt and Littman 2001). Gene expression profiling studies showed that Nef induces a transcriptional program in Jurkat T cells that is highly similar (but not identical) to that of anti-CD3 T cell activation and partly dependent on ZAP-70 and the zeta chain of the TCR (Simmons et al. 2001). In particular, Nef induces transcription factors that transactivate the HIV-1 LTR promoter [NFAT, NFκB, IRF-1/2 (interferon regulatory factor), c-fos, Jun-D] and several cellular co-factors of viral replication, such as the Tat co-factor CDK9, the transcription elongation factor Tat-SF1, the transactivator NFIB-2, and the spliceosome component U1 snRNP (small nuclear ribonucleic protein) (Simmons et al. 2001). Nef also induces the expression of cytokines and chemokines which are thought to favor viral replication (IL-2 IL-4, TGFβ, MIP1α, MIP1β) (Arendt and Littman 2001). At least in HeLa cells, many effects on cellular gene expression were dependent on an intact PxxP motif in HIV-1 Nef (Shaheduzzaman et al. 2002). Relatively little is known about the effect of other primate lentiviral Nef proteins on the transcription of cellular genes, but one study reported that SIVmac Nef down-modulates genes associated with antigen presentation and induces the transcription of genes involved in cell survival and in the synthesis of membrane glycolipids and phospholipids (Ndolo et al. 2006). In contrast to the HIV-1 Nef (Simmons et al. 2001), that of SIVmac did not significantly modulate genes involved in T cell activation (Ndolo et al. 2006). Although all these findings need to be confirmed in virally infected primary T cells, they indicate that Nef exerts profound effects on the transcriptional response of infected T cells to favor viral replication. Notably, HIV-1 Nef also induces the transcription of the genes encoding T cell activation markers and of death receptors, such as the programmed death receptor 1 (PD-1) and the Fas ligand (Xu et al. 1999; Muthumani et al. 2008). These effects of Nef may play a role in the pathogenesis of AIDS since they may cause the death or dysfunction of uninfected bystander CD8+ T cells.

3.1.3 CD4 Down-Modulation

As discussed in Sect. 2.4.2, down-modulation of CD4 is one of the hallmarks of primate lentiviral infections and the best characterized Nef function. However, it is still not well understood which of its multiple consequences is most critical for efficient viral spread in vitro and in vivo (reviewed by Lama 2003; Kirchhoff et al. 2008). It has been reported that cell surface CD4 interferes with viral particle release by interacting with the HIV-1 envelope protein present on budding virions, and that Nef-mediated down-modulation of surface CD4 relieves progeny virions from this block (Arganaraz et al. 2003; Cortes et al. 2002; Lama et al. 1999; Ross et al. 1999). Furthermore, Nef-mediated down-modulation of CD4 may increase the incorporation of functional Env glycoproteins into progeny virus (Lama et al. 1999; Argañaraz et al. 2003). In support of a relevant role in virus replication, it has been

shown that the potency of CD4 down-modulation correlates with the efficiency of viral replication in primary lymphocyte cultures in human lymphoid tissue ex vivo (Glushakova et al. 2001; Lundquist et al. 2002). Moreover, down-modulation by HIV-1 of its own entry receptor reduces superinfection (Benson et al. 1993; Wildum et al. 2006), which would otherwise likely lead to cell death before virus production is accomplished. However, effective down-modulation of CD4 is not sufficient for effective replication in primary T cells (Saksela et al. 1995). To our current knowledge, at least three HIV-1 Nef activities, i.e., CD4 down-modulation, T cell activation, and enhancement of virion infection, contribute to efficient viral replication. Some seeming discrepancies may be due to the fact that the relative importance of these Nef functions for viral replication varies depending on the activation status and CD4 expression level of the target cells. CD4 down-modulation is most likely particularly important for the efficient release of fully infectious progeny virions from primary T cells since they usually express high levels of this receptor (Pham et al. 2004).

3.1.4 Enhancement of Viral Transfer from DCs to T Cells

Although the main target cells for HIV and SIV are CD4+ T cells and macrophages, it has been proposed that immature DCs present in the mucosa can capture virus particles and, following maturation and migration towards secondary lymphoid organs, transmit the virus to T cells (Granelli-Piperno et al. 1999). Although DCs are poorly infected by HIV-1, they can capture HIV-1 virions using the lectin DC-SIGN and maintain them in an infectious state for several days before transmitting them to lymphocytes (Geijtenbeek et al. 2000).

HIV-1 Nef is required for optimal virus production in DC-T cell co-cultures (Petit et al. 2001) and is thought to be implicated in transmission of virus to T cells (Sol-Foulon et al. 2002). Moreover, increased CD4 surface expression in APCs impairs DC-SIGN-mediated transmission by increasing internalization of particles through productive infection, and Nef-mediated CD4 down-modulation in DCs correlates with enhanced viral transmission to T cells (Wang et al. 2007). Together, these studies provide possible mechanisms by which Nef expression converts DCs into more efficient HIV-1 transmitters to T cells.

3.2 Enhancement of Virion Infectivity

Nef also enhances virion infectivity in a CD4-independent manner. Early studies suggested that Nef acts at an early postentry step because viral particles produced in the absence of Nef have an impaired ability to undergo reverse transcription (Aiken and Trono 1995; Chowers et al. 1995; Miller et al. 1995; Schwartz et al. 1995). This Nef-mediated enhancement of infectivity may depend on the route of entry since it is lost with HIV-1 virions pseudotyped with the vesicular stomatits

virus glycoprotein (VSV-G) which targets virions for entry by endocytosis rather than surface fusion (Aiken 1997; Chazal et al. 2001). However, recent data suggest that entry via low pH-dependent Envs does not always bypass the requirement for Nef (Pizzato et al. 2008). Nef does not affect the efficiency of virion fusion with target cells (Tobiume et al. 2003; Cavrois et al. 2004). Instead, it may promote cytoplasmic delivery of HIV-1 virions (Schaeffer et al. 2001), to the detriment of endocytosis, which may lead to non-productive infection (Maréchal et al. 1998). Other reports suggested that Nef enables HIV-1 complexes to cross the cortical actin network underlying the plasma membrane (Campbell et al. 2004) and may reduce the susceptibility of incoming virions to proteasomal degradation in the target cells (Qi and Aiken 2007). Altogether, the results suggest that Nef may help the virus to penetrate the cortical actin barrier and that this function becomes dispensable if entry is mediated by endocytosis (Campbell et al. 2004).

The enhancement of virion infectivity is dependent on the expression of Nef in the virus producer cells, suggesting that Nef alters the molecular composition or properties of the progeny virions. Small quantities of Nef are incorporated into the viral particles and cleaved by the viral protease (Welker et al. 1996; Pandori et al. 1996). However, virion incorporation and cleavage of Nef does not correlate with its ability to enhance virion infectivity (Chen et al. 1998; Miller et al. 1997). Recently, it has been shown that Nef interacts with the GTPase Dynamin-2 (Dyn-2), an essential regulator of clathrin-mediated endocytosis, and that the infectivity enhancement of Nef is dependent on both Nef interaction with Dyn-2 and clathrin-coated pit formation (Pizzato et al. 2007). Nef itself has also been shown to increase clathrin-coated pit formation in an in vitro model (Foti et al. 1997), suggesting that Nef's ability to enhance virion infectivity may be linked to its ability to enhance clathrin-dependent endocytosis. In support of this possibility it has been shown that the dileucine motif in Nef, which acts as an endocytosis signal, is required for optimal viral infectivity (Craig et al. 1998; Madrid et al. 2005).

