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Review

Novel approaches to inhibiting HIV-1 replication

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ABSTRACT

Considerable success has been achieved in the treatment of HIV-1 infection, and more than two-dozen antiretroviral drugs are available targeting several distinct steps in the viral replication cycle. However, resistance to these compounds emerges readily, even in the context of combination therapy. Drug toxicity, adverse drug–drug interactions, and accompanying poor patient adherence can also lead to treatment failure. These considerations make continued development of novel antiretroviral therapeutics necessary. In this article, we highlight a number of steps in the HIV-1 replication cycle that represent promising targets for drug discovery. These include lipid raft microdomains, the RNase H activity of the viral enzyme reverse transcriptase, uncoating of the viral core, host cell machinery involved in the integration of the viral DNA into host cell chromatin, virus assembly, maturation, and budding, and the functions of several viral accessory proteins. We discuss the relevant molecular and cell biology, and describe progress to date in developing inhibitors against these novel targets. This article forms part of a special issue of Antiviral Research marking the 25th anniversary of antiretroviral drug discovery and development, Vol 85, issue 1, 2010.

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1. Introduction

1.1. Overview of HIV-1 replication

The replication cycle of human immunodeficiency virus type 1 (HIV-1) is a complex multi-step process that depends on both viral and host cell factors (Figs. 1 and 2) (Freed, 2007). Replication begins with viral entry into the target cell. Entry proceeds by fusion of the viral lipid envelope and the cellular plasma membrane (Doms, 2000). The viral component that mediates fusion is the envelope (Env) glycoprotein spike, which is composed of a trimeric, non-covalently associated complex of the surface glycoprotein gp120 and the transmembrane glycoprotein gp41 (Roux and Taylor, 2007). Fusion is initiated by binding of gp120 to the cellular receptor CD4 and a subsequent interaction with the CCR5 or CXCR4 coreceptor (Berger et al., 1999; Doms, 2000). Coreceptor binding triggers a series of conformation changes in both gp120 and gp41 that mediate membrane fusion (Doms, 2000; Melikyan, 2008). Fusion delivers the viral core into the cytoplasm of the target cell. The viral core is composed of a capsid (CA) protein shell that encapsidates the single-stranded, dimeric viral RNA genome in complex with the viral nucleocapsid (NC) protein and the viral enzymes reverse transcriptase (RT) and integrase (IN) (Adamson and Freed, 2007; Ganser-Pornillos et al., 2008). The core uncoats (Warrilow and Harrich, 2007) and RT copies the RNA genome into a double-stranded DNA copy (Sarafianos et al., 2009), which is transported into the nucleus where IN stably integrates it into the host cell genome (Delelis et al., 2008; Suzuki and Craigie, 2007; Vandegraaff and Engelman, 2007). The host cell cofactor lens epithelium-derived growth factor/transcriptional co-activator 75 (LEDGF/p75) plays an important role in the integration process by tethering IN to chromatin (Poeschla, 2008).

The integrated proviral DNA is transcribed to generate full-length progeny viral RNA and a number of spliced mRNA transcripts that are translated in the cytoplasm (Rabson and Graves, 1997; Swanstrom and Wills, 1997). Transcription and translation, performed by cellular machinery (Bolinger and Boris-Lawrie, 2009; Nekhai and Jeang, 2006), result in the synthesis of several major structural proteins: (i) the Gag polyprotein precursor, which is composed of four domains – matrix (MA), CA, NC and p6 – and two spacer peptides, SP1 and SP2, (ii) the Gag-Pol polyprotein precursor, which is produced via a −1 ribosomal frameshift during *gag* translation and encodes the viral enzymes protease (PR), RT and IN, and (iii) the Env glycoprotein precursor, gp160, which is cleaved into the gp120 and gp41 subunits by a host protease during trafficking through the Golgi apparatus (Swanstrom and Wills, 1997). These protein components, together with full-length viral genomic RNA, are each transported to the site of virus particle assembly at the plasma membrane (Adamson and Freed, 2007). Assembly is directed by Gag, which coordinates the incorporation of each of

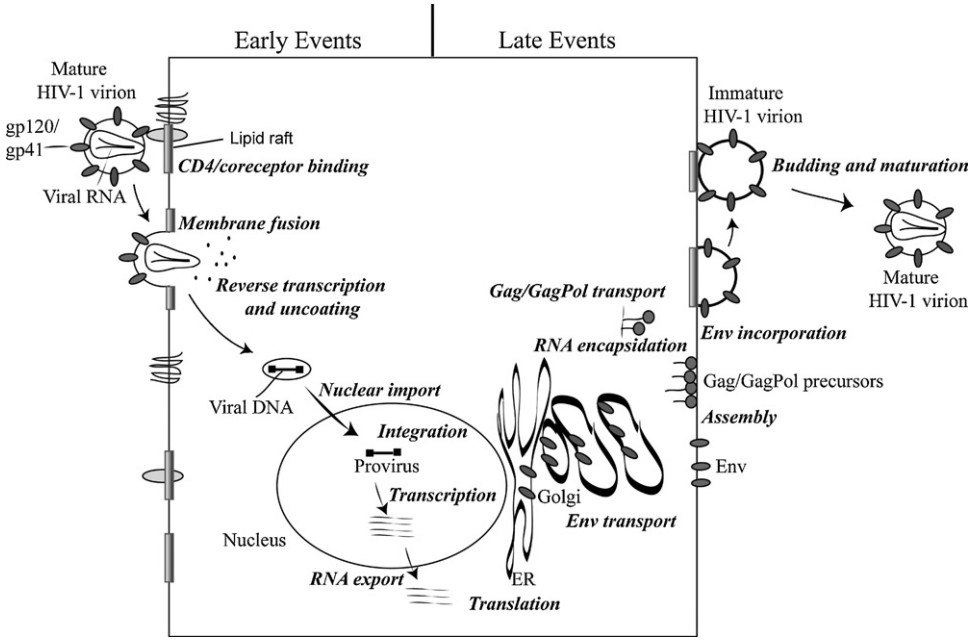

Fig. 1. Schematic representation of the HIV-1 replication cycle. Details are provided in the text. Reprinted with permission from Elsevier (Freed, 2004).

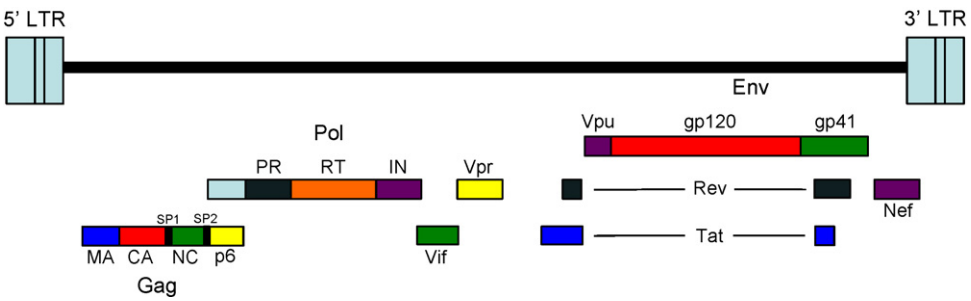

Fig. 2. Organization of the HIV-1 genome. The gene products encoded by HIV-1 include the Gag proteins matrix (MA), capsid (CA), nucleocapsid (NC) and p6 and spacer peptides SP1 and SP2; the Pol proteins protease (PR), reverse transcriptase (RT) and integrase (IN); the surface Env glycoprotein gp120 and the transmembrane Env glycoprotein gp41; the regulatory proteins Tat and Rev; the accessory proteins Vif, Vpr, Vpu, and Nef. Also shown are the 5′ and 3′ long terminal repeats (LTRs).

the viral components, together with a number of host cell factors, into the assembling particle (Adamson and Freed, 2007). Virus particle production is completed upon budding of the nascent virion from the plasma membrane (Adamson and Freed, 2007). To facilitate virus release, the p6 domain of Gag hijacks components of the cellular endosomal sorting machinery, which normally function to promote the budding of vesicles into late endosomes to form multivesicular bodies (MVBs) (Bieniasz, 2009; Demirov and Freed, 2004; Fujii et al., 2007; Morita and Sundquist, 2004). Concomitant with virus release, PR cleaves the Gag and Gag-Pol precursors into their respective protein domains (Swanstrom and Wills, 1997). Gag and Gag-Pol cleavage leads to virion maturation, a reassembly event that produces mature particles containing the condensed, conical core (Adamson and Freed, 2007; Ganser-Pornillos et al., 2008).

In addition to the structural proteins listed above, HIV-1 encodes two regulatory gene products – Tat and Rev – and several accessory proteins: viral infectivity factor (Vif), viral protein U (Vpu), negative factor (Nef), and viral protein R (Vpr) (Fig. 2). Tat transactivates transcription from the HIV long terminal repeat (LTR) by binding to an RNA element (the transactivation response region, or TAR) at the 5′ end of all viral mRNAs. Rev promotes the export of unspliced mRNAs from the nucleus by binding to the Rev responsive element (RRE) in the viral RNA (Freed, 2007). Vif significantly enhances virus infectivity; Vpu stimulates the release of budded particles from the plasma membrane and induces CD4 degradation. Nef, which is an important determinant of viral pathogenesis and disease progression *in vivo*, downregulates surface expression of CD4 and major histocompatibility complex I (MHC-I), modulates cell activation pathways, and enhances particle infectivity. Vpr induces cell-cycle arrest, stimulates transcription from some cellular promoters, influences virus-induced apoptosis, and has been reported to promote nuclear import of the preintegration complex (PIC) following reverse transcription in the newly infected target cell (Freed, 2007).

At virtually every step in its life cycle, HIV-1 takes advantage of host cell factors and pathways to promote successful replication. However, it has become clear in recent years that the host cell has set up antiretroviral barriers in the form of restriction factors that markedly impair specific steps in the replication cycle. For example, tripartite motif protein 5α (TRIM5α) acts at a post-entry step by interacting with CA on the incoming viral core leading to its premature degradation. In some cases, retroviruses have responded by evolving counter-defense mechanisms to overcome these restriction factors. For example, Vif counteracts the cytosine deaminase apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) by inducing its proteasomal degradation, and Vpu counteracts an interferon-induced host protein, variously known as CD317, BST-2, or tetherin, which prevents virus release by "tethering" particles to the plasma membrane.

In this review, we explore a number of unexploited targets for antiretroviral therapy. Many of the approaches discussed here remain hypothetical but may provide future opportunities for drug development. We will cover targets ranging from lipid rafts, RNase H, and LEDGF to budding and maturation, as well as the possibility that information derived from the study of host innate immunity could be applied to the development of novel therapeutics. We will not discuss Tat or Rev as targets as this topic has been treated elsewhere (Baba, 2004; Bannwarth and Gatignol, 2005; Richter and Palu, 2006). We will also not deal with Vpr, as it remains unclear whether this accessory protein is a viable target for drug development.

1.2. Clinically approved antiretroviral drugs

The complex, multi-step HIV-1 replication cycle outlined above offers numerous opportunities for pharmacological intervention. To date, more than 20 antiretroviral drugs have been approved for clinical use. These drugs can be divided into six different mechanistic classes that target distinct steps in the HIV-1 replication cycle. Two drugs have been developed that inhibit virus entry. T20 (enfuvirtide) blocks viral fusion by targeting gp41 and maraviroc acts as a CCR5 antagonist, making it the only currently approved antiretroviral drug that targets a host cell factor. Entry inhibitors are discussed by Doms (2010). The remaining four classes of approved drugs target each of the viral enzymes: RT, PR and IN. Inhibitors targeting RT and PR are the most numerous and successful antiretroviral drugs and combinations of these drugs are the standard initial treatment strategy. RT inhibitors fall into two classes based on their mode of action: the nucleoside-analog RT inhibitors (NRTIs) and non-nucleoside-analog RT inhibitors (NNTRIs), which are discussed by Cihlar (2010) and De Béthune (2010). PR inhibitors (PIs) target the catalytic action of this enzyme (see article by Nijhuis, 2010). The newest antiretroviral drug to achieve widespread clinical use is the IN inhibitor, raltegravir, approved in 2007 (see article by McColl, in this issue).

The antiretroviral drugs mentioned above have significantly extended patient survival (Richman et al., 2009; Simon et al., 2006). Therapy typically consists of a combination of three to four drugs in therapeutic regimens known as highly active antiretroviral therapy (HARRT) (Chen et al., 2007; Simon et al., 2006). The simultaneous use of multiple drugs is required because of the ease with which HIV-1 can acquire drug resistance to any single inhibitor (Emini and Fan, 1997; Simon et al., 2006; Temesgen et al., 2006). Resistance arises due to the high degree of HIV-1 genetic diversity within an individual patient, a consequence of a rapid rate of viral replication combined with the error-prone nature of RT and frequent recombination events (Hu et al., 2003; Simon et al., 2006; Svarovskaia et al., 2003). Despite the positive impact of HAART on patient survival, drug resistance can still emerge even in the face of this multi-drug treatment (Perno et al., 2008; Simon et al., 2006; Temesgen et al., 2006). Drug toxicity, combined with poor patient adherence, can contribute to drug resistance and treatment failure. The serious clinical consequences of multi-drug resistance require the use of alternative treatment regimens, known as salvage therapy (Perno et al., 2008; Temesgen et al., 2006). Salvage therapy is most likely to be effective if new drugs targeting novel sites of action are available (Greene et al., 2008; Perno et al., 2008). As current antiretroviral drugs do not eradicate the virus, patients are required to use HAART on a life-long basis to suppress viral replication (Marsden and Zack, 2009; Richman et al., 2009). Until a cure for HIV infection is achieved, sustained successful treatment of HIV-1-infected patients with antiretroviral drugs may require the development of a continuous pipeline of new drugs. To this end, an intensive research effort into understanding the basic mechanisms governing HIV-1 replication has led to the identification of an array of new therapeutic targets, which have significant potential for future antiretroviral drug development.

