Chapter 10
Molecular Mechanisms of HIV Entry

Craig B. Wilen, John C. Tilton, and Robert W. Doms

Abstract Human immunodeficiency virus (HIV) entry is a complex and intricate process that facilitates delivery of the viral genome to the host cell. The only viral surface protein, Envelope (Env), is composed of a trimer of gp120 and gp41 heterodimers. It is essentially a fusion machine cloaked in a shroud of carbohydrate structures and variable loops of amino acids that enable it to evade the humoral immune response. For entry to occur gp120 sequentially engages the host protein CD4 and then one of two chemokine coreceptors, either CCR5 or CXCR4. CD4 binding facilitates exposure and formation of the coreceptor-binding site, and coreceptor binding then triggers the membrane fusion machinery in the gp41 subunit. Our understanding of HIV entry has led to the development of successful small molecule inhibitors for the clinical treatment of HIV infection as well as insights into viral tropism and pathogenesis.

Abbreviations

6HB	Six-helix bundle
AIDS	Acquired immunodeficiency syndrome
CD4bs	CD4-binding site
Cryo-EM	Cryo-electron microscopy
ECL	Extracellular loop
Env	Envelope
ER	Endoplasmic reticulum
HIV	Human immunodeficiency virus
HR-C	C-terminal heptad repeat
HR-N	N-terminal heptad repeat
R5 HIV	CCR5-tropic HIV
R5X4 HIV	Dual-tropic HIV
SIV	Simian immunodeficiency virus
V3	Variable loop 3
X4 HIV	CXCR4-tropic HIV

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10.1 Introduction

Acquired immunodeficiency syndrome (AIDS) is a devastating disease caused by human immunodeficiency virus (HIV)-mediated destruction of CD4+ T lymphocytes (Barre-Sinoussi et al. 1983; Gallo et al. 1983). Since its emergence over 25 years ago, HIV/AIDS has killed more than 25 million people, and another 33 million are currently infected (UNAIDS 2009). The profound effect of this pandemic has led to tremendous research efforts to elucidate the mechanisms of the HIV life cycle and identify susceptible targets for therapeutic intervention. Here we will focus on the molecular mechanisms of the first stage of the HIV life cycle: viral entry.

HIV is an enveloped, single-strand RNA virus that probably first infected humans via a cross-species transmission from SIV-infected (simian immunodeficiency virus) chimpanzees in the first half of the twentieth century (Gao et al. 1999). For HIV and other known non-human primate lentiviruses to infect and replicate inside their hosts, the viral surface protein Envelope (Env) must first bind the host receptor CD4 and subsequently a coreceptor, most commonly CCR5 or CXCR4 (Alkhatib et al. 1996; Choe et al. 1996; Dalgleish et al. 1984; Deng et al. 1996; Doranz et al. 1996; Dragic et al. 1996; Feng et al. 1996; Oberlin et al. 1996). Coreceptor binding then triggers a conformational change in Env that mediates pH-independent membrane fusion and delivery of the viral payload (Fig. 10.1). Once inside the cell, the viral capsid is uncoated, revealing the viral genome. The viral RNA is then reverse transcribed via error-prone reverse transcriptase into double-strand DNA that is transported into the nucleus of the host cell where it integrates into a host chromosome. The integrated provirus is then transcribed and translated by host machinery to generate a polyprotein that is autocatalytically cleaved and processed to form nascent virions that bud from the host cell, enabling additional rounds of replication (Freed 2001).

Advances in understanding the HIV entry pathway have directly led to the development of specific and effective antiviral agents that prevent virus entry, including the first antiviral agent that targets a host cell molecule needed for virus infection. This provides proof of principle that virus entry in general, including the viral membrane fusion machines, can be targeted. Currently, two small molecule HIV entry inhibitors, the fusion inhibitor enfuvirtide and the CCR5 antagonist maraviroc, are FDA approved for the treatment of HIV, while others are currently under various stages of development (Dwyer et al. 2007; He et al. 2008a, b; Schurmann et al. 2007; Strizki et al. 2005; Tilton and Doms 2010). The success of these compounds emphasizes the potential therapeutic benefit in