The capability of Nef to enhance virion infectivity is highly conserved between different primate lentiviral lineages (Münch et al. 2007), and some evidence suggests that it is relevant for viral spread in vivo (Brenner et al. 2007). However, it is noteworthy that the effect of Nef on virion infectivity does not correlate with its ability to promote viral replication in primary T cells or ex vivo infected human lymphoid tissues (Glushakova et al. 2001; Lundquist et al. 2002) and has mainly been examined using HeLa-derived indicator cell lines. Thus, it will be important to further investigate how Nef modifies progeny virions and enhances virion infectivity in primary producer and target cells.

3.3 Induction of Soluble Factors Facilitating Virus Spread

Nef not only manipulates the infected host cells but may also induce changes in the cellular environment to render it more conducive to viral spread. Specifically, it has been shown that Nef expression in HIV-1-infected macrophages induces the

secretion of the chemokines MIP-1alpha and MIP-1beta (Swingler et al. 1999). These chemokines can promote the chemotaxis of resting T-lymphocytes, thus recruiting them to sites of HIV-1 virion release from infected macrophages. Furthermore, it has been suggested that Nef expression in macrophages leads to the production of the soluble factors sCD23 and sICAM-1, which stimulate the production of accessory surface molecules on neighboring B cells (Swingler et al. 2003), which in turn can render resting T cells permissive for productive HIV-1 infection. These results indicate that HIV and SIV Nef proteins have evolved highly sophisticated ways to manipulate the cross-talk between different cell types, and that some effects that may be highly important for viral spread in vivo can be missed in standard cell cultures.

4 Effects of Nef on Programmed Cell Death

One can conceive how it might be advantageous for primate lentiviruses to inhibit apoptosis of the infected host cell to prolong the time of virus production. No less than five viral proteins, Tat, Nef, Vpr, Vpu, and Env, were reported to modulate the programmed cell death of virally infected T cells (Gougeon 2003; Fackler and Baur 2002). As discussed in Chap. 2, most SIV and HIV-2, but not HIV-1, Nefs inhibit apoptosis by blocking the responsiveness of infected T cells to activation (Schindler et al. 2006). Furthermore, Nef induces the expression of the Fas ligand (CD95L), possibly to induce the apoptosis of "attacking" bystander CD8+ cytotoxic T-cells (Xu et al. 1999; Muthumani et al. 2005). It has also been suggested that Nef directly represses pro-apoptotic signaling in infected cells by inhibiting apoptosis signal-regulating kinase 1 (ASK1) (Geleziunas et al. 2001) and by the inactivation of the pro-apoptotic Bad-2 protein through phosphorylation (Wolf et al. 2001). However, it is controversial whether Nef inhibits or enhances apoptosis. In support of a pro-apoptotic role, it has been described that Nef sensitizes CD4+ T cells to apoptosis by up-regulating CD95 and CD95L (Zauli et al. 1999) and by reducing the expression of the anti-apoptotic proteins Bcl-2 and Bcl-XL (Rasola et al. 2001). Experiments using HIV-1 constructs coexpressing Nef and eGFP from single bicistronic RNAs that allowed to directly correlate the levels of cell death and Nef expression failed to detect a significant effect of Nef on apoptosis in HIV-1-infected primary T cells (Schindler et al. 2005). This suggests that Nef affects the survival of the infected cells mainly indirectly, e.g., by reducing CTL lysis and suppressing T cell activation (in the case of HIV-2 and most SIVs), rather than by direct effects on apoptosis.

5 Interactions of Exogenous Nef with Host Cells

In addition to its expression in virally infected cells, Nef is also present in the extracellular environment and can reach concentrations of up to 10 ng/ml in the sera of HIV-infected individuals (Fujii et al. 1996). Extracellular Nef may activate

various transcription factors in monocytes and macrophages (Alessandrini et al. 2000; Mangino et al. 2007; Olivetta et al. 2003; Varin et al. 2003) and induce apoptosis (Fujii et al. 1996; Okada et al. 1997; Huang et al. 2004). It has been proposed that soluble Nef is internalized and blocks immunoglobulin class switch DNA recombination in B cells by perturbing CD40 ligand activation of B cells by T cells (Qiao et al. 2006). Recently, soluble Nef has also been shown to interact with CD34+ hematopoietic stem cells and to inhibit their clonogenic potential through induction of PPARγ and down-modulation of STAT5A and 5B expression (Prost et al. 2008), suggesting that it may contribute to the hematopoietic abnormalities observed in HIV-infected patients (Marandin et al. 1996; Sloand et al. 1997). These studies suggest that extracellular Nef may play a relevant role in the pathogenesis of AIDS. However, it is currently largely unknown how effectively Nef is released and how it can interact or be taken up by uninfected bystander cells. Furthermore, the significance of some of these findings is difficult to assess since they were obtained using rather artificial experimental conditions, such as high levels of Nef produced by bacteria or insect cells.

6 Conclusion

HIV and SIV Nef proteins perform a large number of interactions and functions that help the virus to persist in the infected host by facilitating immune evasion and by increasing virus spread. Although (or perhaps because) a myriad of Nef effects has been reported, we are still far away from a comprehensive understanding of the role of this "all-rounder" protein in viral persistence and pathogenesis. However, some principles of Nef function have become clear: (1) Nef interacts with the cytoplasmic domains of various cellular receptors to recruit them to the endocytic machinery, or to reroute them to endosomes, for ultimate degradation in lysosomes. The reduced surface expression of MHC-I prevents CTL lysis, that of CXCR4 impairs cellular migration, and that of several others, such as CD4, CD28, and CD3, interferes with TCR signaling. Together, these effects of Nef help the virus to evade the host immune system by making infected cells less "visible" to the immune system and prolonging their survival time and by preventing or deregulating the crosstalk between infected T cells and APCs. (2) Simultaneously, Nef interacts with various cellular factors involved in signaling, trafficking, cell activation and migration to alter the responsiveness of virally infected cells to stimulation and to facilitate the transcription of the viral genome and various cellular genes. The preliminary evidence suggests that HIV and SIV have evolved highly elaborate mechanisms to favor the expression of cellular genes that promote viral spread at the cost of antiviral cellular factors. (3) By reducing the surface levels of CD4 and by another mechanism that is currently incompletely understood, Nef facilitates viral release from primary T cells and enhances the infectivity of progeny virions. (4) Nef may also facilitate viral immune evasion by up-modulating several death receptors and inducing the secretion of cytokines that affect the survival and

function of uninfected bystander CD8 T cells. (5) Finally, by inducing the release of cellular factors, Nef may recruit T cells to the sites of infection and render them susceptible to infection. Thus, although Nef is commonly considered as an early viral gene product, it acts essentially at every stage of the virus life cycle and may even modify the microenvironment of the infected cells to facilitate viral spread. The combination of these Nef interactions and functions allows HIV and SIV to persist efficiently at high levels in their respective hosts. Most likely, the great majority of primate lentiviral Nefs also limits the detrimental effects associated with these high viral loads by efficiently suppressing the responsiveness of virally-infected T cells to activation. Exceptions are *nef* alleles from *vpu*-containing viruses, i.e., HIV-1 and its simian precursors, which increase rather than block the responsiveness of infected T cells to stimulation. These differences in Nef function could contribute to the differential levels of immune activation associated with pathogenic and nonpathogenic primate lentiviral infections and hence play a relevant role in the clinical outcome of infection (Kirchhoff, 2009). Understanding the sophisticated mechanisms that primate lentiviruses have evolved to evade the host immune response and to manipulate cells to their advantage may help us to develop novel preventive and therapeutic strategies.

Note Some SIV Nef Proteins Are Tetherin Antagonists: Very recent data demonstrate that the Nef proteins of some SIVs antagonize the recently identified interferon-inducible host-cell factor Tetherin, also known as BST2, CD317 or HM1.24 (Jia et al., 2009; Zhang et al., 2009). This further adds to the list of Nef functions that facilitate virus spread. Furthermore, these studies identify the second function (beside the suppression of CD4 cell surface expression) that Nef shares with the small accessory viral protein U (Vpu), which is used by HIV-1 to counteract tetherin (Neil et al., 2008).