2. Novel therapeutic approaches

2.1. Lipid microdomains as a target for antiviral therapy

The plasma membrane of mammalian cells is composed of a variety of microdomains with specific lipid and protein compositions. Lipid rafts are a particularly well-studied class of plasma membrane microdomain, characterized by a high concentration of saturated lipids and cholesterol (Brown and London, 1998). While the function, and even the existence, of lipid rafts has been controversial (Munro, 2003), there is now general agreement that cholesterol- and saturated lipid-enriched microdomains do exist and serve a variety of functions in cell signaling, cell motility and

polarization, intercellular synapse formation, and protein trafficking. Membrane rafts have been defined as "small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions" (Pike, 2006).

Studies from many laboratories have demonstrated that a number of viruses, including HIV-1, use lipid rafts as platforms for both viral entry and particle assembly and release (Ono and Freed, 2005; Waheed and Freed, 2009). Lipid rafts can be analyzed biochemically based on their resistance, relative to non-raft membrane, to solubilization in cold, non-ionic detergents (Brown and Rose, 1992). HIV-1 Gag and Env have been reported to associate with such detergent-resistant membrane (DRM), providing support for the hypothesis that HIV-1 assembly takes place in lipid raft microdomains (Nguyen and Hildreth, 2000; Ono and Freed, 2001). Microscopy-based approaches confirm that Gag and Env colocalize with lipid raft markers (Ono and Freed, 2005) and disruption of lipid rafts with cholesterol-depleting agents inhibits both virus release (Ono and Freed, 2001; Pickl et al., 2001) and virion infectivity (Campbell et al., 2002, 2004; Graham et al., 2003; Guyader et al., 2002; Liao et al., 2001; Manes et al., 2000; Nguyen and Taub, 2002; Popik et al., 2002). Cholesterol depletion interferes with virus particle production by inhibiting the ability of Gag to bind the plasma membrane (Ono et al., 2007). Further support of a raft origin for the assembled particle derives from the finding that the lipid composition of HIV-1 virions is high in raft components, specifically cholesterol and saturated lipids (Aloia et al., 1992, 1993; Brugger et al., 2006; Chan et al., 2008). Taken together, these findings demonstrate that both virus entry and particle egress take place in cholesterol-enriched membrane microdomains. Furthermore, cell–cell transmission of HIV-1 occurs at a "virological synapse" that exhibits a concentration of lipid raft markers (Jolly and Sattentau, 2005).

The use of lipid rafts by HIV-1 at multiple steps in the virus replication cycle opens up the possibility that lipid rafts could in some way be targeted as an antiviral strategy. One report suggested that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) inhibitors (statin drugs), widely used to treat high cholesterol *in vivo*, could lower viral loads in HIV-1-infected patients (del Real et al., 2004). However, subsequent studies failed to reproduce these findings (Moncunill et al., 2005; Probasco et al., 2008; Sklar et al., 2005). Systemic treatment with lipid raft disrupting agents would likely be associated with significant toxicity, making topical treatment in the context of chemoprevention a more realistic approach. Indeed, in a humanized severe combined immunodeficient (SCID) mouse model system, treatment of the vaginal mucosa with a cholesterol-depleting cyclodextrin reduced virus transmission resulting from inoculation with HIV-1-infected human peripheral blood lymphocytes (PBLs) (Khanna et al., 2002). Inhibitors of glycosphingolipid synthesis have also been reported to inhibit HIV-1 infection in culture (for reviews see Puri and Blumenthal, 2008; Waheed and Freed, 2009).

Another approach to interfering with HIV-1 replication by targeting lipid rafts entails the use of cholesterol-binding agents that associate with the virion lipid bilayer. One such compound, amphotericin B methyl ester (AME), has been shown to inhibit both virus particle production and virion infectivity (Waheed et al., 2006, 2008). The infectivity of HIV-1 virions bearing heterologous Env glycoproteins (e.g., from murine leukemia virus or vesicular stomatitis virus) or truncated forms of HIV-1 gp41 is not affected by AME, demonstrating that the long gp41 cytoplasmic tail is required for AME-imposed inhibition of infectivity (Waheed et al., 2006). Consistent with this hypothesis, long-term culture of HIV-1 in the presence of AME led to the emergence of AME-resistant variants that acquired mutations in the gp41 cytoplasmic tail (Waheed et al., 2006). Remarkably, the mechanism by which these gp41 mutations conferred resistance to AME involved the cleavage of the gp41 cytoplasmic tail by the viral PR after Env incorporation into the virion (Waheed et al., 2007). Although amphotericin B is used clinically to treat fungal infections it is highly toxic. AME is reportedly less toxic than its parent compound (Parmegiani et al., 1987); however, long-term systemic treatment is unlikely to be well tolerated. Again, use of AME as a chemopreventive agent warrants consideration.

2.2. Post-entry

2.2.1. Uncoating of the viral core as a potential antiviral target

Fusion of the viral and cellular membranes delivers the core of the mature virus particle into the cytoplasm of the target cell (Fig. 1). Following entry: (a) the core partially disassembles (uncoats) to form the reverse transcription complex (RTC), (b) the viral RNA genome is reverse transcribed into a double-stranded DNA copy, (c) the PIC, which contains the viral DNA, translocates through the nuclear pore, and (d) the viral DNA integrates into the host genome to establish the provirus (Delelis et al., 2008; Freed, 2007; Sarafianos et al., 2009; Suzuki and Craigie, 2007; Warrilow and Harrich, 2007).

The reverse transcription and integration steps are relatively well understood at the molecular level (Delelis et al., 2008; Sarafianos et al., 2009). Antiretroviral drugs targeting the RT and IN enzymes have been successfully developed (see the following articles: Cihlar, 2010; De Béthune, 2010; McColl, in this issue). New approaches to target reverse transcription and integration are discussed in Sections 2.2.2 and 2.2.3 below. In contrast, core uncoating, which converts the viral core into the RTC and ultimately the PIC, is poorly understood. The precise order of events and the exact composition, structure, location and transport pathway of the RTC and PIC remain to be defined (Suzuki and Craigie, 2007; Warrilow and Harrich, 2007). This is currently an active and rapidly progressing field of research. While many details await elucidation, it is clear that correct regulation of core uncoating is essential for completion of the early steps of the HIV-1 replication cycle. Mutations in CA that affect core stability but not core formation result in impaired reverse transcription and infection (Brun et al., 2008; Fitzon et al., 2000; Forshey et al., 2002). Furthermore, the vulnerability of core uncoating has been highlighted by the discovery of the host restriction factor, TRIM5α, which appears to target this early step in retroviral replication (Bieniasz, 2004; Luban, 2007; Ozato et al., 2008; Stremlau et al., 2004; Towers, 2007).

The mechanism by which TRIM5α exerts its antiretroviral effect is not fully understood. However, it is known that TRIM5α targets intact or partially uncoated incoming viral cores via a pattern-recognition function that identifies a structure formed by the CA lattice (Forshey et al., 2005; Hatziioannou et al., 2004a; Kar et al., 2008; Langelier et al., 2008; Sebastian and Luban, 2005; Shi and Aiken, 2006; Stremlau et al., 2006) (see Section 2.4.2 for further details of the CA lattice). The block to replication occurs at a post-entry but pre-reverse transcription step, such that completion of reverse transcription is inhibited (Besnier et al., 2002; Cowan et al., 2002; Munk et al., 2002; Shibata et al., 1995; Stremlau et al., 2004; Towers et al., 2000). It has been suggested that TRIM5α blocks retroviral replication either by causing cores to undergo rapid and premature disassembly (Perron et al., 2007; Stremlau et al., 2006) and/or by recruiting cellular proteasomal degradation machinery (Anderson et al., 2006; Campbell et al., 2008; Rold and Aiken, 2008; Wu et al., 2006). Importantly, the antiretroviral effects of TRIM5α are exerted in a species-specific manner (Bieniasz, 2004; Hatziioannou et al., 2004b; Keckesova et al., 2004; Luban, 2007; Ozato et al., 2008; Perron et al., 2004; Stremlau et al., 2004; Towers, 2007; Yap et al., 2004). For example, HIV-1

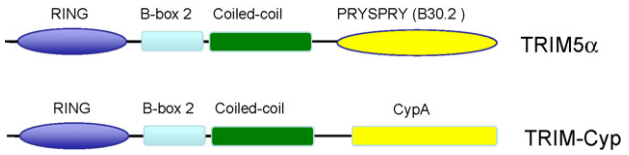

Fig. 3. Domain organization of TRIM5α and TRIM-Cyp. The major domains of TRIM5α – RING, B-box 2, coiled-coil, and PRYSPRY (B30.2) – are indicated. In TRIM-Cyp, the PRYSPRY (B30.2) domain has been replaced with cyclophilin A (Cyp). Details are provided in the text.

replication is potently blocked by TRIM5α from the non-human primate rhesus macaque (TRIM5α_{rh}); in contrast, human TRIM5α (TRIM5α_{hu}) is largely ineffective against HIV-1 but does inhibit other retroviruses.

The specificity of retroviral restriction correlates with the ability of TRIM5α to recognize the incoming viral core and has been mapped to a CA recognition domain in TRIM5α (Bieniasz, 2004; Luban, 2007; Ozato et al., 2008; Towers, 2007). TRIM family proteins are composed of three domains (Fig. 3): (i) a RING-finger, (ii) a B-box, and (iii) a coiled-coil domain, which together form the tripartite RBCC domain (Ozato et al., 2008). The α splice variant of TRIM5 (TRIM5α) contains an additional C-terminal PRYSPRY (or B30.2) domain (Reymond et al., 2001). The PRYSPRY domain is the main determinant of core recognition, CA-binding, and hence restriction specificity (Kar et al., 2008; Langelier et al., 2008; Li et al., 2006; Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2004, 2005, 2006; Yap et al., 2005). The degree of CA-binding and restriction potency correlates with amino-acid variations in the PRYSPRY domain and, remarkably, a single-amino-acid substitution in the PRYSPRY domain is sufficient to enable TRIM5α_{hu} to restrict HIV-1 (Langelier et al., 2008; Li et al., 2006; Stremlau et al., 2005; Yap et al., 2005). Conversely, mutations in CA give rise to differential susceptibility of incoming cores to TRIM5α restriction (Hatziioannou et al., 2004a; Li et al., 2006; Owens et al., 2004; Stremlau et al., 2006).

Interestingly, in some primate species (e.g., owl monkey) the PRYSPRY domain has been replaced by cyclophilin A (CypA) to generate what is referred to as a "TRIM-Cyp" fusion (Luban, 2007; Nisole et al., 2004; Sayah et al., 2004; Stoye and Yap, 2008). The CypA portion of TRIM-Cyp binds CA (Gamble et al., 1996; Luban et al., 1993) and disruption of this interaction abolishes the restriction activity of TRIM-Cyp (Towers et al., 2003). Indeed, fusion of CypA to the C-termini of non-restricting TRIM proteins (e.g., TRIM1, 18 or 19) generated functional HIV-1 restriction factors (Yap et al., 2006). The CypA portion of TRIM-Cyp therefore replaces the CA-binding function of the PRYSPRY domain in TRIM5-mediated retroviral restriction.

CA binding recruits the N-terminal tripartite RBCC domain to the viral core. Each of the RBCC domains contributes to efficient retroviral restriction. TRIM proteins assemble in cells into low molecular weight oligomers (dimers or trimers), as well as large aggregations known as cytoplasmic bodies (Kar et al., 2008; Langelier et al., 2008; Mische et al., 2005; Stremlau et al., 2004). The primary function of the coiled-coil domain is to mediate oligomerization to increase CA-binding (Javanbakht et al., 2006; Mische et al., 2005; Rold and Aiken, 2008; Yap et al., 2007). The role of the B-box is the least well understood but it has been implicated in several functions, including the capacity to induce higher-order self-association of TRIM5α oligomers to promote cooperative binding to the multimeric retroviral CA (Li and Sodroski, 2008). The ring domain possesses E3 ubiquitin ligase activity and is thought to recruit cellular proteasomal degradation machinery that plays an important role in TRIM5α restriction (Anderson et al., 2006; Campbell et al., 2008; Rold and Aiken, 2008; Wu et al., 2006; Xu et al., 2003; Yamauchi et al., 2008).

TRIM5α provides a conceptual framework for the development of novel antiretroviral strategies that target the vulnerable incoming viral core and disrupt early events in HIV-1 replication. As TRIM5α_{hu} is ineffective against HIV-1, simple use of interferon to induce its expression (Asaoka et al., 2005; Sakuma et al., 2007a) is unlikely to be therapeutically effective. An alternative strategy is to use gene delivery to express an isoform of TRIM5α that potently inhibits HIV-1. As described above, expression of non-human primate TRIM5α's, such as TRIM5α_{rh}, in human cells restricts HIV-1. However, these non-human TRIM5α's are likely to be recognized as foreign *in vivo* and to provoke an immune response. The restriction specificity of TRIM5α can be manipulated by genetically engineering the CA-recognizing PRYSPRY domain. Chimeric human-rhesus TRIM5α molecules may therefore be more immunologically tolerated for a gene therapy application (Anderson and Akkina, 2008). The gene therapy approach has yielded initial promising results *in vitro* in a range of human cell types including $CD34^+$ hematopoietic progenitor stem cells that give rise to both T cells and macrophages, and *in vivo* in SCID-hu mouse-derived thymocytes (Anderson and Akkina, 2005, 2008; Sakuma et al., 2007b). While theoretically possible, a TRIM5α-mediated gene therapy approach is not currently practical for treating HIV-1 infected patients.