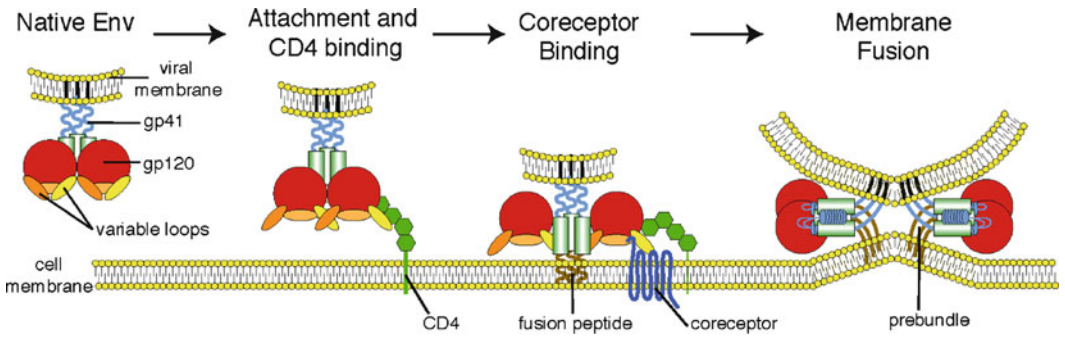

Fig. 10.1 HIV entry schematic. The HIV entry process can be divided into three key steps, all mediated by the viral surface protein Env, which is a trimer of gp120 and gp41 heterodimers. First, the virion binds to the host cell which can be facilitated by nonspecific cell attachment factors and allows Env to bind CD4. Second, CD4 binding induces conformational changes in Env that enable engagement of a coreceptor, most commonly CCR5 or CXCR4. This step triggers membrane fusion. Third, the gp41 subunits of Env enable fusion of the viral and host membranes allowing delivery of the viral payload

elucidating the mechanisms of viral entry. In addition, these drugs serve as critical tools in studying the mechanics of entry machines.

In addition to the identification of new drug targets, characterizing the HIV entry pathway has led to the identification of genetic polymorphisms in the human population that help account for the variable progression to AIDS seen after HIV infection (Gonzalez et al. 2005; McDermott et al. 1998). The most significant of these polymorphisms is a 32 base-pair deletion in *ccr5* called *ccr5Δ32*. Homozygosity for this mutation renders individuals highly resistant to HIV infection due to the functional loss of this viral coreceptor (Dean et al. 1996; Liu et al. 1996; Samson et al. 1996). People heterozygous for the *ccr5Δ32* mutation have somewhat reduced susceptibility to infection, and if they do get infected, they tend to progress to AIDS more slowly (Blanpain et al. 2002; Dean et al. 1996; Hoffman et al. 1997; Huang et al. 1996). In addition to the *ccr5Δ32* mutation, segmental duplications of a natural CCR5 ligand, CCL3L1 (MIP1α), may be associated with both decreased risk of HIV acquisition and disease progression. The gene duplications are thought to result in increased CCL3L1 expression and thus reduced CCR5 cell surface expression (Gonzalez et al. 2005). While genetic polymorphisms influencing CCR5 expression contribute significantly in explaining the variable disease progression observed in HIV-infected individuals, much of this variability cannot yet be explained and thus calls for additional investigation.

Despite the tremendous advances in understanding HIV entry, many questions remain unanswered. To penetrate cells, enveloped viruses must fuse with cells either directly at the plasma membrane or in intracellular compartments (Marsh and Helenius 2006). Historically, many have assumed that HIV fuses at the plasma membrane due to early studies demonstrating that Env can mediate cell–cell fusion both in vitro and in vivo, and because HIV entry can be augmented, but not inhibited, by lysomotropic agents (Maddon et al. 1988; McClure et al. 1988). However, the fact that HIV entry is pH independent provides no spatial information regarding the site of entry: pH-independent viruses can conceivably fuse at the cell surface or from within endocytic vesicles, albeit in a pH-independent fashion (Stein et al. 1987). Identifying the cellular site at which viral membrane fusion occurs is not a trivial process, but recent evidence using sophisticated live cell imaging to follow single virions suggests that productive membrane fusion may actually occur in endosomal compartments (Miyauchi et al. 2009; Uchil and Mothes 2009). Regardless of where fusion occurs, the mechanics of entry will likely be the same, and entry inhibitors clearly function both in vivo and in vitro.

In addition to questions regarding the site of membrane fusion during infection of primary cells and the implications this may hold for viral entry, several additional questions remain unanswered. Our knowledge of Env structure remains incomplete, and this complicates our understanding of how receptor binding induces such dramatic structural rearrangements in Env. It is also not clear how the fusion pore forms and expands, or how many trimers are needed for fusion. This chapter will review what is known about the mechanisms of the HIV fusion machine and how this informs our understanding of viral tropism, pathogenesis, and the development of antiviral therapies.