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Vpr and Its Interactions with Cellular Proteins

Vicente Planelles and Serge Benichou

Contents

Abstract Like most viral regulatory proteins, HIV-1 Vpr and homologous proteins from primate lentiviruses are small and multifunctional. They are associated with a plethora of effects and functions, including induction of cell cycle arrest in the G_2 phase, induction of apoptosis, transactivation, enhancement of the fidelity of reverse transcription, and nuclear import of viral DNA in macrophages and other nondividing cells. This review focuses on the cellular proteins that have been reported to interact with Vpr and their significance with respect to the known functions and effects of Vpr on cells and on viral replication.

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Abbreviations

ANT	Adenine nucleotide transporter
APOBEC3	Apolipoprotein B mRNA-editing, catalytic polypeptide-like
ATM	Ataxia telangiectasia-mutated
ATR	ATM and Rad3-related
Cdc	Cell division cycle
Cdk	Cyclin-dependent kinase
Chk1	Checkpoint protein 1
CREB	Cyclic AMP response element binding protein
Cul4	Cullin 4
CypA	Cyclophilin A
DCAF1	DDB1 and Cul4 associated factor-1
DDB1	Damaged DNA-binding protein-1
GADD45α	Growth arrest and DNA damage protein alpha
GR	Glucocorticoid receptor
HHR23	Human homolog of Rad23
HIV	Human immunodeficiency virus
Hsp70	Heat shock protein 70
LTR	Long terminal repeat
MDM	Monocyte-derived macrophages
NE	Nuclear envelope
PTPC	Permeability transition pore complex
Rad23	Radiation-sensitive 23
Roc1	Ring of cullins
SAP	Splicing-associated protein
SIV	Simian immunodeficiency virus
TFIIB	Transcription factor IIB
UNG	Uracil-N glycosylase
Vpr	Viral protein regulatory
VprBP	Vpr-binding protein

1 Introduction

Vpr was originally deemed "viral protein, regulatory" because, when its open reading frame was disrupted by mutagenesis, the resulting virus replicated with a slower kinetics (Hattori et al. 1990; Ogawa et al. 1989; Wong-Staal et al. 1987). HIV-1 Vpr is a small, 96-amino acid (14 kDa) protein that appears at two different times during the virus life cycle. Vpr is packaged in the virus particles via a direct interaction with the C-terminal p6 region of the Gag precursor, and is consequently present in the cytoplasm of newly infected cells (reviewed in

Tungaturthi et al. 2003). Vpr is then expressed de novo by the provirus, from a late mRNA (Schwartz et al. 1991).

Despite its small size, Vpr is a multifunctional protein. A plethora of effects and functions have been ascribed to Vpr, including induction of cell cycle arrest in the G_2 phase, transactivation, nuclear import of preintegration complexes in macrophages and other non-dividing cells, induction of apoptosis, and enhancement of the fidelity of reverse transcription (reviewed in Andersen and Planelles 2005; Le Rouzic and Benichou 2005).

This review will focus on the cellular proteins that have been reported to interact with Vpr and their significance with respect to the known functions and effects of Vpr on cells and on viral replication. A previous review on the topic, also including interactions of Vpr with viral components, is recommended for additional details (Kino and Pavlakis 2004).

2 Structure of Vpr

The molecular structure of Vpr consists of three bundled α-helices spanning residues 17–33, 38–50, and 55–77, and flanked by flexible, unstructured N- and C-terminal domains that are negatively and positively charged, respectively (Fig. 1) (Morellet et al. 2003). Four conserved prolines (positions 5, 10, 14, and 35), which are subjected to *cis*/*trans* isomerization, are found in the N-terminal domain

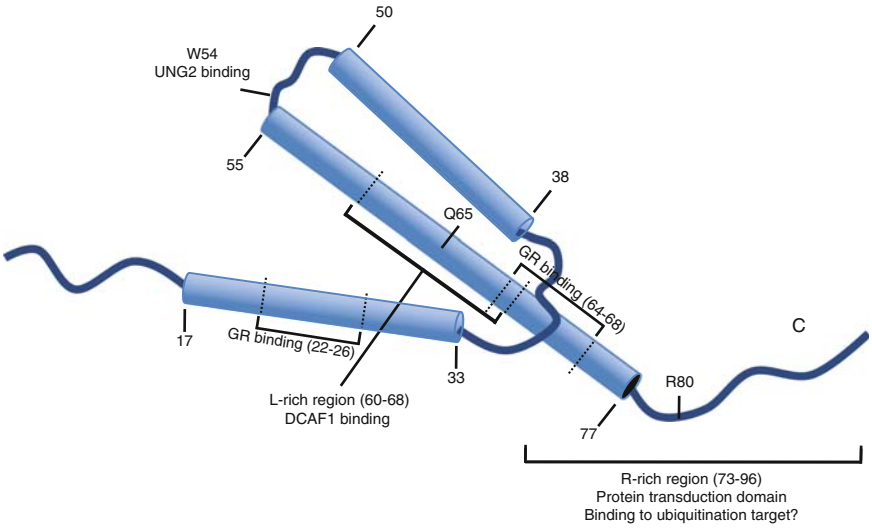

Fig. 1 Diagramatic structure of Vpr as determined by nuclear magnetic resonance (adapted from Morellet et al. 2003). *Cylinders* denote regions of alpha helix comprised between residues indicated by *numbers*. *N* Amino-terminus, *C* carboxy-terminus, *GR* glucocorticoid receptor. Selected regions are shown with associated functions

(Bruns et al. 2003). It was reported that the cellular peptidyl-propyl isomerase cyclophilin A was able to interact with Vpr via prolines at position 14 and 35, to ensure the correct folding of the viral protein (Zander et al. 2003). The carboxy-terminus of Vpr contains six arginine residues between positions 73 and 96 (Fig. 1). This domain shows similarity with those of arginine-rich protein transduction domains, and may explain the transducing properties of Vpr, including its ability to cross the cell membrane lipid bilayer (Coeytaux et al. 2003; Kichler et al. 2000; Sherman et al. 2002). In addition, the third helix of Vpr is rich in leucine residues (Schuler et al. 1999), and one side of the helix presents a stretch of hydrophobic side chains that can form a leucine-zipper like motif (Schuler et al. 1999). This region accounts for the formation of Vpr oligomers (Fritz et al. 2008; Mahalingam et al. 1997; Schuler et al. 1999; Wang et al. 1996) and for the interaction with certain cellular partners (see below).

How the dimeric or multimeric state of Vpr affects its function is unclear. A recent study using a flow cytometric fluorescence resonance energy transfer has elegantly confirmed in real time that Vpr self-associates within live cells (Bolton and Lenardo 2007). Self-association was dependent on the hydrophobic patch that is located on the third α-helix (Fig. 1), and mutations in this region, such as I63E and I70S, did not impair Vpr's ability to induce G_2 arrest (Bolton and Lenardo 2007). In addition, mutations in the arginine-rich domain, such as R80A and R87/88A, did not impair selfassociation but were unable to induce G_2 arrest (Bolton and Lenardo 2007). Therefore, it appears that Vpr does not require dimerization toward induction of cell cycle disruption.

3 Interactions of Vpr with Cellular Proteins

3.1 Interactions of Vpr with the DNA Repair Machinery

3.1.1 Uracil DNA Glycosylase

Vpr was shown to bind the nuclear form of uracil DNA glycosylase/uracil N-glycosylase (UNG2) (Bouhamdan et al. 1996). UNG2 is involved in the repair of cellular DNA that contains uracil residues and, initially, appeared to be an adequate candidate to explain how Vpr induced a DNA damage-like signal. However, subsequent mutagenesis analysis demonstrated that the interaction of Vpr with UNG2 and the induction of G_2 arrest were separable and independent effects of Vpr (Selig et al. 1997).