Small molecules are a more realistic and cost-effective strategy for antiretroviral therapy. TRIM5α-like molecules that restrict HIV-1 could potentially be developed; however, the requirements for restriction are complex, involving multiple protein domains that function as part of an oligomer. Indeed, a study to design artificial restriction factors concluded that multimerization of a CA-binding domain could be the common minimal design feature for CA-dependent retroviral restriction (Yap et al., 2007). The design of a peptide with restriction activity that is capable of delivery into the cell therefore seems unrealistic. However, it is worth noting that in a recent study the peptide CAI that inhibits virus assembly and maturation by binding to CA (see Sections 2.3.2 and 2.4.2) was also observed to dismantle CA tubes preassembled *in vitro*, suggesting that CAI binding to CA may also affect core stability (Barklis et al., 2009). An alternative approach could exploit the fact that a single-amino-acid substitution is sufficient to enable TRIM5α_{hu} to inhibit HIV-1, suggesting that a conformational change can transform TRIM5α_{hu} into an effective restriction factor (Li et al., 2006). Binding of a small molecule to TRIM5α_{hu} could perhaps induce such a conformational change. This approach would present a major challenge, as most drugs are designed to either disrupt an interaction or inhibit an enzymatic activity rather than to elicit a gain-of-function interaction between two binding partners. Also, a suitable high-throughput screen to identify such molecules would need to be developed. Further elucidation of the mechanism of restriction will be required to advance these and other future therapeutic strategies. A significant advance will be provided by high-resolution structures of TRIM5α or TRIM-Cyp bound to the viral core.

2.2.2. *RNase H inhibitors*

RT plays an essential role in HIV-1 replication, as it copies viral RNA into double-stranded DNA for integration into the host cell genome (Freed, 2007). This is achieved through the use of two distinct enzymatic activities (Champoux and Schultz, 2009; Sarafianos et al., 2009; Schultz and Champoux, 2008). First, as a DNA polymerase, RT copies an RNA and then a DNA template to generate minus- and plus-strand viral DNA, respectively. Second, RT possesses an RNase H activity (Fig. 4) that degrades the RNA strand within the RNA–DNA duplex(es) formed during minus-strand DNA synthesis. This activity also serves to create the plus-strand primers required for initiation of plus-strand DNA synthesis, as well as to remove the minus- and plus-strand primers once synthesis is complete. Inhibitors that target RT polymerase activity have

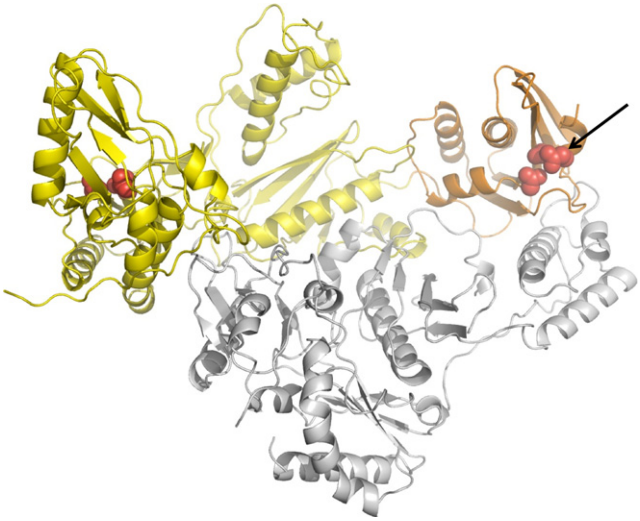

Fig. 4. Structure of HIV-1 RT. The polymerase domain is in yellow, RNase H in orange, and p51 subunit in gray. The polymerase and RNase H active sites are highlighted in red and the RNase H active site is also indicated with an arrow (Das et al., 2008). We thank Kalyan Das and Eddy Arnold for providing the figure.

been highly successful, with multiple drugs routinely used to treat HIV-1-infected patients (see Cihlar, 2010; De Béthune, 2010). Development of drugs targeting RNase H activity, however, has proven to be more difficult, and no RNase H inhibitors (RNHIs) have been approved for clinical use (Jochmans, 2008; Klumpp and Mirzadegan, 2006; Yu et al., 2008). Nevertheless, RNase H remains an attractive drug target, as its activity is essential for HIV-1 replication (Tisdale et al., 1991).

Efforts to develop RNHIs have been hampered by a lack of therapeutic value, with the limited number of promising candidates tested *in vitro* frequently encountering problems of poor cellular uptake or cytotoxicity or the targeting of activities other than RNase H (Jochmans, 2008; Klumpp and Mirzadegan, 2006; Yu et al., 2008). Despite these setbacks, recent development of high-throughput RNase H assays, as well as advances in our understanding of the mechanisms of RNase H activity, have reinvigorated RNHI development efforts. The RNase H domain was the first fragment of HIV-1 RT for which the structure was solved (Davies et al., 1991). Structures of RNase H's from several sources in complex with RNA/DNA hybrid substrates have provided potential clues for future antiretroviral drug exploration (Nowotny et al., 2005, 2007; Sarafianos et al., 2001). Structural information has also contributed to an increased understanding of the molecular mechanisms of RNase H activity (Nowotny et al., 2005, 2007; Nowotny and Yang, 2006; Yang et al., 2006). Specifically, it has become clear that HIV-1 RNase H utilizes a two metal ion-dependent mechanism of catalysis, and residues critical for positioning the RNA strand within the RNase H active site, as well as the catalytic residues themselves, have been identified (Cristofaro et al., 2002; Davies et al., 1991; Klumpp et al., 2003).

An important class of RNHIs targets the metal ion requirement of RNase H. Small molecules that act by this mechanism can be divided into three major groups: (i) N-hydroxyimides (Hang et al., 2004; Klumpp et al., 2003), (ii) diketo acids (Shaw-Reid et al., 2003; Tramontano et al., 2005) and (iii) hydroxylated tropolones (Beilhartz et al., 2009; Budihas et al., 2005; Didierjean et al., 2005). Other classes of RNHIs with alternative or undefined mechanisms of action include: (i) hydrazones (Borkow et al., 1997; Himmel et al., 2006), (ii) vinologous ureas (Wendeler et al., 2008), (iii) napthoquinones (Min et al., 2002) and (iv) small nucleic acid fragments (aptamers) (Hannoush et al., 2004; Somasunderam et al., 2005). Cellular uptake and cytotoxicity issues remain challenging; however, a small number of molecules with acceptable levels of cytotoxicity have been shown to exhibit antiviral activity in cells (Borkow et al., 1997; Somasunderam et al., 2005; Tramontano et al., 2005). Moreover, inhibitor screening has recently become more sophisticated, as lead compounds are scored not only according to the degree with which they inhibit retroviral RNase H, but also on whether they inhibit human RNase H (Budihas et al., 2005). Despite recent progress, none of the current RNHIs have entered clinical trials and an outstanding lead candidate whose antiviral properties can be definitively linked to RNase H activity has yet to be identified (Jochmans, 2008; Klumpp and Mirzadegan, 2006; Yu et al., 2008).

2.2.3. LEDGF/p75 as a novel target for integration inhibitors

As mentioned above, after reverse transcription and nuclear import of the newly synthesized viral DNA, IN catalyzes the insertion of the viral DNA into the host cell genome. While purified IN can mediate most aspects of the integration reaction *in vitro*, numerous studies have demonstrated that other proteins of both viral and cellular origin enhance the efficiency of integration and are likely to be important for integration in the context of a viral infection. The host factor that has received the most attention in recent years is LEDGF/p75 (for review see Engelman and Cherepanov, 2008). This chromatin-associated protein is thought to normally function in transcriptional regulation in stress response and apoptosis pathways (Ganapathy et al., 2003). A role for LEDGF/p75 in lentiviral integration was first suggested by studies that identified an interaction between this protein and HIV-1 IN (Cherepanov et al., 2003; Emiliani et al., 2005). Depletion of LEDGF/p75 shifted the localization of exogenously expressed IN from the nucleus to the cytoplasm, implying a functional relevance for this interaction. Initial studies on the effect of LEDGF/p75 depletion on HIV-1 infectivity produced conflicting and inconsistent results. However, more complete knock-downs, or the use of mouse embryo fibroblasts derived from LEDGF/p75 knock-out mice, revealed a significant loss in HIV-1 infectivity in the absence of this host factor (Llano et al., 2006; Shun et al., 2007). The defect imposed by LEDGF/p75 depletion is at the level of integration, and infectivity of retroviruses that do not interact with LEDGF/p75 is not affected by LEDGF/p75 disruption. Furthermore, overexpression of the IN-binding domain (IBD) of LEDGF/p75 also imposes a severe defect in HIV-1 infectivity (De Rijck et al., 2006; Llano et al., 2006). The preferential targeting of HIV-1 integration to actively transcribed regions of the host cell genome (Schroder et al., 2002) is to a large extent eliminated by LEDGF/p75 knock-down (Ciuffi et al., 2005; Marshall et al., 2007; Shun et al., 2007).

LEDGF/p75 is a multidomain protein that not only binds IN via its IBD but also bears several motifs that function in DNA and chromatin binding (Fig. 5). These include a nuclear localization signal (NLS) and two AT hooks (so named for their preferential binding to AT-rich DNA), and a PWWP motif that binds chromatin (reviewed in Engelman and Cherepanov, 2008). Structural studies have elucidated the basis for the interaction between IN and the IBD of LEDGF/p75. IN is known to form multimers (dimers and tetramers) and an interhelical loop in the LEDGF/p75 IBD fits into a cleft formed by the dimer interface of IN (Cherepanov et al., 2005). While the catalytic core of IN is primarily responsible for binding to LEDGF/p75, the N-terminal domain of IN contributes charge–charge interactions (Hare et al., 2009).

The demonstrated importance of the association between IN and LEDGF/p75 in HIV-1 integration raises the possibility that this interaction could be exploited as an antiviral target. Indeed, overexpression of the LEDGF/p75 IBD was shown to significantly inhibit HIV-1 replication (De Rijck et al., 2006). Resistance to the IBD fragment arose during virus passaging; this resistance was conferred by mutations in IN that map to the IN/IBD interface. Interestingly,

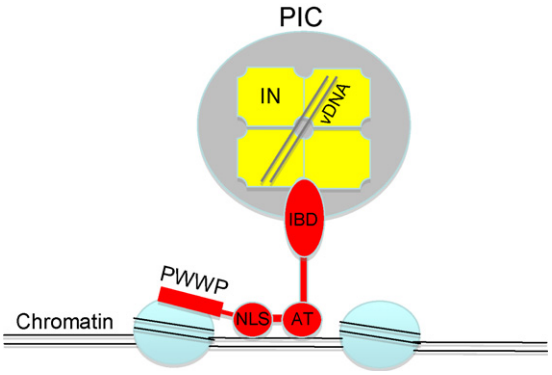

Fig. 5. Schematic representation of newly synthesized viral DNA tethered to chromatin by LEDGF. The preintegration complex (green) containing a tetramer of IN (yellow) and double-stranded viral DNA (vDNA) is shown. The IN-binding domain (IBD) of LEDGF is shown in association with the IN tetramer. The nuclear localization signal (NLS) and AT hooks (AT) are shown bound to DNA; the PWWP domain is depicted bound to histone proteins. Note that it remains to be established to what extent the PIC is intact at the stage of chromatin tethering.

replication of the IBD-resistant virus was even more sensitive to LEDGF/p75 depletion than was that of the WT virus, indicating that the resistant mutant did not replicate in a LEDGF/p75-independent manner (Hombrouck et al., 2007). To identify small molecules that disrupt the interaction between IN and LEDGF/p75, Du et al. (2008) performed a small-scale screen based on yeast and mammalian two-hybrid assays in which reductions in IN-LEDGF/p75 binding could be detected. A benzoic acid derivative, 4-[(5-bromo-4-{2,4-dioxo-3-(2-oxo-2-phenylethyl)-1,3-thiazolidin-5-ylidenemethyl}-2-ethoxyphenoxy)methyl]benzoic acid (D77) scored positive in these assays. Surface plasmon resonance (SPR) analysis demonstrated a direct interaction between D77 and the catalytic core domain of IN (with K_d ~6 μM). Molecular docking and mutational analyses suggested that D77 binds at the dimer interface of IN. In cell-based assays, D77 disrupted the nuclear localization of IN and displayed some antiviral activity (EC_{50} ~20 μg/ml).

A larger screen for inhibitors of the IN-LEDGF/p75 interaction was performed by using a luminescent proximity (AlphaScreen™) assay with the LEDGF/p75 IBD and the IN catalytic core domain (Hou et al., 2008). A library of 700,000 small molecules was screened and ~90 compounds were shown to selectively inhibit IN-LEDGF/p75 binding. One representative compound disrupted integration *in vitro*. The ability of the putative inhibitors to interfere with integration in cell-based assays and to elicit antiviral activity awaits further testing.

Hayouka et al. (2007) used a more directed approach by synthesizing peptides derived from LEDGF/p75. These peptides bound to IN, reportedly shifting the IN multimer from the dimeric form, which is competent to bind DNA, to the tetramer, which is not DNA-binding proficient (Faure et al., 2005). These peptides disrupted *in vitro* integration and inhibited HIV-1 infectivity and replication in cell culture (Hayouka et al., 2007).

As with any strategy that targets a cellular gene, the toxicity of LEDGF/p75-based inhibitors is an important concern. Although LEDGF/p75 knock-out mice display a high level of embryonic lethality (Sutherland et al., 2006), LEDGF/p75 disruption in cell culture appears to be well tolerated. More importantly, LEDGF/p75-based antiviral strategies currently being developed (see above) are aimed at disrupting the IN-LEDGF/p75 interaction rather than suppressing expression of the host factor itself; such inhibitors could, in principle, target either the host (LEDGF/p75) or viral (IN) protein. The LEDGF/p75 IBD may engage in interactions with vital cellular partners, and indeed, the C-terminus of LEDGF/p75 has been shown to interact with the host factor JP02 (Bartholomeeusen et al., 2007; Maertens et al., 2006). However, IN and JP02 appear to utilize non-overlapping interfaces in their interactions with LEDGF/p75 (Bartholomeeusen et al., 2007). It is therefore likely that inhibitors of IN-LEDGF/p75 binding can be developed that do not disrupt the interaction between LEDGF/p75 and its cellular partners.