10.2 Key Players in HIV Entry

10.2.1 Env

The Env protein is a type I integral membrane protein first synthesized in the endoplasmic reticulum (ER) as a precursor termed gp160. While in the ER, gp160 folds, forms non-covalently associated trimers, is extensively glycosylated, and establishes critical disulfide bonds (Earl et al. 1991; Land and Braakman 2001; Land et al. 2003). The protein then traffics to the Golgi where the host protease furin cleaves it into gp120 and gp41 subunits. This cleavage event renders the protein fusion-competent by

generating the N-terminus of gp41 that is composed of a stretch of hydrophobic amino acids termed the fusion peptide (Hallenberger et al. 1992). After cleavage, the soluble surface gp120 and the transmembrane gp41 subunits continue to interact noncovalently forming native Env, a trimer of heterodimers.

Each gp120 subunit contains a highly conserved inner domain and a more variable outer domain. Within these are five highly conserved regions (C1–C5) and five hypervariable loops (V1–V5) with all but V5 defined by a disulfide bridge at its base. The five conserved regions are predominantly located in the inner domain, or gp120 core, while the variable loops predominate at the protein surface. The conserved domains are critical to gp120 folding and function, while the surface-exposed variable loops play roles in immune evasion by presenting a constantly moving target for the host immune system. In addition, the V3 region plays a critical role in coreceptor binding, as will be discussed later (Hartley et al. 2005; Poignard et al. 2001).

The gp41 subunit contains an ectodomain followed by a transmembrane domain and C-terminal cytoplasmic tail. The ectodomain encodes an N-terminal, hydrophobic fusion peptide that inserts into the target cell membrane and two heptad repeat regions (HR-N and HR-C) separated by a hinge region. The transmembrane domain anchors Env in the viral membrane, and the very long cytoplasmic tail plays a complex role in packaging, infectivity, and cell pathogenicity (Yang et al. 2010).

10.2.2 CD4

CD4 is an immunoglobulin superfamily member that contains four extracellular immunoglobulin domains (D1–D4), a single transmembrane domain, and a short intracellular cytoplasmic tail. CD4 plays a key signaling role that assists the T-cell receptor in activating cells in response to immunologic stimuli. It is expressed on macrophages, dendritic cells, and the eponymous CD4+ T cells, which include naive, central, and effector memory subtypes. Depletion of CD4+ T cells, and in particular the memory subsets, is indicative of disease progression and a poor clinical outcome.

10.2.3 Coreceptor

Most Env strains utilize one of two chemokine receptors, either CCR5 or CXCR4, for viral entry. Both CCR5 and CXCR4 are seven-transmembrane G-protein coupled receptors. Each has an extracellular N-terminus, three extracellular loops (ECLs), three intracellular loops, and a cytoplasmic C-terminus.

10.2.3.1 CCR5

CCR5 has three known natural ligands: CCL3, CCL4, and CCL5 formerly known as MIP-1α, MIP-1β, and RANTES, respectively. CCR5 is expressed on macrophages, microglia, and central and effector memory CD4+ T cells (Combadiere et al. 1996; Raport et al. 1996; Zaitseva et al. 1997). Of note, CCR5 is broadly expressed in the gut lamina propria contributing to destruction of the gut integrity during infection (Brenchley and Douek 2008; Kotler et al. 1984). Before CCR5 was discovered as the predominant HIV coreceptor, it was demonstrated that increased levels of its three chemokine ligands reduced susceptibility to HIV infection (Cocchi et al. 1995). This is due to both direct competition with Env and ligand-induced receptor internalization (Alkhatib et al. 1997). As previously discussed, variation in ligand expression may significantly affect disease progression (Gonzalez et al. 2005).