The HIV-1 reverse transcriptase enzyme is an error-prone RNA-dependent DNA polymerase. Interestingly, the in vivo rate of HIV-1 mutation per replication cycle was estimated to be about four-fold higher in the absence of Vpr than in its presence, when assayed in dividing cells (Mansky 1996; Mansky and Temin 1995). This phenotype was exacerbated in primary monocyte-derived macrophages

(MDM), where the increase in the HIV-1 mutation rate in the absence of Vpr is about 18-fold (Chen et al. 2004). This activity correlated with the interaction of Vpr with UNG2 (Mansky et al. 2000). Vpr interacts with UNG2 using the Trp residue at position 54, located in the loop that connects the second and third α-helices of Vpr (Fig. 1). The interacting moiety in UNG2 is near its C-terminus and includes a WXXF motif (BouHamdan et al. 1998). Three distinct Vpr interactors have so far been shown to contain a WXXF motif: the TFIIB transcription factor (Agostini et al. 1999), the mitochondrial adenosine nucleotide translocator (Jacotot et al. 2000), and UNG2 (Mansky et al. 2000). Mansky et al. proposed that the Vpr/UNG2 interaction in virus-producing cells allows the incorporation of a catalytically active UNG2 into virus particles where UNG2 may directly influence the reverse transcription fidelity, and that this recruitment may contribute to the ability of HIV-1 to replicate in primary macrophages (Chen et al. 2004; Mansky et al. 2000).

The role of the Vpr-UNG interaction has been recently reexamined in light of the observation that Vpr led to proteasomal degradation of UNG2 (Schrofelbauer et al. 2005). Schrofelbauer and collaborators observed that HIV-1 virions encapsidated UNG2, and that the amount of UNG2 detectable in virions was surprisingly higher in Vpr-deleted viruses (Schrofelbauer et al. 2005). The authors proposed that UNG2 encapsidation has a detrimental effect on virus replication by increasing the number of abasic sites within viral reverse transcripts. Even though APOBEC3 proteins are targeted by Vif, residual APOBEC3 activity could leave a few uracil residues which, when recognized by the host DNA repair machinery, could be converted to abasic sites. Abasic sites could be detrimental to virus replication at two different levels. First, abasic sites could block synthesis of the plus sense DNA strand by the reverse transcriptase enzyme. Second, abasic sites may be targets for host apurinic/apyrimidinic nucleases (Yang et al. 2007). In addition, the presence of uracil residues in reverse transcription intermediates, in the absence of UNG activity, was shown to hinder priming of plus-strand synthesis (Klarmann et al. 2003). Therefore, the models proposed by Schrofelbauer et al. and Mansky et al. to explain the Vpr/UNG2 interaction are contradictory as they propose deleterious and beneficial roles, respectively, for encapsidation of UNG2 into HIV-1 virions (Chen et al. 2004; Schrofelbauer et al. 2005).

A recent study by Kaiser and Emerman examined whether depletion of UNG2 from cells, or its inhibition, would impact HIV-1 replication (Kaiser and Emerman 2006). The authors found no role for the presence of UNG2 or its activity in retroviral replication (Kaiser and Emerman 2006). Therefore, the role of the Vpr-UNG interaction on HIV-1 replication remains controversial. Since most of the studies mentioned above were performed with overexpressed UNG2, additional experiments relying on endogenous UNG2 will be required.

3.1.2 The Human Homolog of *S. cerevisiae* Rad23

Vpr was also shown to interact with the human homolog of Rad23A (HHR23A) (Withers-Ward et al. 1997). HHR23A functions in the nucleotide excision DNA

repair pathway as part of a multiprotein complex associated with the xeroderma pigmentosum complementation group C protein (van der Spek et al. 1996). It was initially suggested that Vpr binding to HHR23A could account for the Vpr-induced G_2 arrest (Gragerov et al. 1998; Withers-Ward et al. 1997). However, subsequent analyses indicated that there was no correlation between the association of Vpr with HHR23A and the G_2 block (Mansky et al. 2001). The binding between Vpr and HHR23A did not influence the HIV-1 mutation rate (Mansky et al. 2001). Therefore, the role of HHR23A in HIV-1 replication remains unclear.

3.1.3 Vpr Induces a Signal That Resembles Genotoxic Stress

Some of the effects of Vpr on the cell cycle resemble those of DNA damage. More specifically, the presence of hyper-phosphorylated Cdk1, and the ability of methylxanthines, such as caffeine, to relieve the cell cycle block, suggest that the underlying stimulus is DNA damage. It was perplexing, however, that the ataxia telangiectasia-mutated (ATM) protein and the tumor supressor, p53, two checkpoint proteins known at that time, were dispensable for the activity of Vpr (Bartz et al. 1996; Shostak et al. 1999; Stewart et al. 1999). Therefore, opinions were initially divided as to whether or not the effects of Vpr were mediated by a signaling pathway that normally responded to DNA damage. The cloning and functional characterization of the human ATM paralog, the ataxia telagiectasia-mutated and Rad3-related protein (ATR) (Cimprich et al. 1996; Keegan et al. 1996), and its role in detecting a specialized form of genotoxic stress known as replication stress, provided a new candidate to explain the effects of Vpr (see Vpr and ATR).

The issue of whether Vpr causes actual DNA damage or, alternatively, it simply activates signaling events in the absence of damage has also been controversial in the literature. Although this issue falls outside the scope of this review, it merits a brief mention. Vpr has been shown to cause double-stranded breaks and chromosomal numerical and structural aberrations (Siddiqui et al. 2008; Tachiwana et al. 2006). Using pulsed field gel elecrophoresis of mammalian cell DNA, other investigators have found no apparent DNA damage in cells expressing Vpr (Lai et al. 2005). In agreement with the lack of detectable DNA damage in the previous study, Lai et al. found ATM not to be phosphorylated at residue Ser1981 (Lai et al. 2005). Another category of cytopathic effects resulting from Vpr expression in mammalian cells includes mitotic abnormalities, such as supernumerary centrosomes and aberrant multipolar spindles (Chang et al. 2004; Watanabe et al. 2000).

3.2 Interactions of Vpr with Cell Cycle Regulatory Elements

The ability of Vpr to manipulate the cell cycle and, more specifically, to induce arrest at the G_2-to-M transition was first reported in 1995 (He et al. 1995; Jowett et al. 1995; Re et al. 1995; Rogel et al. 1995). About a year earlier, Zhao and collaborators described the first cellular protein found in association with Vpr in