2.3. Assembly and release

The HIV-1 Gag precursor protein, $Pr55^{Gag}$, is the sole viral component required to form immature, non-infectious VLPs in Gag-expressing cells. The production of infectious particles requires coexpression of Gag with the Env glycoproteins, and the *pol*-encoded enzymes PR, RT, and IN. Assembly takes place in a series of discrete steps. The MA domain directs Gag to the host cell plasma membrane where it anchors Gag in the inner leaflet of the lipid bilayer. The MA domain also plays a crucial role in recruiting the viral Env glycoproteins into nascent virus particles. The CA domain, together with SP1 and NC, mediates Gag–Gag interactions that promote particle assembly. NC also interacts with the full-length viral genomic RNA to package two RNA copies into each virus particle. Sequences in p6 known as "late domains" interact with cellular endosomal sorting machinery to promote virus budding and release from the infected cell. Concomitant with virus release, PR cleaves the Gag and GagPol precursor proteins to initiate the maturation process.

There are currently no approved drugs that target Gag or any aspect of the virus assembly pathway. However, as our understanding of assembly has increased, a variety of assembly- and maturation-based targets have emerged as potential targets for novel antiviral therapies.

2.3.1. MA

The MA domain of Gag (Fig. 2) serves several important functions in HIV-1 particle production. It provides the signals necessary to target Gag to the site of assembly, which in most instances is the plasma membrane. The N-terminus of MA is modified with a covalently attached myristic acid moiety that directly inserts into the lipid bilayer. Membrane binding is also promoted by a highly basic patch of amino-acid residues near the N-terminus of the MA domain (residues ~17–31); this positively charged region interacts with negatively charged phospholipids on the inner leaflet of the lipid bilayer. Mutations that block myristylation abolish Gag-membrane binding (Bryant and Ratner, 1990; Freed et al., 1994; Gottlinger et al., 1989), whereas disruption of the basic residues causes Gag to be relocalized to late endosomes or MVBs (Ono et al., 2004). The localization of a number of cellular proteins that contain bipartite membrane binding domains composed of one or multiple acyl groups and a highly basic patch of amino acids is regulated by a family of lipid molecules known as the phosphoinositides. These lipids form a family of molecules that differ from one another in the position and number of phosphates on the inositol headgroup (De Camilli et al., 1996). Different phosphoinositides are enriched on specific membranes within the cell; for example, phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$] is predominantly localized on the inner leaflet of the plasma membrane; PI(3)P is found on early endosomal membranes, and $PI(3,5)P_2$ is concentrated on late endosomes (De Matteis and Godi, 2004). To test whether plasma membrane-localized $PI(4,5)P_2$ plays a role in HIV-1 Gag targeting, $PI(4,5)P_2$ was depleted from the plasma membrane by overexpressing enzymes involved in phospholipid metabolism (Ono et al., 2004). This $PI(4,5)P_2$ disruption induced a relocalization of Gag to late endosomes, recapitulating the phenotype of MA basic residue mutations, and markedly inhibited particle production (Ono et al., 2004). These findings raised the possibility that the basic residues in MA engage in direct inter-

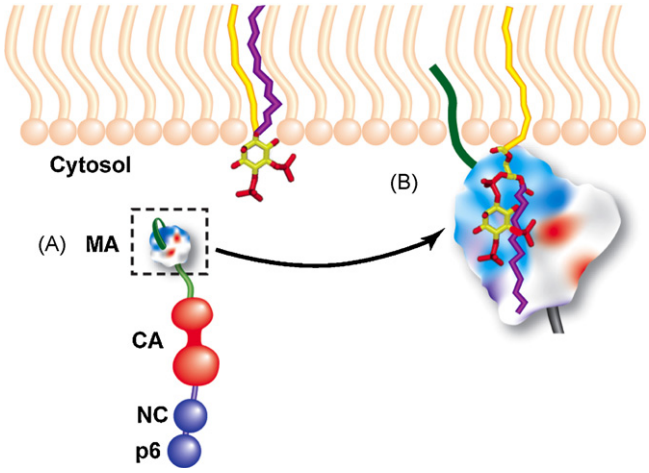

Fig. 6. Model for MA binding to PI(4,5)P_2. (A) shows the unbound Gag monomer, with the myristic acid moiety (dark green) in the sequestered conformation. Unbound PI(4,5)P_2 is shown with both 1′- and 2′-acyl chains (yellow and purple, respectively) embedded in the inner leaflet of lipid bilayer. (B) shows MA bound to PI(4,5)P_2, with the myristic acid in the exposed conformation and embedded in the lipid bilayer, basic residues of MA (blue) engaged in electrostatic interactions with negative charges on PI(4,5)P_2, and the 2′-acyl chain extruded from the bilayer and packed into a hydrophobic groove in MA. This model is based on the NMR study of Saad et al. (2006). Reprinted from Freed (2006).

actions with PI(4,5)P_2. This hypothesis was validated by nuclear magnetic resonance (NMR) spectroscopy (Saad et al., 2006) and by a mass spectrometric protein footprinting approach (Shkriabai et al., 2006). In the Saad et al. study, the structure of the MA domain bound to a soluble, truncated PI(4,5)P_2 derivative was determined (Fig. 6); two particularly interesting observations were made: (1) binding of MA to PI(4,5)P_2 induces the exposure of the N-terminal myristate, which in its membrane-unbound state equilibrates between an exposed and a sequestered conformation, and (2) the 2′-acyl chain of PI(4,5)P_2 packs into a hydrophobic cleft in MA. Whether such a conformation would be energetically favorable in the context of Gag bound to membrane-embedded PI(4,5)P_2 remains to be determined. If so, the hydrophobic cleft into which the 2′-acyl chain packs could be targeted by small molecules that would block PI(4,5)P_2 binding. HIV-2 and EIAV MA proteins have also been found to bind PI(4,5)P_2 (Chen et al., 2008; Saad et al., 2008) suggesting that interaction with this phosphoinositide may be a general strategy used by retroviruses to target the plasma membrane.

As mentioned above, the myristic acid moiety switches between an exposed and a sequestered conformation; in the sequestered conformation it packs into a hydrophobic pocket distinct from the putative PI(4,5)P_2-binding cleft (Tang et al., 2004). Successful targeting of this hydrophobic cavity with small molecules would in theory deregulate the myristyl switch, leading to defects in virus assembly. Finally, MA promotes the incorporation of the Env glycoprotein complex into virions, either via a direct gp41–MA interaction or with the assistance of a host factor (e.g., TIP-47; Diaz and Pfeffer, 1998; Lopez-Verges et al., 2006) that bridges gp41 and MA. Although we currently have limited structural information about how the gp41 cytoplasmic tail fits in the assembled MA lattice, any molecule that would be capable of disrupting the gp41–MA interaction, and thereby inhibit Env incorporation, would be predicted to display significant antiviral activity.

2.3.2. CA

The CA domain (Fig. 2), together with SP1 and NC, mediates Gag–Gag interactions that are required for immature virus particle assembly (Adamson and Freed, 2007). Approximately 2400-5000 Gag molecules multimerize to form a spherical shell (Briggs et al., 2004; Carlson et al., 2008). Cryo- and high-resolution electron microscopy have allowed visualization of Gag within the immature particle. Gag molecules are rod-shaped and packed side-by-side in a radial arrangement (Fuller et al., 1997; Wilk et al., 2001; Yeager et al., 1998). The N-terminal MA domain is associated with the membrane and the C-terminus of Gag is orientated towards the particle center (Fuller et al., 1997; Wilk et al., 2001; Yeager et al., 1998). In the immature VLP, the Gag molecules form a continuous yet incomplete hexameric lattice with irregular defects that accommodate curvature (Briggs et al., 2009; Wright et al., 2007). The CA domain is the major determinant of the ordered lattice (Briggs et al., 2009; Wright et al., 2007).

CA is composed of two structurally independent and largely helical domains, known as the CA N-terminal (CA_{NTD}) and C-terminal (CA_{CTD}) domains, which are separated by a short flexible interdomain linker (Fig. 7A) (Adamson et al., 2009a; Ganser-Pornillos et al., 2008). Mutagenesis of the CA_{NTD} suggests that it does not play a major role in driving Gag assembly (Accola et al., 1998; Borsetti et al., 1998; von Schwedler et al., 2003). However, the CA_{CTD} plays an important role in Gag multimerization. CA_{CTD} forms a dimer (Gamble et al., 1997; Rose et al., 1992; Worthylake et al., 1999), the disruption of which leads to a significant reduction in Gag intermolecular interactions *in vitro* (Burniston et al., 1999) and particle production in cells (Gamble et al., 1997; von Schwedler et al., 2003). However, assembly is not completely abolished by disruption of the CA_{CTD} dimer, demonstrating that the CA_{CTD} dimer interface is not absolutely required for VLP assembly. Near the N-terminus of the CA_{CTD} is the major homology region (MHR), a stretch of 20 residues highly conserved across retroviral genera (Gamble et al., 1997; Wills and Craven, 1991) that forms an intricate array of hydrogen bonds in the CA crystal structure (Gamble et al., 1997). Amino-acid substitutions in the MHR lead to defects in assembly (Mammano et al., 1994; von Schwedler et al., 2003). A high degree of structural homology between the MHR and a mammalian SCAN domain led to the hypothesis that the MHR forms a domain-swapped dimer (Ivanov et al., 2005; Kingston and Vogt, 2005). However, limited experimental evidence has so far corroborated this model. Therefore, the contribution of the MHR to Gag multimerization and assembly remains ill defined.

The last 12 residues of CA and the adjoining SP1 project from the base of the globular CA_{CTD}. This region is highly flexible and thus disordered in crystal structures (Gamble et al., 1997; Newman et al., 2004; Worthylake et al., 1999) but appears to possess helical character (Accola et al., 1998; Morellet et al., 2005; Newman et al., 2004). A recent cryo-electron tomography study of immature particles proposed that this domain forms a six-helix bundle that stabilizes the CA hexamer (Wright et al., 2007). Indeed, genetic studies have demonstrated that this region of Gag forms a critical assembly domain, which mediates strong Gag–Gag interactions that lead to higher-order multimerization (Abdurahman et al., 2004; Accola et al., 1998, 2000; Guo et al., 2005; Krausslich et al., 1995; Liang et al., 2002, 2003; Melamed et al., 2004; Morikawa et al., 2000; Ono et al., 2000; von Schwedler et al., 2003).

In principle, assembly could be disrupted therapeutically by small molecules that bind to critical assembly domains within CA. The mature CA protein also drives a second assembly event to form the viral core following Gag proteolytic cleavage (see Section 2.4.2). Inhibitors that bind CA could therefore disrupt the assembly of both immature VLPs and cores. One such dual inhibitor, CA assembly inhibitor (CAI), has been reported. CAI is a peptide identified in a phage display screen that used both full-length CA and a protein fragment consisting of CA_{CTD}/SP1/NC (C-CANC) as bait (Sticht et al., 2005). CAI inhibits *in vitro* assembly of spherical particles that are structurally analogous to immature VLPs (Sticht et al., 2005). Assembly of tubular structures, in which CA is organized as in the mature viral core, was also disrupted

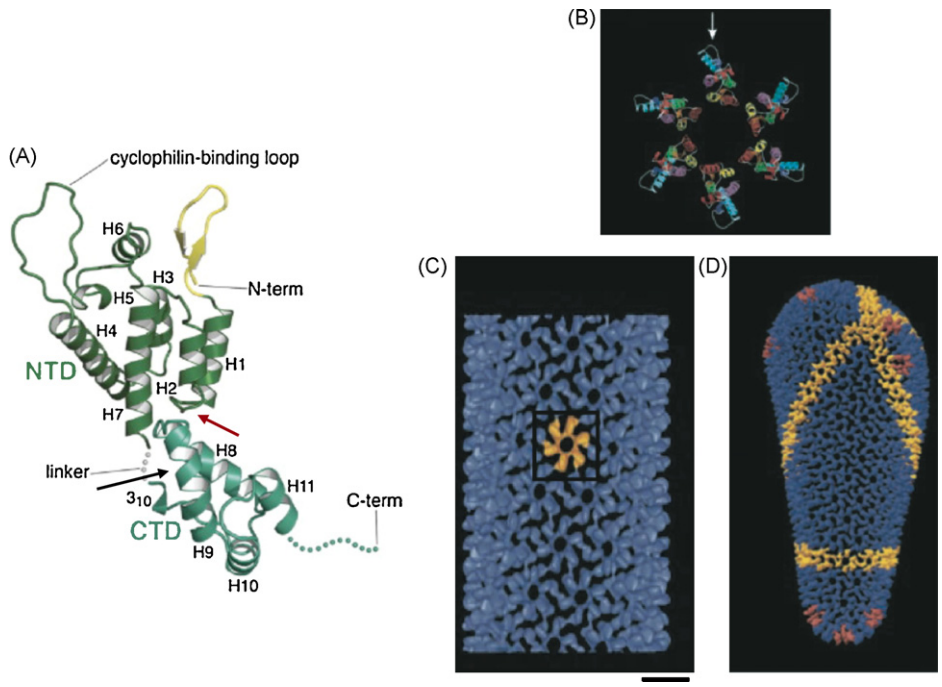

Fig. 7. Structure of HIV-1 CA. (A) Structure of monomeric CA, with the CA_{NTD} (green) and CA_{CTD} (blue/green) indicated. The interdomain linker, N- and C-termini, and cyclophilin A binding loop are shown. Helices 1–11 and the N-terminal β-hairpin (yellow) are labeled. Binding sites for CAP1 and CAI/NYAD-1/NYAD-13 are indicated by red and black arrows, respectively. Reprinted with permission from Elsevier (Ganser-Pornillos et al., 2008). (B) Molecular model of the CA_{NTD} hexameric ring; cyclophilin A binding loop indicated with an arrow. (C) Outside view of an assembled CA tube, showing the CA hexameric lattice. One CA hexamer is shown in yellow. Scale bar = 100 Å. (D) Molecular model of an HIV-1 conical core. A line of hexamers is shown in yellow; pentamers are depicted in red at each end of the conical core. Adapted with permission from Macmillan Publishers Ltd: [Nature], (Li et al., 2000), http://www.nature.com/nature/index.html .

(Sticht et al., 2005). CAI had no effect on virus assembly in cells due to its inherent cell impermeability. This problem was resolved by using hydrocarbon stapling to generate cyclical cell-penetrating derivatives of CAI, termed NYAD-1 and -13 (Bhattacharya et al., 2008; Zhang et al., 2008). In cells, NYAD-1 exhibited an antiviral effect and assembly of both immature and mature-like virus particles was disrupted (Zhang et al., 2008).