10.2.3.2 CXCR4

Compared to CCR5, CXCR4 is broadly expressed on the vast majority of hematopoietic cells and on many parenchymal cells as well. Genetic disruption of either CXCR4 or its only known natural ligand, CXCL12 (SDF-1), is embryonic lethal in mice and leads to defects in B-cell, cardiac, and cerebellar development, leukocyte migration, and bone marrow colonization by hematopoietic stem cells (Bleul et al. 1996; Nagasawa et al. 1996; Oberlin et al. 1996; Zou et al. 1998). Small molecule inhibitors of CXCR4 advanced to clinical trials for the treatment of HIV when it was noticed that the compounds induced peripheral mobilization of hematopoietic stem cells (Hendrix et al. 2004). Recently, the CXCR4 antagonist plerixafor was FDA approved for this very purpose: to harvest hematopoietic stem cells prior to bone marrow transplantation. Unlike for CCR5, there are no known CXCR4 mutations that contribute to HIV resistance, presumably due to the embryonic lethality of such defects. However, a gain-of-function mutation in CXCR4 has been reported that causes excessive signaling by preventing ligand-induced receptor internalization. This genetic lesion leads to WHIM syndrome, characterized by warts, hypogammaglobulinemia, increased susceptibility to infections, and myelokathexis, or retention of neutrophils in the bone marrow (Gorlin et al. 2000; Hernandez et al. 2003).

While the vast majority of transmitted virions use CCR5 (R5 HIV), viruses capable of using CXCR4 either alone (X4 HIV) or in combination with CCR5 (R5X4 HIV) emerge in approximately 50% of infected individuals in the developed world, typically 5–7 years after infection (Connor et al. 1997). Such a coreceptor switch expands viral target cell tropism and heralds a poor prognosis; however, the reasons it occurs and why only in later stages of disease remain unclear. It is of note that some exceedingly uncommon HIV isolates have been reported to use alternative coreceptors; however, the clinical relevance of alternative coreceptor use remains to be elucidated (Choe et al. 1996; Deng et al. 1997; Doranz et al. 1996; Edinger et al. 1998; Farzan et al. 1997).

10.3 The Structure of Env

Since HIV establishes a chronic infection, the viral Env protein must employ mechanisms to evade the ever-evolving humoral immune response. First, the shroud of N-linked carbohydrate structures and flexible variable loops act as a malleable shield masking more conserved and potentially neutralizable epitopes. Second, as a result of the error-prone reverse transcriptase, the HIV genome is highly diverse (Saag et al. 1988). On average, between one and ten of the 10,000 nucleotides comprising the HIV genome are mutated during each round of replication, and with some individuals producing as many as ten billion new virions per day, each nucleotide site in the viral genome can be theoretically mutated to each of the other three nucleotides at every site in the genome each day (Drosopoulos et al. 1998; Keulen et al. 1997; Lal et al. 2005). This variability enables Env to escape potentially neutralizing antibodies, partially explaining the difficulty in developing an effective neutralizing antibody vaccine (Stamatatos et al. 2009).

10.3.1 Env Crystal Structures

Several crystal structures have revealed detailed pictures of the HIV-1 gp120 monomer in complex with soluble CD4 (sCD4) and various antibodies (Huang et al. 2005; Kwong et al. 1998), though in all cases portions of gp120 were removed and the resulting purified protein deglycosylated to assist in crystallization efforts. An unliganded, glycosylated form of SIV gp120 has also been solved

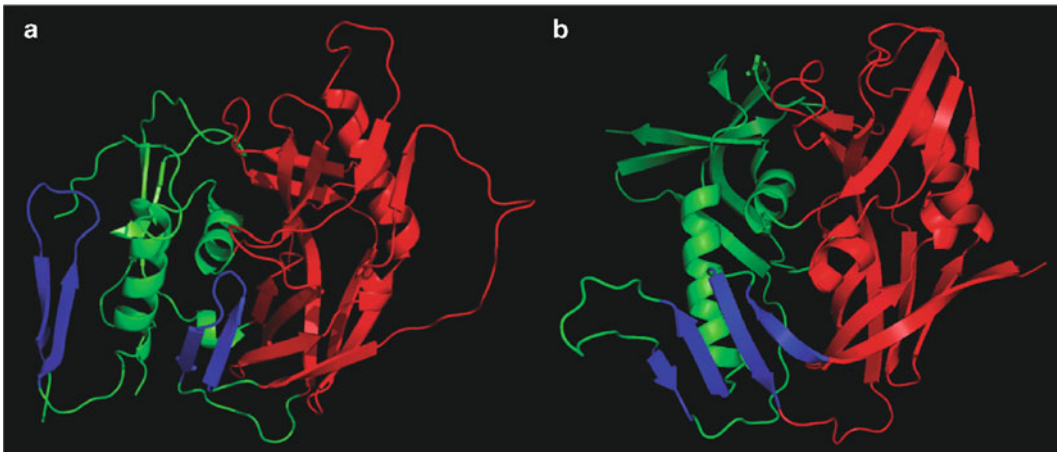

Fig. 10.2 Liganded and unliganded gp120 structures. CD4 binding induces massive structural rearrangements in gp120 that result in exposure and formation of the coreceptor-binding site. (**a**) Depicts the unliganded SIV gp120 monomer structure. The inner domain is in *green*, the outer domain is in *red*, and the bridging sheet is in *blue* (PDB ID: 2BF1) (Chen et al. 2005). (**b**) Depicts the CD4-bound HIV gp120 structure. The color scheme is the same as in (**a**). CD4 binding induces movement of the V3 loop that partially reveals the coreceptor-binding site. In addition, it causes the two pairs of β-strands to form the four-stranded β-sheet that enables coreceptor binding (PDB ID: 1GC1) (Kwong et al. 1998)