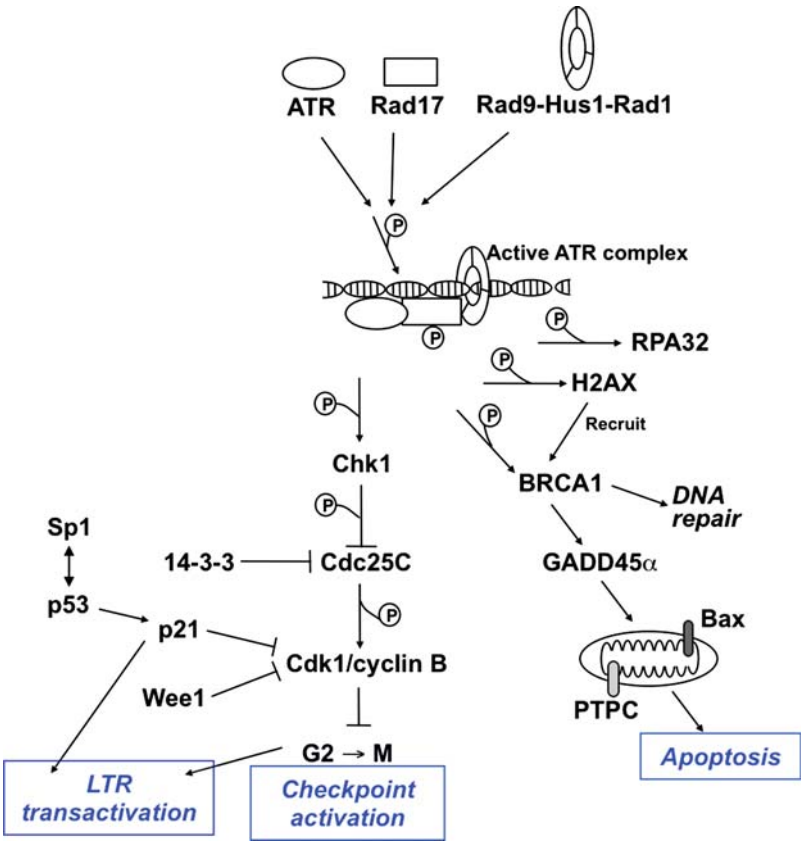

Fig. 2 Signaling pathways proposed to mediate induction of G_2 arrest, apoptosis, and LTR transactivation by HIV-1 Vpr

co-precipitation experiments (Zhao et al. 1994). This was a novel cellular protein of unknown function, and was named Vpr-binding protein (VprBP) (Zhao et al. 1994). Initial studies did not link VprBP to the cell cycle effects of Vpr, and it was only recently that a direct link was found (see Vpr and the Ubiquitin/Proteasome System). In the meantime, a plethora of other cell cell cycle regulators were found to interact with Vpr (Fig. 2).

3.2.1 Vpr and 14-3-3 proteins

Proteins from the 14-3-3 family of cell cycle regulators were found in association with Vpr in a yeast two-hybrid screen (Kino et al. 2005). 14-3-3 proteins bind to phosphorylated serine/threonine residues of target proteins, such as Chk1, Cdc25C, Wee1 and Cdk2, and regulate their activities by changing their subcellular

localization, stability, and/or by preventing dephosphorylation (reviewed in Hermeking and Benzinger 2006). Kino et al. reported that Vpr binds to the carboxy-terminus of 14-3-3 proteins, a region that is responsible for the binding specificity to target phosphoproteins (Hermeking and Benzinger 2006). These authors also found that Vpr-induced cell cycle arrest was reduced in cells lacking 14-3-3σ, and that overexpression of 14-3-3σ enhanced Vpr-induced cell cycle arrest (Kino et al. 2005). Vpr binding to 14-3-3 enhanced the interaction of 14-3-3σ to Cdc25C, resulting in cytoplasmic sequestration and inactivation of Cdc25C (Kino et al. 2005). Of note, a subsequent report found that 14-3-3σ was not expressed in lymphocytes and, instead, 14-3-3 isoforms β, γ and θ were the key isoforms involved in Vpr-induced G_2 arrest in lymphocytes (Bolton et al. 2008). Bolton et al. failed to detect any redistribution of Cdc25C between the nucleus and the cytoplasm in the presence of Vpr or in HIV-1 infected cells (Bolton et al. 2008). Therefore, delineation of the precise roles of 14-3-3 proteins in Vpr-induced G_2 arrest will require further investigation.

3.2.2 Vpr and ATR

The discovery of a new upstream checkpoint kinase known as ATR (Cimprich et al. 1996; Keegan et al. 1996) prompted studies to ascertain whether this new kinase could account for the cytostatic properties of Vpr (Roshal et al. 2003). Roshal et al. showed that Vpr-induced G_2 arrest via activation of ATR (Roshal et al. 2003). ATR is a sensor for replication stress, a cellular condition that involves the stalling of replication forks, and can be induced by deoxyribonucleotide depletion, topoisomerase inhibition, or ultraviolet light-induced DNA damage (reviewed in McGowan and Russell 2004). ATR can activate the G_2 checkpoint (Fig. 2) concomitant with inhibitory phosphorylation of Cdk1 in a manner that is independent of p53 and ATM, and is inhibited by caffeine. ATR exerts its effects via phosphorylation of a number of target proteins (Cimprich and Cortez 2008). The ATR target that controls G_2 checkpoint activation is Chk1. In agreement with this idea, Roshal et al. also showed that depletion of Chk1 or ATR relieved Vpr-induced G_2 arrest (Roshal et al. 2003). These results provided a satisfactory explanation for earlier observations that suggested that Vpr stimulated the DNA damage-sensing machinery and excluded known checkpoint proteins, such as p53 and ATM (see Vpr Induces a Signal That Resembles Genotoxic Stress). However, a direct interaction between Vpr and ATR has not so far been reported.

3.2.3 Cdc25

Goh et al. reported that Vpr binds and inactivates Cdc25C the positive regulator of Cdk1, and that mutation of the Cdc25-binding region within Vpr rendered Vpr deficient in G_2 arrest induction (Goh et al. 2004). The observation that Vpr inhibits

Cdc25C could be interpreted as a consequence of the upstream signaling whereby Chk1 can phosphorylate Cdc25C at Ser 216 leading to Cdc25 inhibition. However, based on the observation that Vpr interacts directly with Cdc25C, it is formally possible that Vpr acts at two different, apparently redundant levels. As mentioned above (See Vpr and 14-3-3 proteins), Vpr has also been proposed to inhibit Cdc25C indirectly, by interacting with 14-3-3 proteins (Kino et al. 2005).

3.2.4 Wee1

Consistent with a model in which Vpr activates a DNA damage response pathway, Yuan et al. found that Vpr activates the negative regulator of Cdk1, the Wee1 kinase (Yuan et al. 2004). They further showed that Vpr is unable to induce G_2 arrest in cells in which Wee1 has been depleted by siRNA (Yuan et al. 2004). The ability of Vpr to upregulate Wee1 activity, ultimately resulting in inhibitory phosphorylation of Cdk1, required the direct binding of Vpr to the "N" lobe of the Wee1 kinase domain (Kamata et al. 2008).

3.2.5 $p21^{WAF}$

Vpr has also been found to bind to the cyclin dependent kinase inhibitor, $p21^{WAF}$ (Cui et al. 2006). The authors showed that this interaction was required for optimal transactivation capacity of the HIV-1 LTR by p21 and p300 (Felzien et al. 1998; Parker et al. 1996). Interestingly, the Vpr-$p21^{WAF}$ interaction prevented the ability of p21 to induce G_1 arrest, but had little or no effect on Vpr-induced G_2 arrest (Cui et al. 2006). A separate, functional relationship between Vpr and p21 has also been described (see Sp1).

3.2.6 Sp1

Sawaya et al. reported the formation of a ternary complex including Vpr, the tumor supressor protein, p53, and the transcription factor, Sp1 (Sawaya et al. 1998). The formation of this complex resulted in activation of the $p21^{WAF}$ promoter (Sawaya et al. 1998). The enhancement of $p21^{WAF}$ expression induced by Vpr has independently been reported by Tripp and colleagues (Tripp et al. 2005). The $p21^{WAF}$ protein arrests cell growth by modulating the activity of cyclin-dependent kinases and down-modulates DNA synthesis by interacting with the proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase δ (Cooper et al. 1999). The interplay between Vpr and $p21^{WAF}$ was also proposed to be responsible for transactivation of the HIV-1 LTR (Amini et al. 2004). The above observations, taken together, prompt a model by which induction of G_2 arrest and transctivation of the LTR by Vpr are mediated by $p21^{WAF}$ (Fig. 2).