CAI and its derivatives target the CA_{CTD} (Fig. 7A) (Bhattacharya et al., 2008; Sticht et al., 2005; Ternois et al., 2005). A high-resolution X-ray structure of CAI in complex with CA_{CTD} reveals that the peptide binds to a hydrophobic groove in an α-helical conformation (Ternois et al., 2005). Binding of CAI appears to allosterically disrupt the CA dimer interface and interfere with contacts between the CA_{NTD} and CA_{CTD} (Bartonova et al., 2008; Bhattacharya et al., 2008; Sticht et al., 2005; Ternois et al., 2005). Understanding the consequences of CAI binding for assembly of viral cores has been possible due to detailed structural information on the interactions involved (see Section 2.4.2 and Adamson et al., 2009a; Ganser-Pornillos et al., 2008). However, the effect of CAI binding on immature particle assembly is less clear given that Gag–Gag interfaces required for VLP assembly are not fully defined. It is tempting to speculate that the effect of CAI binding at the dimer interface may be important, given the role of this region of CA_{CTD} in immature particle assembly.

Although the issue of CAI cell permeability has been addressed by the generation of NYAD-1 and 13, these molecules bind with relatively low affinity (Sticht et al., 2005; Zhang et al., 2008) and are therefore not likely to progress to the clinic. They do, however, represent significant leads for the development of future HIV-1 assembly inhibitors. Given that Gag–Gag interactions appear to involve several distinct domains, it might be considered surprising that the screen by Sticht et al. identified a group of related peptides that bind to a single reactive site in CA (Sticht et al., 2005). The fact that CAI binds to a hydrophobic groove might explain the favorability of this site, as defined pockets to which an inhibitor can bind are more likely to yield successful drug candidates. The fact that assembly inhibitors will target interacting surfaces that may be relatively flat and cover large areas, and are therefore problematic for drug discovery, represents a significant future challenge for the development of this class of antiretrovirals (Arkin and Wells, 2004).

2.3.3. NC

HIV-1 NC (Fig. 2) is a small (7 kDa), highly basic protein characterized by the presence of two zinc-finger motifs. These zinc fingers are critical to several of NC's functions; indeed, the presence of one or two zinc fingers in NC is one of the most highly conserved features of retroviral Gag proteins. The consensus zinc-coordinating motif found in retroviral NC proteins is Cys-X_2-Cys-X_4-His-X_4-Cys (CCHC); this sequence is somewhat atypical for cellular zinc-finger motifs. Mutations that disrupt the NC zinc fingers abolish genomic RNA encapsidation and virus infectivity (Gorelick et al., 1988, 1990). NC performs several important functions during the virus replication cycle (Levin et al., 2005). As a domain of $Pr55^{Gag}$, it binds and packages the viral RNA genome into nascent virions. This nucleic acid binding property also allows NC to promote Gag–Gag interactions during assembly. After cleavage of NC from the Gag precursor by the viral PR, NC functions as a nucleic acid chaperone to stimulate reverse transcription and integration.

A number of NC-based HIV-1 inhibitors have been reported, most of which target the zinc fingers. Early efforts focused on cell-permeable oxidizing agents that caused zinc to be "ejected" from the zinc fingers (Rice et al., 1993, 1995). One such compound, 2,2-dipyridyl disulfide (Aldrithiol-2 or AT-2), induces extensive cross-linking and has been used in a number of studies as a tool to inactivate HIV-1 virions for experimental purposes (e.g., Lifson et al., 2004). Another compound, N-ethylmaleimide (NEM), inactivates virus infectivity by alkylating the zinc-bound thiols in NC (Chertova et al., 1998). More selective inactivation of NC zinc fingers was observed with the sulfhydryl compound 4-vinylpyridine (4-VP), which, when combined with a membrane-permeable diva-

lent cation chelating agent, potently inactivated HIV-1 infectivity (Morcock et al., 2008). Appella and coworkers have focused on a series of zinc-finger-based inhibitors in the 2-mercaptobezamide thioester class that display low micromolar antiviral activity in culture (Song et al., 2002; Turpin et al., 1999). One of these compounds reduced infectious virus production in an HIV transgenic mouse model system (Schito et al., 2003). Although specificity continues to be an issue with the zinc-ejecting compounds, recent studies have reported specific binding between the 2-mercaptobezamide thioester compounds and NC (Jenkins et al., 2006).

In an alternative strategy that does not involve zinc ejection from the zinc fingers, Shvadchak et al. (2009) screened a chemical library for compounds that block the nucleic acid chaperone activity of NC; specifically, by preventing NC from unfolding a doubly labeled DNA stem-loop structure. This screen identified several compounds that bind the NC zinc fingers and prevent the protein from interacting with the DNA stem-loop. Other studies have focused on compounds in the aminoglycosidic antibiotic class that interact not with NC itself but with the genomic RNA packaging signal, a series of stem-loop structures at the 5′ end of the HIV-1 genome that direct RNA encapsidation into virions (Turner et al., 2006).

2.3.4. p6

The budding of HIV-1, like that of other retroviruses, proceeds via the hijacking of cellular endosomal sorting machinery (Fig. 8) (Bieniasz, 2006; Demirov and Freed, 2004; Morita and Sundquist, 2004). This machinery normally promotes the inward budding of vesicles into late endosomes to generate MVBs and also functions during the membrane fission, or "budding-off", step of cytokinesis (McDonald and Martin-Serrano, 2009). Because virus particle budding from the plasma membrane is topologically equivalent to vesicle budding into late endosomes (oriented away from the cytosol), retroviruses evolved to usurp endosomal sorting machinery to drive the pinching-off of virions from the plasma membrane. At the core of this machinery are three multiprotein complexes known as the endosomal sorting complexes required for transport (ESCRT) I, II, and III. An additional complex, sometimes referred to as ESCRT-0, sorts cargo proteins from early to late endosomes (Hurley and Emr, 2006). Several accessory molecules associate with the ESCRT complexes, including ALG-2-interacting protein X (Alix) and the AAA ATPase Vps4.

The interaction between viral Gag proteins and cellular endosomal sorting machinery is mediated by small motifs in Gag known as "late domains" that directly engage their cellular partners. In the case of HIV-1, two late domains are found in the p6 portion of Gag. The major HIV-1 late domain, Pro-Thr/Ser-Ala-Pro (PTAP) binds to the ESCRT-I component Tsg101; a secondary late domain, Tyr-Pro-X_n-Leu (YPX_nL, where X is a variable amino-acid and n is 1–3 residues) interacts with Alix. Deletion of p6 (Gottlinger et al., 1991) or mutation of the PTAP motif (Huang et al., 1995) is highly detrimental to virus budding and abolishes or attenuates virus replication in most cell types (Demirov et al., 2002b). Mutations in the Alix binding site in p6 do not induce a marked defect in virus budding but nevertheless delay virus replication in T-cell lines and in primary T cells and macrophages (Fujii et al., 2009).

The importance of Tsg101 in HIV-1 budding was demonstrated by the findings that (i) siRNA-mediated depletion of this ESCRT-I component profoundly reduces virus budding (Garrus et al., 2001), (ii) Tsg101 fusion to Gag compensates for p6 mutation (Martin-Serrano et al., 2001), and (iii) overexpression of the N-terminal, Gag-binding domain of Tsg101 exerts a dominant-negative inhibition of virus budding (Demirov et al., 2002a). The inhibitory activity of this N-terminal Tsg101 fragment (referred to as TSG-5′) requires a direct TSG-5′–Gag interaction and is PTAP-dependent (Goila-Gaur et al., 2003; Shehu-Xhilaga et al., 2004). Budding and replication of feline immunodeficiency virus (FIV), a feline lentivirus that also encodes a PTAP-type late domain, are strongly inhibited by TSG-5′ (Luttge et al., 2008), demonstrating that this Tsg101 fragment can inhibit a spreading lentiviral infection. Overexpression of the Gag-binding domain of Alix (known as the "V-domain") also imposes a potent block to HIV-1 budding (Lee et al., 2007; Munshi et al., 2007). It is important to note that disrupting the interaction between HIV-1 Gag late domains and their cellular partners has a more profound effect on virus infectivity than on particle release, since many of the particles that are produced in the absence of a functional late domain are morphologically aberrant and poorly infectious.

While tissue culture experiments demonstrate that TSG-5′ and the Alix V domain are potent inhibitors of virus budding, they are not viable therapeutics. However, high-resolution structural information is available for the p6–Tsg101 and p6–Alix interaction sites (Fig. 9); (Pornillos et al., 2002a,b), making the rational design of budding inhibitors possible. As a first step toward this goal, Liu et al. engineered PTAP-based peptoid mimetics that display significantly increased affinity for Tsg101 relative to peptides bearing the native PTAP motif (Liu et al., 2006, 2008). These peptoids could, if rendered sufficiently cell-permeable, act as competitive inhibitors of the p6–Tsg101 interaction. By using a reverse two-hybrid system designed to detect disruption of a protein–protein interaction, Tavassoli et al. (2008) screened a large library of cyclic peptides to identify a small number of peptides that interfered with the interaction between p6 and Tsg101. One of these peptides was able to inhibit HIV-1 budding several-fold in cell culture. Clearly, additional work needs to be done to identify small molecules capable of disrupting virus budding. Also to be determined is whether blocking the primary late domain of p6 (PTAP) would be sufficient to confer potent antiviral activity or whether simultaneous disruption of both p6–Tsg101 and p6–Alix interactions would be required for full efficacy.

2.4. Maturation

2.4.1. Maturation inhibitors that target Gag processing

HIV-1 particle maturation occurs concomitant with virus release (Fig. 1). PR initiates the maturation process by proteolytically processing the Gag and Gag-Pol polyprotein precursors. Gag is cleaved to generate the MA, CA, NC and p6 proteins and the SP1 and SP2 spacer peptides (Fig. 10A); the Pol portion of Gag-Pol is cleaved into the PR, RT and IN enzymes (Fig. 2) (Adamson and Freed, 2007; Vogt, 1996). Gag cleavage follows a sequential cascade of events that is kinetically controlled by the differential rate of processing at each of the five cleavage sites in Gag (Erickson-Viitanen et al., 1989; Krausslich et al., 1988; Mervis et al., 1988; Pettit et al., 1994; Tritch et al., 1991; Wiegers et al., 1998) (Fig. 10A). Disrupting cleavage at any of the sites in Gag, or altering the order in which the sites are cleaved, results in the formation of particles with an aberrant morphology and significantly reduced infectivity (Accola et al., 1998; Kaplan et al., 1993; Krausslich et al., 1995; Lee et al., 2009; Li et al., 2003; Pettit et al., 1994, 2002; Wiegers et al., 1998; Zhou et al., 2004b). Disrupting Gag processing thus represents an attractive therapeutic strategy for inhibiting HIV-1 replication.

PIs, which have been in the clinic for more than a decade, block Gag and Gag-Pol processing by directly inhibiting the enzymatic activity of PR (see article by Nijhuis, 2010). An alternative approach is to target individual Gag cleavage sites. While this strategy could potentially be used to block any of the Gag cleavage sites, thus far only the CA-SP1 cleavage site has been successfully targeted (Adamson et al., 2009a; Aiken and Chen, 2005; Salzwedel et al., 2007). The small molecule 3-O-(3′,3′-dimethylsuccinyl)betulinic acid (DSB), also known PA-457 or bevirimat (BVM), potently inhibits HIV-1 replication by specifically blocking CA-SP1 cleavage (Fig. 10A–C) (Li et al., 2003; Zhou et al., 2004b). Processing at the

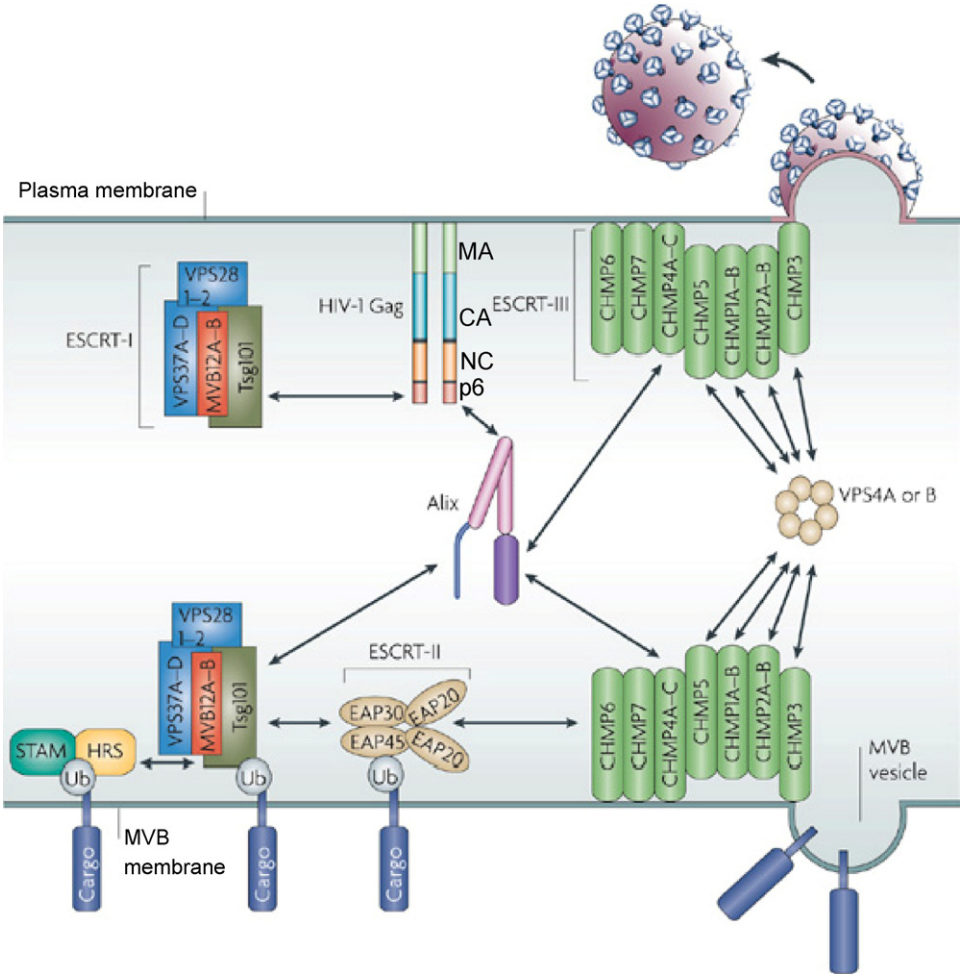

Fig. 8. Role of ESCRT and associated machinery in the sorting of cargo proteins to multivesicular bodies (MVBs) and in virus release. At the bottom is depicted the interaction of ubiquitinated (Ub) cargo protein with the STAM/Hrs complex and ESCRT-I, II, and III and the delivery of the cargo protein into a vesicles budding inwardly into the MVB. At the top is depicted the interaction of Gag with ESCRT-I and Alix and the involvement of ESCRT-III and the ATPase Vps4 in HIV-1 budding from the plasma membrane. The major Gag domains – MA, CA, NC, and p6 – are indicated. For additional details, see text, and Fujii et al. (2007). Adapted with permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology], (Fujii et al., 2007), http://www.nature.com/nrmicro/index.html .

CA-SP1 cleavage site occurs late in the Gag cleavage cascade and when inhibited results in the formation of particles that are non-infectious because they fail to complete maturation (Wiegers et al., 1998). BVM-treated particles exhibit an aberrant morphology typified by an acentric core and an electron-dense Gag crescent inside the viral membrane (Fig. 10B) (Li et al., 2003). This morphology mirrors that induced by mutations at the CA-SP1 cleavage site (Wiegers et al., 1998). Due to its novel mechanism of action, BVM is the first in a new mechanistic class of antiretroviral drugs termed maturation inhibitors, which are defined as compounds that target the substrate of the viral PR rather than the enzyme itself (Adamson et al., 2009a; Aiken and Chen, 2005; Salzwedel et al., 2007).

The precise mechanism by which BVM inhibits CA-SP1 cleavage has not been established. However, it is hypothesized that BVM binds to the CA-SP1 junction in Gag and prevents cleavage either by directly inhibiting access of PR to the processing site or by altering the conformation, exposure or flexibility of this region such that it is less efficiently cleaved by PR (Adamson et al., 2009a). Several lines

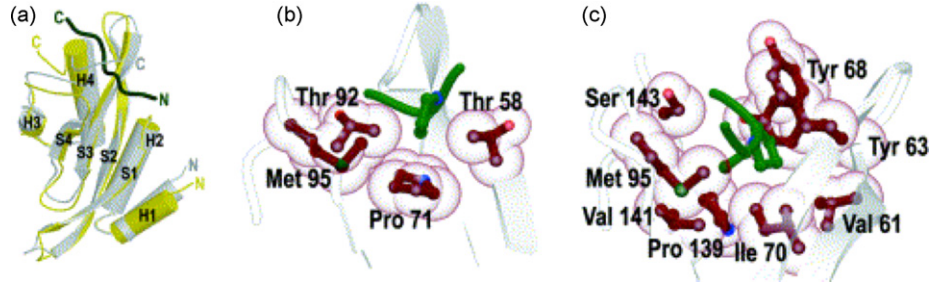

Fig. 9. Structure of the ubiquitin enzyme 2 variant (UEV) domain of Tsg101 bound to a PTAP-containing peptide. (a) The structure of the UEV domain is shown in yellow and gray, the PTAP-containing peptide (C, C-terminus; N, N-terminus) is shown in dark green. High-resolution structure of the first Pro (b) and Ala-Pro (c) of PTAP docked in the PTAP-binding groove of Tsg101, viewed from the N-terminus of the peptide. Reprinted with permission form Macmillan Publishers Ltd: [Nature Structural and Molecular Biology], (Pornillos et al., 2002a), http://www.nature.com/nsmb/index.html .

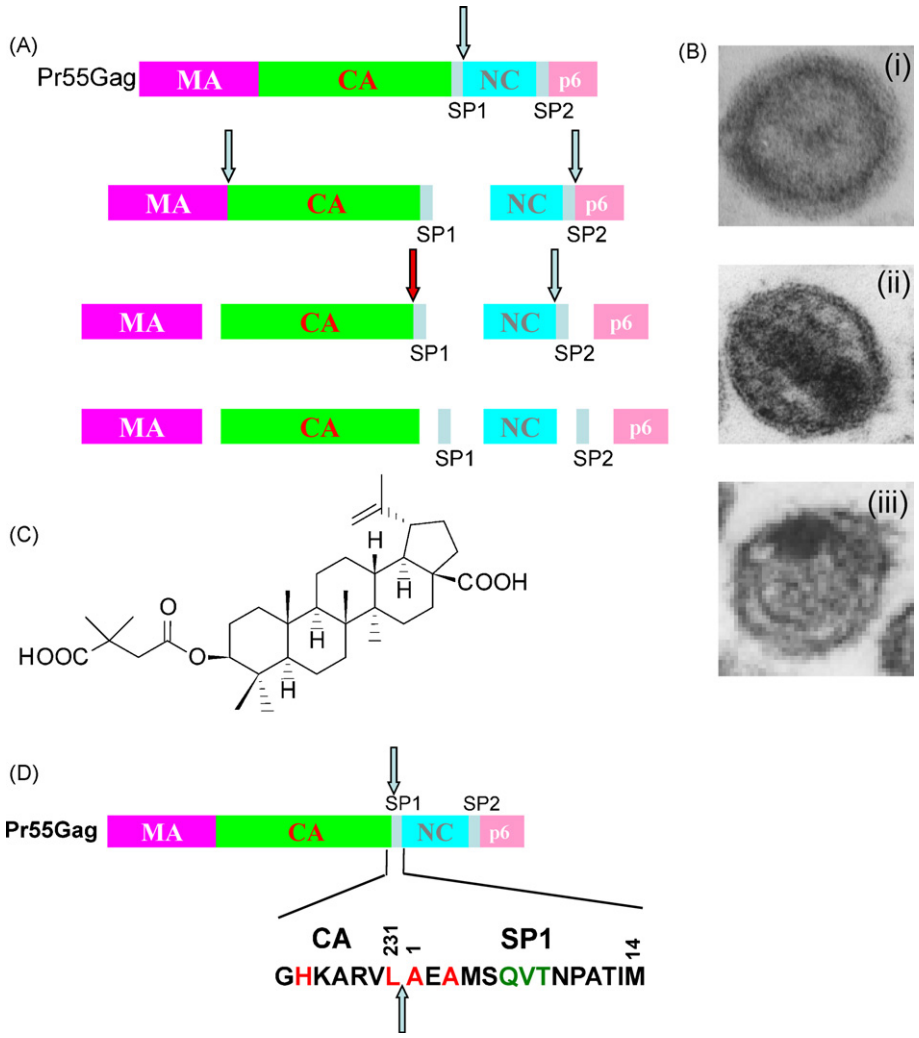

Fig. 10. Inhibition of HIV-1 maturation by bevirimat. (A) Gag processing cascade, illustrating the order in which the Gag precursor ($Pr55^{Gag}$) is cleaved by the viral protease. Red arrow depicts the cleavage event blocked by bevirimat, leading to an accumulation of the CA-SP1 cleavage intermediate. (B) Virion morphology visualized by transmission electron microscopy. Immature (i), mature (ii), and bevirimat-treated (iii) particles are shown. (C) Structure of bevirimat. (D) Amino-acid sequence at the CA-SP1 boundary region; the final residue of CA (residue 231) and the first (1) and final (14) residues of SP1 are shown. Amino acids highlighted in red indicate those at which BVM resistance arises *in vitro* (Adamson et al., 2006); the highly polymorphic SP1 residues 6, 7, and 8 are highlighted in green. Arrows denote the site of CA-SP1 processing. Adapted with permission from Elsevier (Adamson and Freed, 2008).

of evidence (discussed below) support this hypothesis, although structural information about BVM bound to its substrate has not yet been obtained. Mapping of residues that confer BVM resistance to the CA-SP1 region (Fig. 10D), and not elsewhere in Gag or in PR, strongly suggests that this region of Gag is the primary molecular target of this compound (Adamson et al., 2006; Fun et al., 2009; Li et al., 2003; Van Baelen et al., 2009; Zhou et al., 2004b). Inherent BVM resistance of HIV-2 and simian immunodeficiency virus from rhesus macaques (SIVmac) is due to variability in the amino-acid sequence at the CA-SP1 junction (Zhou et al., 2004b). Swapping residues between the CA-SP1 junctions of HIV-1 and SIVmac results in exchange of BVM sensitivity between these two viruses (Zhou et al., 2004a). In addition, some sequence divergence between HIV-1 and SIVmac occurs at residues to which BVM resistance maps *in vitro* (Adamson et al., 2006; Zhou et al., 2004b).

BVM has been shown to bind immature but not mature HIV-1 particles, suggesting that Gag processing leads to disruption of the BVM binding site (Zhou et al., 2005). Further, BVM binds to a pocket formed during Gag oligomerization, as BVM does not inhibit CA-SP1 processing in the context of monomeric Gag in solution (Li et al., 2003), but rather requires Gag assembly for its activity (Li et al., 2003; Sakalian et al., 2006; Zhou et al., 2005). A reduction in Gag binding to immature particles has been observed in the presence of several mutations that confer resistance to BVM, suggesting that resistance is acquired by prevention of BVM–Gag binding (Zhou et al., 2005, 2006). However, blocking BVM binding may not be the only mechanism by which resistance is acquired as some binding capacity is retained by two of the mutants tested (Zhou et al., 2006), and a degree of drug dependence is associated with other mutations that confer resistance to BVM (Adamson et al., 2006).

Characterization of the putative BVM binding pocket has been hindered by the lack of high-resolution structural information for the CA-SP1 junction. This region of Gag, while disordered in CA crystals, has been proposed to be α-helical (Accola et al., 1998; Gamble et al., 1997; Morellet et al., 2005; Newman et al., 2004; Worthylake et al., 1999; Wright et al., 2007). The oligomeric state of CA-SP1 region of Gag in immature particles remains unresolved, although a recent cryo-electron tomography study proposed that it forms a six-helix bundle (Wright et al., 2007). Further structure-function analysis of the CA-SP1 region of Gag will be required to more fully understand the mechanism of action of BVM. Such information may also provide a rational basis for the design of additional compounds that target this cleavage site.

The potent *in vitro* activity of BVM and its novel mechanism of action have encouraged its clinical development. Testing in HIV-1-infected patients was initiated following promising pharmacological and safety studies in animal models and in Phase I clinical trials (Martin et al., 2008). Statistically significant, dose-dependent viral load reductions were demonstrated in initial Phase II clinical trials (Smith et al., 2007). However, further Phase II studies showed that, despite optimal BVM plasma concentrations, not all BVM-treated patients exhibited significant viral load reductions. In a Phase IIb functional monotherapy trial, ~50% of patients receiving BVM were defined as non-responders with vial load reductions of <0.5 log (McCallister et al., 2008). Virological parameters were hypothesized to be responsible for the observed variable clinical outcome; the presence of base-line polymorphisms at SP1 residues 6, 7 and 8 (Fig. 10D) appeared to correlate with a patient's failure to respond (Margot et al., 2009; McCallister et al., 2008; Salzwedel et al., 2008, 2009; Van Baelen et al., 2009). Residues 6–8 are located in the relatively non-conserved C-terminal half of SP1 and the occurrence of polymorphisms at these positions appears to be independent of prior treatment with antiretrovirals (Knapp et al., 2009; Yebra and Holgun, 2008). *In vitro* testing demonstrated reduced BVM-susceptibility associated with key polymorphisms located at residues 7 and 8 but not residue 6 (Van Baelen et al., 2009) (Adamson et al., unpublished data). Further studies are required to understand the relationship between these polymorphisms and HIV-1 susceptibility to BVM. However, a genotypic assay is now readily available to identify those patients most likely to respond to BVM treatment.

The type of resistance acquired by those patients who respond to BVM remains an outstanding question. Virus isolates with key polymorphisms at SP1 residues 6–8 are known to replicate in patients and a recent study has shown the acquisition of resistance mutations at SP1 residues 7 and 8 in *in vitro* selection studies with BVM (Fun et al., 2009). Therefore, it is likely that mutations at these positions will contribute to BVM resistance in patients. However, it is noteworthy that *in vitro* selection studies have identified a panel of six other BVM-resistance mutations that arise at positions different from SP1 residues 6–8 (Adamson et al., 2006; Li et al., 2003; Zhou et al., 2004b) (Fig. 10D). Specifically, these mutations mapped to highly conserved residues at or near the C-terminus of CA (CA-H226Y, CA-L231M, CA-L231F) and the first and third residues of SP1 (SP1-A1V, SP1-A3V, SP1-A3T). It has been predicted that, of this panel, the SP1-A1V substitution is most likely to arise *in vivo* because it was selected most frequently and replicated robustly even at a high BVM concentration (Adamson et al., 2006, 2009b). The highly conserved nature of SP1 residue 1 among HIV-1 isolates suggests that a fitness cost could be associated with mutations at this position *in vivo*. However, the SP1-A1V mutant replicates efficiently in primary macrophages (Adamson et al., unpublished data) and in SCID-hu Thy/Liv mice (Stoddart et al., 2007). Furthermore, SP1-A1V has been observed in isolates from two of the 46 patients participating in BVM Phase II clinical trials (Adamson et al., 2009a). It is also noteworthy that the CA-L231M substitution has been reported in the context of one PI-experienced patient sample and the SP1-A3T mutation is present in one viral isolate listed in the Los Alamos sequence database (Malet et al., 2007; Salzwedel et al., 2007).