3.2.7 p300

Vpr was shown by several groups to interact with p300/CREB-binding protein (Felzien et al. 1998; Parker et al. 1996). This interaction was proposed to be involved in regulating the basal transcriptional machinery to increase the levels of HIV-1 gene expression. Kino et al. further proposed that the Vpr-p300 complex regulated transcription of the HIV-1 LTR and glucocorticoid-responsive promoters (see The Glucocorticoid Receptor) (Kino et al. 2002).

3.2.8 Vpr and the Ubiquitin/Proteasome System

The upstream event(s) of the signaling pathway activated by Vpr, specifically how Vpr induces activation of the ATR kinase, remained obscure for quite some time. Progress in the last 2 years has yielded some exciting clues. First, Schrofelbauer et al. reported the interaction of Vpr with cullins 1 and 4 (Cul1, Cul4), components of modular ubiquitin ligases. Then in 2006, several groups identified a family of proteins that were associated with the damaged-DNA specific binding protein 1 (DDB1), a Cul4 adaptor (Angers et al. 2006; He et al. 2006; Higa et al. 2006a; Jin et al. 2006). This novel family of proteins, which include VprBP, act as the substrate specificity modules in Cul4- and DDB1-based ubiquitin ligase complexes or E3 (Angers et al. 2006; He et al. 2006; Higa et al. 2006a; Jin et al. 2006). VprBP was, accordingly, renamed DDB1- and Cul4A-associated factor (DCAF) -1. Shortly thereafter, it was shown that, through its interaction with VprBP/DCAF1, Vpr is capable of binding to a larger complex (Fig. 3) that includes Cul4A, DDB1, and, presumably, Rbx1/Roc1 and a ubiquitin-conjugating enzyme or E2 (Belzile et al. 2007; DeHart et al. 2007; Hrecka et al. 2007; Le Rouzic et al. 2007; Schrofelbauer et al. 2007; Tan et al. 2007; Wen et al. 2007).

DDB1 links Cul4 to a number of possible substrate specificity subunits, collectively referred to as DCAFs. The ubiquitination substrates for several DCAFs have been identified. For example, CDT2 (DCAF2) recruits the origin of replication licensing factor, CDT1 (Higa et al. 2003; Hu et al. 2004), to prevent rereplication of DNA. The damaged DNA-binding 2/xeroderma pigmentosum complementation group E protein (DDB2/XPE) is another DCAF that interacts with DDB1-Cul4A to promote degradation of XPC (Sugasawa et al. 2005), and the histones 3 and 4 (Wang et al. 2006), as part of the response to DNA damage. Cul4A- and Cul4B-containing E3 ligases are also responsible for destruction of the cyclin-dependent kinase inhibitor, p27, and cyclin E, respectively (Higa et al. 2006b). Thus, the general roles of Cul4-DDB1 E3 ligases appear to involve genome stability, DNA replication, and cell cycle checkpoint control. Merlin, a tumor suppressor protein, is the only substrate of DCAF1 found to date (Huang and Chen 2008). In vitro, Merlin is degraded in response to serum stimulation, and this induces cellular proliferation. In this capacity, DCAF1 confers the Cul4-DDB1 ubiquitin ligase a pro-proliferative role (Huang and Chen 2008).

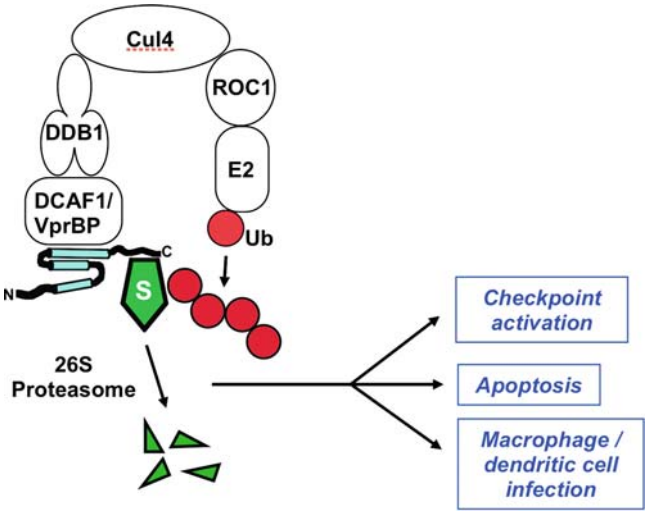

Fig. 3 Diagramatic representation of the ubiquitin ligase complex that is recruited by Vpr. The prevailing model states that Vpr recruits an unknown cellular substrate (S) to the Cul4/DDB1/DCAF1 ubiquitin ligase; it is thought that the cellular substrate protein subsequently becomes ubiquitinated and degraded by the 26 S proteasome, leading to various effects of Vpr

Based on the above evidence, a model has emerged in which Vpr binds to a Cul4-DDB1-DCAF1 E3 ligase, to trigger polyubiquitination and subsequent degradation of a putative cellular protein, resulting in activation of the G_2 checkpoint (reviewed in DeHart and Planelles 2008). This model predicts that Vpr would be using two different interfaces to bind to VprBP/DCAF1 and the putative ubiquitination substrate protein. Site-directed mutagenesis of Vpr confirmed this prediction as follows. The domain of Vpr that binds to DCAF1 was mapped to the leucine-rich (LR) motif 60-LIRILQQLL-68 within the third α-helix of HIV-$1_{89.6}$ Vpr (Zhao et al. 1994). A Vpr mutant disrupting the DCAF1 interaction, Vpr(Q65R), was described by Le Rouzic et al. (Le Rouzic et al. 2007). Consistent with the idea that DCAF1-Vpr interaction is required for Vpr function, Vpr(Q65R) failed to induce G_2 arrest (Le Rouzic et al. 2007). Truncation of the c-terminal 18 residues of Vpr [Vpr(1-78)] or replacement of arginine at position 80 by alanine, Vpr(R80A), resulted in Vpr proteins with unaltered binding to DCAF1, but also unable to induce G_2 arrest (DeHart et al. 2007; Le Rouzic et al. 2007). In addition, co-expression of either Vpr(1-78) or Vpr(R80A) (Fig. 1) with wild-type Vpr resulted in a dominant-negative effect by the mutant.

The interaction of Vpr with a ubiquitin ligase could result in two theoretical outcomes: inhibition or activation of the enzymatic activity. In the first case, one would predict that RNAi-mediated depletion of DCAF1 would mimic the activity of Vpr. On the other hand, if Vpr promoted activation of the E3 ligase, then depletion of DCAF1 should counteract the effect of Vpr. RNAi-mediated depletion of DCAF1 restores a normal cell cycle profile in the presence of Vpr (DeHart et al.

2007; Hrecka et al. 2007; Le Rouzic et al. 2007; Wen et al. 2007), providing support for the idea that Vpr requires or triggers the activity of the ubiquitin ligase. Together, these observations indicate that (1) binding of Vpr to DCAF1 is necessary, but not sufficient, for induction of G_2 arrest, and (2) the carboxy-terminal domain of Vpr is likely required for the recruitment of a cellular protein, whose ubiquitination leads to G_2 arrest.

Vpr-induced G_2 arrest has two known downstream effects that likely contribute to the pathogenesis of HIV-1. First, the transcriptional activity of the viral promoter is increased by several-fold during G_2/M (Goh et al. 1998; Hrimech et al. 1999; Zhu et al. 2001), leading to enhanced production of viral particles (Goh et al. 1998). It has also been suggested that accumulation of infected cells in G_2 may favor selective translation of viral products due to the presence of a putative internal ribosome entry site in the HIV-1 genome (Brasey et al. 2003). Secondly, G_2 arrest leads to the commitment of infected cells to death by apoptosis (see Interactions of Vpr with the Apoptosis Machinery reviewed in Andersen et al. 2008).