Two studies have also investigated the *in vitro* acquisition of BVM resistance in the context of viral isolates with preexisting mutations in PR that confer resistance to PIs (Adamson et al., 2009b; Fun et al., 2009). This is a significant question as BVM is likely to be used as salvage therapy for patients failing first-line drug regimens due to multi-drug resistance and are therefore likely to be PI-experienced. The impact of the PR mutations on the temporal acquisition of BVM resistance compared to wild-type (WT) virus differed between the two studies and may be dependent on the type of PR mutations or the study systems used. In both studies, however, the SP1-A1V substitution was acquired frequently. A spectrum of other mutations were also frequently acquired in the study by Fun et al. Interestingly, these mutations mapped to SP1 residues 5, 7 and 8 and to CA residue 230 (Fun et al., 2009). Ongoing clinical trials will ultimately reveal the types of resistance mutations that arise in patients who respond to BVM. The emergence of A1V, as well as the preexistence of polymorphisms at SP1 residues 6–8, will present a challenge for the successful development of BVM as a clinically effective antiretroviral drug.

BVM is the first Gag-targeted compound that has undergone clinical development. Thus, BVM opens the way for the discovery and development of other maturation inhibitors. These may include second- and third-generation inhibitors that target the CA-SP1 cleavage site. Other cleavage sites in Gag may also be viable targets. A recent study explores this possibility by introducing mutations that independently block each of the five Gag processing sites and analyzing their dominant-negative effect on virus maturation and infectivity when mixed at different ratios with the WT counterpart (Lee et al., 2009). Blocking cleavage at the MA-CA cleavage site resulted in the most potent inhibition, with very small amounts of the uncleaved MA-CA protein poisoning correct virus maturation and thus significantly suppressing viral infectivity (Lee et al., 2009). The MA-CA cleavage site therefore represents a promising future drug development target.

2.4.2. Maturation inhibitors that target core assembly

HIV-1 maturation generates a condensed conical core composed of a CA lattice (Fig. 7B–D) surrounding the viral RNA genome in complex with NC, RT, and IN. The function of the core is to facilitate the delivery and reverse transcription of the viral RNA genome following infection of the target cell. The core is formed by a CA reassembly event triggered by the liberation of the CA domain from Gag upon proteolytic processing (Adamson et al., 2009a; Ganser-Pornillos et al., 2008). Correct core formation and stability are essential for virus infectivity (Fitzon et al., 2000; Forshey et al., 2002; Ganser-Pornillos et al., 2004; Lee et al., 2009; Li et al., 2003; Reicin et al., 1996; Tang et al., 2003; von Schwedler et al., 1998, 2003; Wiegers et al., 1998). Disrupting the CA–CA interactions required for core formation thus represents a potential therapeutic strategy for inhibiting HIV-1.

A detailed understanding of HIV-1 core morphology, organization of the CA lattice, and the CA–CA interactions required for core formation (Fig. 7B–D) has laid a solid foundation for the identification and development of maturation inhibitors that disrupt core formation (Adamson et al., 2009a; Ganser-Pornillos et al., 2008). This understanding originates from the important observation that purified CA assembles *in vitro* to form long tubes organized in a manner similar to that of authentic cores (Ganser et al., 1999; Li et al., 2000). Furthermore, the *in vitro* assembly systems offer a tractable model to screen for CA-based inhibitors of core formation (Sticht et al., 2005; Zhang et al., 2008).

HIV-1 cores are usually cone-shaped, although a range of related structures have been observed (Benjamin et al., 2005; Briggs et al., 2003, 2006; Welker et al., 2000). The cone is formed by a curved hexagonal CA lattice, which is closed by the strategic positioning of 12 pentamers at the ends of the cone (Fig. 7D). This model is based on the geometrical principles of a fullerene-cone (Ganser et al., 1999) and has been validated *in vitro* by elegant crytallographic structural and modeling studies of the CA lattice (Ganser et al., 1999; Ganser-Pornillos et al., 2004, 2007; Li et al., 2000; Pornillos et al., 2009) and by imaging of authentic, mature retroviral particles (Benjamin et al., 2005; Briggs et al., 2003, 2006; Butan et al., 2008). The *in vitro* structure-based studies have provided significant insights into the CA–CA interactions that are required for core formation.

Both the CA_{NTD} and CA_{CTD} play a role in CA hexameric lattice assembly. The lattice is constructed from rigid rings of six CA_{NTD}'s connected to the neighboring hexameric rings via more flexible CA_{CTD} dimer interactions (Ganser-Pornillos et al., 2007; Li et al., 2000; Pornillos et al., 2009). This organization requires three types of interfaces: (i) a CA_{NTD}–CA_{NTD} sixfold symmetric interface that creates the hexameric rings, (ii) a CA_{NTD}–CA_{CTD} intermolecular interface between the two domains that reinforces the hexamer and (iii) a CA_{CTD}–CA_{CTD} dimeric interface that links the hexameric rings to form the lattice (Ganser-Pornillos et al., 2007; Pornillos et al., 2009). Various genetic, biochemical and biophysical studies support the existence of these interacting interfaces (Dorfman et al., 1994; Fitzon et al., 2000; Reicin et al., 1996; Scholz et al., 2005; Tang et al., 2001; von Schwedler et al., 1998, 2003). Each of the three major CA–CA interfaces represents a possible target for disrupting core formation.

Two key studies used different approaches to identify molecules that target CA. One study used *in silico* modeling to identify compounds that potentially bind clefts on the CA surface, followed by a binding screen using NMR titration spectroscopy (Tang et al., 2003). The other study, already discussed in Section 2.3.2, used *in vitro* assembly assays to conduct high-throughput screening of a random peptide phage display library (Sticht et al., 2005). These studies identified two lead candidates, CAP-1 (N-(3-chloro-4-methylphenyl)-N′-{2-[({5-[(dimethylamino)-methyl]-2-furyl}-methyl)sulfanyl]ethyl}urea) and CAI, respectively. The primary mode of action of CAP-1 is to disrupt core formation (Tang et al., 2003) whereas CAI and its cell-permeable derivatives NYAD-1 and NYAD-13 inhibit both core formation and assembly of immature particles (Barklis et al., 2009; Sticht et al., 2005; Zhang et al., 2008).

High-resolution structural studies of CAP1, CAI, and NYAD-13 in complex with CA have provided valuable insights into their mechanisms of action (Bhattacharya et al., 2008; Sticht et al., 2005). CAP-1 interacts with the base of the CA_{NTD} (Fig. 7A). Binding induces a conformational change that creates the CAP-1 binding site by displacement of CA_{NTD} residue F32 from its buried position in the protein core (Kelly et al., 2007). The aromatic ring of CAP-1 is sequestered within a hydrophobic pocket vacated by F32 (Kelly et al., 2007). The position of CAP-1 and F32 suggests that CAP-1 binding inhibits the formation of the CA_{NTD}–CA_{CTD} interface of the CA lattice (Ganser-Pornillos et al., 2007, 2008; Kelly et al., 2007; Pornillos et al., 2009; Tang et al., 2003). Mutagenesis of key CA residues that interact with CAI suggests that this peptide inhibits core formation by two mechanisms (Bartonova et al., 2008). First, CAI blocks the CA_{NTD}–CA_{CTD} interaction by competing for the natural binding region in the CA_{NTD} and, second, CAI alters the CA_{CTD}–CA_{CTD} dimer interface that is important for connecting the hexameric rings in the CA lattice (Bartonova et al., 2008; Ganser-Pornillos et al., 2007, 2008; Pornillos et al., 2009; Ternois et al., 2005). As CAP-1 and CAI (and derivatives NYAD-1 and NYAD-13) interfere with the CA_{NTD}–CA_{CTD} interface, this region of CA may act as a 'hot-spot' for inhibitors targeting CA–CA interactions. Despite the fact that neither CAP1 nor CAI and related peptides are drug candidates, the extensive knowledge gained in studying these inhibitors and their molecular targets will likely be exploited for future rational design of more effective inhibitors that target CA–CA interactions and core formation.

2.5. Accessory proteins as targets

In addition to the structural proteins and *pol*-encoded enzymes, HIV-1 encodes several accessory proteins—Vif, Vpu, Nef, and Vpr (Fig. 2). Under some circumstances, these proteins are dispensable for replication in culture (hence their designation as accessory proteins); however, *in vivo* these proteins appear to be crucial for virus propagation and disease induction. Recent progress has greatly increased our understanding of the mechanism by which the HIV-1 accessory proteins function, providing new opportunities for the development of antiviral agents. As mentioned in the Introduction, HIV-1 also encodes two regulatory proteins, Rev and Tat. Efforts to develop inhibitors against these proteins will not be discussed here, as this topic is covered elsewhere (Baba, 2004; Bannwarth and Gatignol, 2005; Richter and Palu, 2006).

2.5.1. Vif and APOBEC-family proteins

As mentioned in the Introduction, HIV-1 and a number of other lentiviruses encode an accessory protein known as Vif that markedly enhances virus infectivity. Early studies demonstrated that this infection-stimulating effect was producer-cell-type dependent; virus derived from Vif-permissive cells was fully infectious regardless of whether or not it encoded Vif, whereas the infectivity of virus produced from Vif-non-permissive cells displayed a strong requirement for Vif expression (Gabuzda et al., 1992). Importantly, relevant primary cell types (PBMCs and macrophages) are Vif-non-permissive. Heterokaryons formed between Vif-permissive and Vif-non-permissive cells exhibited the Vif-non-permissive phenotype, suggesting that Vif enhances infectivity by counteracting a dominant restriction factor expressed in the virus-producing cell (Madani and Kabat, 1998; Simon et al., 1998). This restriction factor was ultimately identified as APOBEC3G (A3G), a member of a large family of cytosine deaminases that functions in mRNA-editing and immunoglobulin gene diversification (Harris and Liddament, 2004; Sheehy et al., 2002). The related deaminase, APOBEC3F (A3F), also displays antiviral activity that is counteracted by Vif. In the absence of Vif expression, A3G is incorporated into virus particles in the producer cell, and, during reverse transcription in the next round of infection, converts cytosines to uracils (Fig. 11). This cytosine deamination leads to G-to-A hypermutation in the newly synthesized viral DNA, potentially leading to its instability (Malim, 2009). A3G also induces defects in reverse transcription and DNA integration (Bishop et al., 2008; Mbisa et al., 2007). Vif counteracts this cellular defense mechanism by recruiting components of the proteasomal pathway, e.g., cullin 5 and elongins B and C, to induce the polyubiquitination and degradation of A3G (Yu et al., 2003). The net effect of Vif-induced A3G degradation is to prevent the incorporation of A3G into virions.

The potent antiviral activity of A3G raises the possibility that inhibitors could be developed that prevent Vif from counteracting this cellular factor. To this end, Nathans et al. (2008) devised a cell-based screen in which A3G fused to yellow fluorescent protein (YFP) was expressed in cells in the presence of Vif. A library of 30,000 compounds was tested for their ability to counteract Vif-mediated degradation of A3G and thereby increase the A3G-YFP fluorescent signal. After performing secondary screens designed to exclude compounds that simply increase fluorescence or elevate gene expression, several dozen compounds were obtained. A set of these molecules was then tested for antiviral activity in permissive (A3G-deficient) or non-permissive (A3G-expressing) cells. Two compounds were identified that displayed antiviral activity only in non-permissive cells, suggesting that they act by preventing Vif from counteracting A3G. The more potent compound, termed RN-18, was selected for further study. Analysis of Vif and APOBEC levels demonstrated that RN-18 decreased Vif expression in the presence but not the absence of a Vif–APOBEC interaction. This APOBEC-specific loss of Vif expression led to increased levels of A3G in virions and inhibition of virus infectivity. Additional studies will be required to define in more detail the mechanism by which RN-18 leads to reduced Vif expression. In an earlier, independent report, it was observed that the membrane-permeable zinc chelator N,N,N′,N′-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) inhibited cullen 5 recruitment by Vif and Vif-mediated A3G degra-

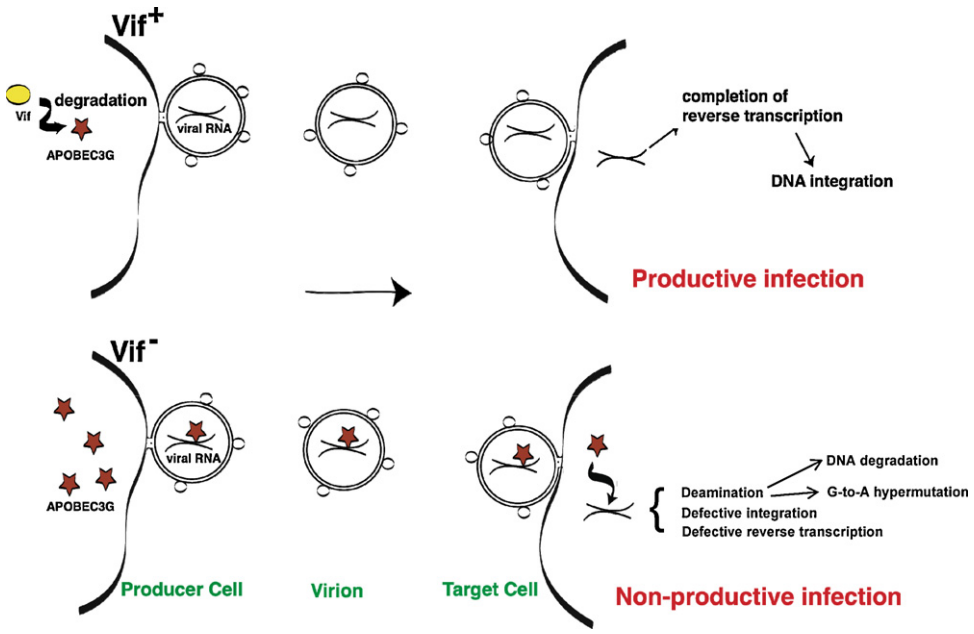

Fig. 11. Schematic representation of the counteraction of APOBEC3G by Vif. In the Vif+ setting (top), Vif (yellow) induces the proteasomal degradation of APOBEC3G (red star) in the virus-producing cell (left), enabling productive infection to occur in the target cell (right). In the absence of Vif expression (bottom), APOBEC3G is packaged into virus particles, and in the next round of infection induces the deamination of cytosines to uracils, resulting in G-to-A hypermutation. The presence of APOBEC3G also impairs reverse transcription and integration. Reprinted with permission from Elsevier (Freed, 2004).

dation (Xiao et al., 2007). Gabuzda and colleagues have developed a scalable fluorescence resonance energy transfer (FRET)-based Vif-A3G binding assay and have used it to identify peptides and monoclonal antibodies that block this protein–protein interaction (Mehle et al., 2007; Pery et al., 2009). This assay is useful not only for mapping the determinants of Vif-A3G binding but also, potentially, for developing a high-throughput screen for inhibitors that block this binding event.

APOBEC-mediated restriction is not confined to lentiviruses. Mice encode four APOBEC family members (as compared to 11 in humans) one of which, APOBEC3, blocks infection by mouse mammary tumor virus (MMTV). APOBEC3 knock-out mice support increased MMTV replication relative to their WT litter mates (Okeoma et al., 2007) demonstrating the importance of this host antiviral defense mechanism *in vivo*. Treatment of mice with lipopolysaccharide (LPS) or interferon increases APOBEC3 expression, leading to suppression of MMTV replication (Okeoma et al., 2009). Interestingly, the murine leukemia virus resistance factor encoded by the recovery from Friend virus 3 (Rfv3) allele (Chesebro and Wehrly, 1979) was recently identified as APOBEC3 (Miyazawa et al., 2008; Santiago et al., 2008). Together, these studies indicate that modulation of APOBEC expression can elicit an antiviral effect *in vivo*. However, it remains to be determined whether such an approach would be practical in humans, and there is concern that increased expression of APOBEC family proteins could be accompanied by host cell DNA damage (Harris et al., 2002). Nevertheless, the Vif–APOBEC axis holds much promise for the development of novel antiretroviral agents and will likely continue to be the focus of active investigation.

2.5.2. *Vpu and tetherin*

In addition to A3G and TRIM5α, a third component of the innate immune response functions at the level of virus particle release from the cell surface. Early studies on the HIV-1 accessory protein Vpu demonstrated that Vpu-deficient mutants exhibit an accumulation of mature virus particles apparently stuck to the plasma membrane of the virus-producer cell and in internal vesicles. As observed for Vif, the requirement for Vpu is producer cell-dependent, and fusions between Vpu-permissive and Vpu-non-permissive cells exhibit the non-permissive phenotype (Varthakavi et al., 2003). These results suggested that Vpu counteracts a dominant factor that in some way retains virus particles on the cell surface after they have budded from the plasma membrane. It was also noted that treatment of Vpu-permissive cells with interferon recapitulates the Vpu-non-permissive phenotype, suggesting that the putative restriction factor is interferon-inducible. Two groups independently identified a protein known as CD317 or bone marrow stromal cell antigen 2 (BST-2) as the host factor counteracted by Vpu (Neil et al., 2008; Van Damme et al., 2008). Depletion of CD317/BST-2 from Vpu-non-permissive cells greatly stimulated the release of Vpu(−) HIV-1; conversely, in Vpu-permissive cells that do not express CD317/BST-2, overexpression of this factor inhibited the release of Vpu(−) HIV-1 (Neil et al., 2008; Van Damme et al., 2008). In accordance with its apparent function as a virus-tethering factor, CD317/BST-2 was given the name tetherin (Neil et al., 2008). A second major function for Vpu is to induce the proteasomal degradation of CD4 (Bour and Strebel, 2003).

Shortly after the identification of tetherin as the host factor counteracted by Vpu, several interesting observations were made that increased our understanding of the tetherin-mediated restriction: (1) tetherin proteins encoded by a variety of non-human primates and from rodents inhibit virus release but are not counteracted by HIV-1 Vpu (Goffinet et al., 2009; Gupta et al., 2009; Jia et al., 2009; McNatt et al., 2009; Rong et al., 2009; Sato et al., 2009) and (2) tetherin restricts the release of not only HIV-1 but also of a wide range of retroviruses (Jouvenet et al., 2009) and non-retroviral enveloped viruses (e.g., Ebola; Kaletsky et al., 2009). Because Vpu expression is limited to HIV-1 and a small number of closely related SIVs, other viruses have likely evolved distinct approaches to counteracting this host restriction factor. For example, the Env glycoprotein of some strains of HIV-2 possesses Vpu-like activity (Bour et al., 1996; Ritter et al., 1996) and the Nef proteins of several SIVs antagonize tetherin (Jia et al., 2009).

The precise mechanism by which tetherin retains virus particles on the cell surface remains to be defined. Tetherin displays a somewhat unusual topology (Kupzig et al., 2003) (Fig. 12); it

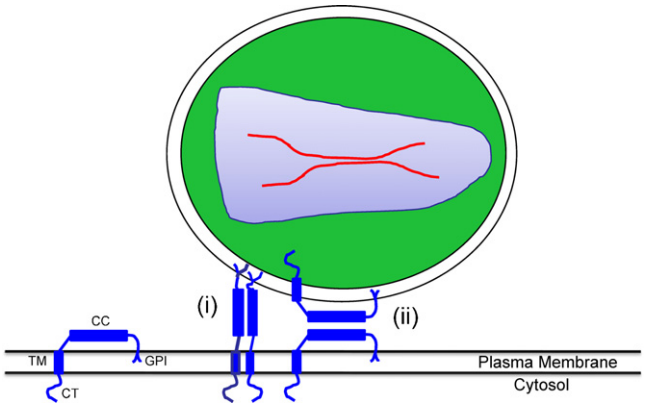

Fig. 12. Hypothetical models for the tethering of HIV-1 virions to the cell surface by CD317/BST-2/tetherin. The virion is shown in green, with core in blue and viral RNA in red. BST-2/CD317/tetherin is shown on the left anchored in the lipid bilayer of the plasma membrane, with the cytoplasmic tail (CT), transmembrane (TM), and coiled-coil (CC) domains and the GPI anchor indicated. In model (i), two molecules of BST-2/CD317/tetherin are aligned in parallel, with the TM domains in the producer cell plasma membrane and the GPI anchors in the viral membrane. In model (ii), one molecule is embedded in the plasma membrane, the other in the viral membrane. The two molecules associate via their coiled-coil domains.

bears an N-terminal cytosolic domain, a transmembrane anchor sequence, and an extracellular coiled-coil domain. The transmembrane domain reportedly determines whether or not a particular tetherin protein can be antagonized by Vpu (Jia et al., 2009; McNatt et al., 2009; Rong et al., 2009). At its C-terminus, tetherin possesses another membrane anchor, a glycosylphosphatidylinositol (GPI) moiety, which serves to recruit the protein to lipid rafts (Kupzig et al., 2003). If tetherin does in fact function as a protein tether, the presence of two membrane anchors allows one to envision several distinct topologies that the protein could adopt to retain virions on the cell surface (Fig. 12). Additional studies will be required to determine whether tetherin does act as a tethering molecule and, if so, to define the orientation of the protein in both cellular and viral membranes.

Also unclear is how Vpu counteracts tetherin to stimulate virus release. Initial reports either did not detect significant reductions in tetherin levels upon Vpu expression (Neil et al., 2008), or observed a Vpu-induced downregulation of tetherin from the cell surface (Van Damme et al., 2008). Subsequent studies, in contrast, were able to measure a significant reduction in total tetherin levels resulting from Vpu expression (Mitchell et al., 2009; Rong et al., 2009). Vpu reportedly removes tetherin from the cell surface and induces its degradation in the lysosome by serving as an adaptor between tetherin and the β-TrCP/SCF E3 ubiquitin ligase complex (Mitchell et al., 2009) or induces its degradation in the proteasome (Goffinet et al., 2009). Despite its ability to induce tetherin internalization and degradation, it remains to be established how critical this downregulation is in Vpu's ability to counteract tetherin and promote virus release; some evidence has been presented suggesting that Vpu can antagonize tetherin even in the absence of clear downregulation of either surface or total expression of the host factor (Miyagi et al., 2009).

Several approaches could be attempted in developing Vpu as a therapeutic target. Vpu has been reported to form ion channels, an activity that has been implicated in its virus-release activity (Bour and Strebel, 2003). Compounds that block Vpu ion channel activity have been reported to display anti-Vpu activity (Ewart et al., 2002; Park and Opella, 2007). Stephens and colleagues demonstrated that the presence of the *vpu* gene in an SIV/HIV-1 (SHIV) chimera contributes to SHIV pathogenesis in macaques (Stephens et al., 2002). Interestingly, substitution of the transmembrane domain of Vpu with that of the influenza A viroporin M2, or mutation of a key residue in the Vpu transmembrane domain, rendered virus replication sensitive to M2 ion channel blockers (Hout et al., 2006a,b). These findings illustrate the potential for developing anti-HIV agents that act by blocking Vpu ion channel activity. An alternative approach to disrupting Vpu function is to target the Vpu-tetherin interface with small molecules or transmembrane peptide decoys that bind the Vpu membrane-spanning domain and block its interactions with tetherin (Montal, 2009). The interface between Vpu and host factors that help mediate the lysosomal or proteasomal degradation of tetherin (e.g., β-TrCP) could also be targeted. The cholesterol-binding compound AME, discussed above (Section 2.1), was shown to inhibit virus release in a Vpu-dependent fashion, suggesting that it might disrupt the ability of Vpu to antagonize tetherin (Waheed et al., 2008). The relative importance of Vpu's two major functions – tetherin antagonism and CD4 downregulation – in HIV-1 pathogenesis remains to be defined. This question will be important in determining which function and domain of Vpu to target in developing inhibitors.

2.5.3. Nef

The *nef* gene encodes a 27-kDa membrane-associated protein that is expressed at high levels in infected cells and is incorporated into virus particles. Nef was initially considered to be a negative regulatory factor (hence the name negative factor) but was ultimately shown to be an important positive determinant of lentiviral pathogenesis. A role for Nef in disease induction was demonstrated by two key observations: (1) deletion of the *nef* gene in SIV_{mac} profoundly impairs progression to AIDS in infected rhesus macaques (Kestler et al., 1991), and (2) individuals infected with *nef*-defective strains of HIV-1 in some cases exhibit very low viral loads and a long-term non-progressor phenotype in which they remain healthy for extended periods of time postinfection (Deacon et al., 1995; Kirchhoff et al., 1995). The apparent importance of Nef in disease induction implies that disruption of Nef function would provide antiretroviral activity.

A confounding factor in developing Nef-based inhibitors is the complexity and diversity of the reported functions of this protein. A number of Nef activities have been reported, including: (1) downregulation of surface expression of the major histocompatibility complex I (MCH-I), (2) downmodulation of CD4 expression, (3) alteration of cellular activation pathways, including stimulation of p21-activated protein kinase 2 (Pak2), and (4) enhancement of virus infectivity (for reviews, see Foster and Garcia, 2007, 2008). These four major activities appear to be genetically separable (Foster and Garcia, 2008), suggesting the presence of multiple potential drug targets. It remains to be determined which of these functions is critical for the ability of Nef to increase viral loads and stimulate disease induction *in vivo*, although there is some evidence that CD4 downregulation is important for HIV-1 pathogenesis (Carl et al., 2000). Nef has been reported to interact with a large number of cellular partners, including CD3, CD4, the clathrin adapter protein complex 2 (AP-2), the vacuolar ATPase, COP1 coatomers, ADP ribosylation factor 1 (Arf1), p53, actin, $p56^{lck}$, Pak2, and Hck (for reviews see Foster and Garcia, 2007, 2008; Freed, 2007).

The structure of the Nef core has been determined in its unliganded state and in complex with Src homology 3 (SH3) domains or the cytoplasmic tail of CD4 (Arold et al., 1997; Grzesiek et al., 1996a,b, 1997; Lee et al., 1996). Nef contains an unstructured N-terminus, followed by a poly-Pro type II helix that bears the Pro-X-X-Pro motif responsible for binding SH3 domains, several helical regions, and a five-stranded antiparallel β-sheet. Dileucine and diacidic motifs in a 30-amino-acid flexible loop in Nef contribute to AP-2 binding. In addition to the Pro-X-X-Pro motif, SH3 binding also requires a hydrophobic groove referred to as the RT loop-binding region (RTLBR); this region interacts with the RT loop of the SH3 domain (Arold et al., 1997; Lee et al., 1996). Betzi and

colleagues performed an *in silico* screen to identify compounds that bind Nef and prevent its interaction with the SH3 domain of Hck (Betzi et al., 2007). This virtual screen was followed up with binding studies and a mammalian-cell-based two-hybrid assay to identify compounds that block Nef-SH3 binding. This study identified two drug-like compounds that bind Nef in the micromolar range and effectively compete for SH3 binding. It remains to be determined whether these compounds will move forward to clinical development; however, the importance of Nef in HIV disease progression will continue to make this protein an interesting target for drug discovery.

3. Conclusions

The ultimate objectives of HIV research are a safe and effective vaccine to prevent viral infection, and the ability to eradicate HIV from infected patients. However, achieving these goals, if they are even possible, may take many years if not decades. The most feasible approach to managing HIV infection will therefore continue to center on combination antiretroviral therapy aimed at suppressing viral loads in infected individuals. The remarkable ability of HIV-1 to evade antiretroviral agents, even when administered in combination, together with problems of drug toxicity, will likely necessitate the continued development of novel therapeutics. Success will require a sustained and multidisciplinary effort involving virology and chemical, structural, molecular, and cell biology. As drug discovery expands from its focus on purely viral targets (e.g., RT, PR, IN, gp41) to include viral–host protein interactions or cellular targets, a more in-depth understanding of the cell biology of HIV-1 replication will be required. High-throughput screens will have to be devised to identify small-molecule inhibitors of still-uncharacterized viral–host and host–host interactions and medicinal chemistry efforts will be required to translate hits in these screens to effective and non-toxic drugs. In this article, we provide an overview of recent progress in understanding fundamental aspects of HIV-1 replication and discuss how this information is being used, and could, in theory, be extended in the future, to develop to new drugs. These efforts will enhance our ability to treat HIV-1 infection and will also reap enormous benefits in terms of fundamental new insights into the molecular and cell biology of retroviral replication and pathogenesis.

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