3.3 Interactions of Vpr with the Apoptosis Machinery

Vpr was found to be a potent inducer of cell death both when expressed alone or in the context of HIV-1. Vpr-induced cell death has the hallmarks of apoptosis (Andersen et al. 2005; Muthumani et al. 2002; Shostak et al. 1999; Stewart et al. 2000). However, other reports in the literature propose that the type of cell death induced by Vpr rather conforms to necrosis (Bolton et al. 2002; Lenardo et al. 2002).

An exciting model to explain the pro-apoptotic activity of Vpr stemmed from the observation that recombinant Vpr associates with purified mitochondria. This interaction was mediated via binding to the adenine nucleotide transporter (ANT), a component of the permeability transition pore complex (PTPC) that resides at the inner mitochondrial membrane (Jacotot et al. 2000; Vieira et al. 2000) (Fig. 2). The addition of recombinant Vpr to purified mitochondria triggered mitochondrial membrane permeabilization and release of pro-apoptotic proteins, such as cytochrome c (Jacotot et al. 2000; Vieira et al. 2000).

Recent studies of ANT and cyclophilin D (an essential regulatory element of ANT; Tsujimoto and Shimizu 2007) in knockout mice suggest that these mitochondrial pore components may promote necrotic, but not apoptotic cell death (Baines et al. 2005; Kokoszka et al. 2004; Nakagawa et al. 2005). In recent studies, siRNA-mediated depletion of ANT did not appreciably affect Vpr-induced apoptosis, whereas depletion of another mitochondrial pore-forming protein, Bax (Fig. 2), effectively blocked apoptosis (Andersen et al. 2006). Induction of apoptosis by Vpr correlated with a conformational change in Bax that is concomitant with Bax activation (Andersen et al. 2006; Rathmell et al. 2003). The association between Vpr and ANT remains intriguing, especially in light of the fact that multiple groups have independently reported this interaction (Hrecka et al. 2007; Jacotot et al. 2000).

An alternative model to explain Vpr-induced apoptosis proposes that apoptosis is a direct consequence of the prolonged G_2 arrest (Andersen et al. 2006; Jacquot et al. 2007; Yuan et al. 2003). More specifically: (a) the pro-apoptotic activity of Vpr paralleled the G_2 arrest; (b) and both Vpr activities required the activation of ATR; and (c) apoptosis was shown to be dependent on ATR-mediated phosphorylation of Brca1 leading to GADD45α upregulation (Andersen et al. 2006) (Fig. 2).

Tissue macrophages infected with HIV-1 are relatively resistant to the viral cytopathic effects (Gartner et al. 1986; Gorry et al. 2005; Kedzierska and Crowe 2002). Consequently, macrophages are viewed as one of the reservoirs for HIV-1 infection, and as being capable of disseminating the virus to various tissues including the brain (Ghorpade et al. 1998; Orenstein et al. 1997). The ability of Vpr to induce ATR activation and apoptosis in primary monocyte-derived macrophages (MDM) was recently tested (Zimmerman et al. 2006). Not surprisingly, Vpr did not alter the DNA content of MDM, due to their non-dividing status. However, macrophages also proved refractory to Vpr-induced apoptosis. Western blot demonstrated the absence of protein expression for, at least, three essential proteins in the ATR signaling pathway: ATR itself, Chk1, and Rad17 (Zimmerman et al. 2006). In view of these results, it is tempting to speculate that the differences in cytopathicity associated with infection of lymphocytes and that of macrophages may be due, at least in part, to the absence of ATR signaling in the latter cell type.

3.4 Interactions of Vpr with Nuclear Pore and Nuclear Transport Elements

Despite the lack of a canonical nuclear localization signal (NLS), Vpr displays evident karyophilic properties and is rapidly targeted to the host cell nucleus after infection (Depienne et al. 2000; Di Marzio et al. 1995; Kamata and Aida 2000; Lu et al. 1993). As shown for proteins containing a basic-type NLS, it was initially proposed that Vpr uses an importin α-dependent pathway to access the nuclear compartment (Popov et al. 1998b; Vodicka et al. 1998). However, it has also been proposed that Vpr nuclear import is mediated by an unidentified pathway, which is distinct from the classical NLS- and M9-dependent pathways (Jenkins et al. 1998). The interaction of Vpr with importin α has recently been reexamined (Kamata et al. 2005; Nitahara-Kasahara et al. 2007). In these studies, it was proposed that Vpr is transported into the nucleus by importin α alone, in an importin β-independent manner, suggesting a novel nuclear import process (Kamata et al. 2005). However, Vpr is a dynamic mobile protein able to shuttle between the nucleus and cytoplasmic compartments (Jenkins et al. 2001; Le Rouzic et al. 2002; Sherman et al. 2001, 2003). This shuttling activity has been linked to the distal leucine-rich region, which may form a classical nuclear export signal recognized by the CRM1-dependent machinery (Sherman et al. 2001, 2003).

It is noteworthy that Vpr also possesses a great affinity for the nuclear envelope (NE), and several groups reported that Vpr specifically interacts with components

of the nuclear pore complex (Fouchier et al. 1998; Le Rouzic et al. 2002; Popov et al. 1998a; Vodicka et al. 1998). While initial studies revealed that Vpr could bind to the FG-rich region of several nucleoporins (Nups), including the human p54 and p58 Nups, the rodent POM121, and the yeast NUP1P (Fouchier et al. 1998; Popov et al. 1998a; Vodicka et al. 1998), a direct interaction with the human CG1 nucleoporin was also reported (Le Rouzic et al. 2002). The interaction with hCG1 is not mediated by the FG-repeat region of this Nup but, rather, via a region without consensus motif located at the N-terminus of hCG1. The specific role of Vpr at the NE is still unclear, but it was recently proposed that Vpr targeting to the nuclear pore complex (NPC) is not absolutely required, but can facilitate HIV-1 replication in macrophages (Jacquot et al. 2007). These observations could be related to the targeting of the PIC containing the viral DNA to the NPC before or during its translocation into the nuclear compartment.

Vpr was shown to promote herniations and transient ruptures of the NE leading to inappropriate redistribution of key cell cycle regulators, including Wee1, Cdc25, and cyclin B into the cytoplasm of the host cell (de Noronha et al. 2001). The molecular mechanism behind the local bursting induced by Vpr is not known. It is possible that the interaction of Vpr with nucleoporins may cause misassembly of the nuclear pore complex leading to alterations of the NE architecture. Alterations of the subcellular localization of compartmentalized cell cycle regulators could explain the G_2 arrest induced by Vpr. Alternatively, nuclear herniations could affect chromatin structure or integrity, perhaps leading to the activation of ATR. In support for a direct cause–effect link between NE accumulation of Vpr and G_2 arrest induction, mutants of Vpr that would separate these two effects have not been described thus far. This idea led Jacquot et al. to propose that Vpr accumulation at the NE may constitute an early step toward Vpr-induced G_2 arrest and subsequent apoptosis (Jacquot et al. 2007).

3.5 Vpr Binds to the Splicing Regulator, SAP145

Vpr interacts with the splicing-associated protein (SAP) 45, a subunit of the splicing factor 3b (SF3b), which is required for the splicing of precursor mRNAs (Terada and Yasuda 2006). In their study, the authors found that full-length Vpr, but not a carboxy-terminal truncated mutant of Vpr, bound SAP145, and that Vpr-SAP145 binding prevented the association between SAP145 and SAP49 (Terada and Yasuda 2006). It was also found that depletion of either SAP145 or SAP49 caused G_2 arrest and the induction of γH2AX and BRCA1 nuclear foci (Terada and Yasuda 2006), as has been observed in the context of expression of Vpr (Zimmerman et al. 2004). It is unclear how the Vpr/SAP145 interaction may lead to G_2 arrest, as inhibition of SAP145 did not alter the splicing of genes required for cell cycle progression such as Cyclin B1 or Cdk1 (Terada and Yasuda 2006). In a subsequent report, the Vpr/SAP145 interaction was confirmed, and it was also found that such an interaction was required for Vpr-induced inhibition of processing of certain pre-mRNAs, such

as those for immunoglobulin M and β-globin (Hashizume et al. 2007). However, the authors found that Vpr(R80A), a mutant defective for G_2 arrest (DeHart et al. 2007), still interacted with SAP145 and inhibited pre-mRNA processing (Hashizume et al. 2007). These results suggest that inhibition of splicing and cell cycle arrest may be genetically separable functions of Vpr.

3.6 Heat Shock Protein 70

Hsp70 is an abundant stress-regulated cellular protein that exhibits several anti-apoptotic activities (reviewed in Arya et al. 2007). Hsp70 has been shown to inhibit translocation of Bax into mitochondria, release of cytochrome c to the cytoplasm, formation of the apoptosome, and activation of initiator caspases. Bukrinsky and Zhao reported that Hsp70 interacted with Vpr resulting in inhibition of G_2 arrest (Bukrinsky and Zhao 2004). It was also shown that Hsp70 inhibited replication of Vpr-positive, but not Vpr-negative HIV-1 (Iordanskiy et al. 2004a, b). These observations led the authors to propose a model by which Hsp70 opposes the pathogenic effects of Vpr, and is possibly induced by HIV-1 infection as part of an innate antiviral response.

3.7 Cyclophilin A

Cyclophilin A is a member of a family of cellular proteins that share a peptidyl prolyl cis-trans isomerase activity. This activity is important for the maintenance of proper conformation of numerous cellular proteins (Fischer et al. 1989; Takahashi et al. 1989; Wang and Heitman 2005). A role for CypA in the life cycle of primate lentiviruses emerged in 1993 with its isolation as a yeast two-hybrid partner of the HIV-1 core protein, p24 (Goff 2004; Luban et al. 1993). Later, Vpr was also shown to interact with Cyclophilin A (CypA), and it was proposed that CypA was required for the stability and function of Vpr, specifically for Vpr-induced G_2 arrest (Zander et al. 2003). However, a subsequent report demonstrated that ablation of the Vpr-CypA interaction (either by using CypA-deficient cells, by incubation with Cyclosporin A, or by mutating Vpr at proline 35) had no effect on Vpr's cell cycle disruption (Ardon et al. 2006). Therefore, it was proposed that binding to CypA and induction of G_2 arrest are independent activities of Vpr, and the role of the interaction of Vpr with CypA, if any, remains enigmatic (Ardon et al. 2006).

3.8 The Glucocorticoid Receptor

Vpr was found in complex with the glucocorticoid receptor (GR) (Refaeli et al. 1995), and to enhance the activity of corticosteroids by acting as a coactivator

(Kino et al. 1999; Refaeli et al. 1995; Sherman et al. 2000). Interaction with the GR is mediated by a double LXXLL motif present in Vpr (Fig. 1), which is shared with steroid receptor coactivator-1 and p300/CREB-binding protein (Kino et al. 1999; Sherman et al. 2000). Sherman et al. found that G_2 arrest and GR-binding are independent phenotypes of Vpr based on experiments in which Vpr mutants defective for G_2 arrest retained their ability to bind the GR (Sherman et al. 2000). Furthermore, the transactivation activity of Vpr, which had been previously attributed to its ability to induce G_2 arrest, was shown to be at least in part independently effected via its GR coactivator ability (Sherman et al. 2000).

3.9 Vpx as a Paralog of Vpr

HIV-1 Vpr has two homologous proteins in the HIV-2/SIVsm/SIVmac lineage, Vpr and Vpx. While HIV-2 Vpr shares the ability to induce G_2 arrest with HIV-1 Vpr (Fletcher et al. 1996; Planelles et al. 1996), HIV-2 Vpx has no effect on the cell cycle and, instead, is required for efficient infection of non-dividing cells such as macrophages and dendritic cells (Fletcher et al. 1996; Guyader et al. 1989; Pancio et al. 2000; Yu et al. 1991). Given the common evolutionary origin, the high degree of homology, and the divergent functions of Vpx with respect to HIV-1 Vpr, Vpx can be considered as a paralog of HIV-1 Vpr.

Vpx positively affects the lentiviral life cycle in nondividing cells by facilitating the nuclear import of preintegration complexes and/or by promoting the accumulation of full-length viral DNA (Fletcher et al. 1996; Fujita et al. 2008; Goujon et al. 2007; Sharova et al. 2008; Srivastava et al. 2008). To explain the ability of Vpx to enhance lentiviral infection of dentritic cells, it has been proposed that Vpx overcomes an unknown restriction factor (Goujon et al. 2007). It has also been proposed that the restriction mechanism involves the ubiquitin/proteasome system, since treatment with proteasome inhibitors has a similar effect to that with Vpx expression (Goujon et al. 2007). Restriction factors are typically genetically dominant. In agreement with that, when Sharova et al. fused permissive cells (infection of which did not require Vpx) with restricting ones, the resulting heterokaryons had the restricting phenotype (Sharova et al. 2008).

The finding that HIV-1 Vpr manipulates the Cul4/DDB1DCAF1 ubiquitin ligase (Fig. 3) prompted studies to examine the interaction of the Vpr homologs with DCAF1. It was shown that SIV_{MAC} and HIV-2 Vpr interacted with DCAF1 (Le Rouzic et al. 2007; Wen et al. 2007). Surprisingly, Le Rouzic et al. found that SIVmac Vpx, which is unable to manipulate the cell cycle, also binds to DCAF1 (Le Rouzic et al. 2007). Binding of lentiviral Vpr and Vpx proteins to DCAF1 is mediated by a highly conserved leucine-rich motif (Fig. 1) (Le Rouzic et al. 2007). It is tempting, then, to speculate that Vpr and Vpx have preserved through evolution the ability to recruit DCAF1, although for different purposes. Two recent reports address whether the interaction of Vpx with DCAF1 is required for the enhancement of infectivity of non-dividing cells (Sharova et al. 2008;

Srivastava et al. 2008). These reports show that depletion of DCAF1 via RNA interference (Sharova et al. 2008; Srivastava et al. 2008) or expression of a Vpx mutant, Q76A, devoid of DCAF1 binding (Srivastava et al. 2008) ablated the enhancement of infectivity by Vpx. The role of DCAF1 is still controversial, however, as a recent report by Goujon et al. argues that an HIV-2_{ROD} Vpx clone devoid of DCAF1-association ability (Wen et al. 2007) is fully capable of enhancing infectivity (Goujon et al. 2008). Goujon et al. also showed that depletion of DCAF1 in differentiated THP-1 monocytoid cells had no effect on Vpx function (Goujon et al. 2008).

4 Conclusions and Future Directions

Small, regulatory viral proteins are generally multifunctional. Vpr clearly has the ability to interact with a large number of cellular components, and functional effects for most of these interactions can be documented. Many of these interactions appear specific and have independently been reported by several groups. Yet, our ability to separate those that are relevant to lentiviral replication and pathogenesis from those that are not has only begun to produce results in recent years. An additional complicating factor toward understanding Vpr is that it is dispensable, to a large degree, for viral replication in vitro, while it certainly is maintained and conserved in vivo. Future studies will hopefully define more clearly the structural elements of Vpr that determine each interaction, possibly including the production of co-crystals for x-ray diffraction. Detailed knowledge of these interactions will permit fine structure-function analyzes via mutagenesis. We envision that much future work will also be devoted to the exploration of cellular functions that are manipulated by viral proteins, and how such manipulations lead to the induction of disease, virus propagation, and immune evasion.

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