(Chen et al. 2005) (Fig. 10.2). In addition, portions of gp41 in its postfusion state have been solved (Chan et al. 1997; Weissenhorn et al. 1997). However, no crystal structures of trimeric Env have yet been reported.

Each gp120 monomer is comprised of a relatively conserved inner, or core, domain and a more variable outer domain. In addition, the outer domain contains the stems of the V3, V4, and V5 loops. While all the variable loops are thought to confer some degree of protection against neutralizing antibodies, the V3 loop is of particular interest due to its interactions with coreceptor. The V3 loop is almost always 35 residues in length and extends 30 Å from the gp120 surface (Huang et al. 2005, 2007). It is comprised of a relatively conserved disulfide-linked base, a long flexible stem, and a tip defined by a β-hairpin.

10.3.2 Cryo-Electron Microscopy

While crystallography has yielded atomic snapshots into Env subunit structure, cryo-electron tomography (see chapter 4) has revealed intriguing and though at times somewhat controversial structural properties of native Env trimers. By fitting known crystal structures into density maps of native Env obtained by cryo-electron tomography, detailed insights can be made into the native structure. The viral spike contains three propeller-like globular domains displaying threefold symmetry. The globular domains share a common density at their apex and are perched on a 50-Å stalk, most likely composed of gp41. The entire structure extends 120–140 Å from the viral membrane and is 110–120 Å in diameter (Sougrat et al. 2007; Zanetti et al. 2006; Zhu et al. 2006, 2008).

The abundance of structural studies has at times yielded conflicting results about several aspects of Env, with most of these revolving around how to place the known crystal structures within the tomographic densities. For instance, the position of the V1/V2 loop at the apex of Env proposed by Liu et al. (2008) is in contrast to that proposed using the unliganded SIV gp120 monomer, which

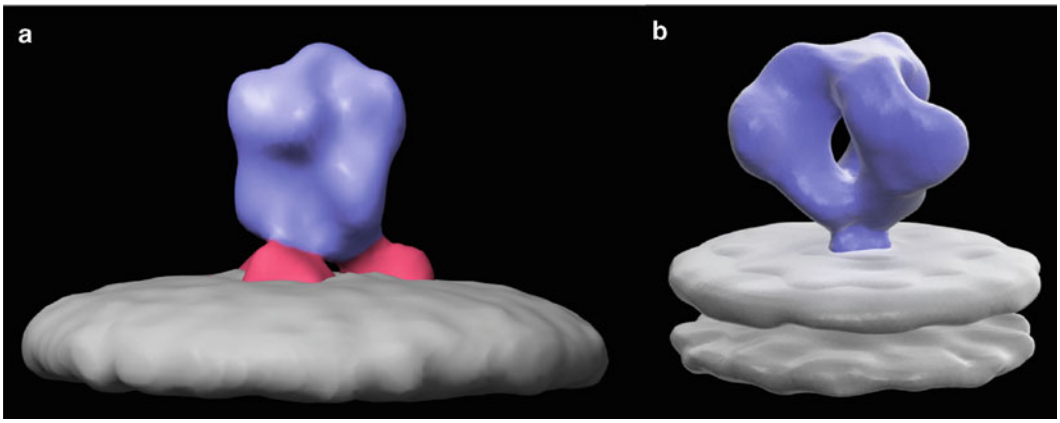

Fig. 10.3 Cryo-EM structures of the gp41 stalk. (**a**) Depicts the tripod-like stalk (highlighted in *red*) of native Env. Image courtesy of Kenneth Roux, Florida State University, 2010. In contrast, (**b**) Depicts native Env with a compact stalk. Reprinted by permission from Macmillan Publishers Ltd: Nature (Liu et al. 2008), copyright 2008

suggested that the V1/V2 loop is located nearer to the base of the trimer (Zanetti et al. 2006; Zhu et al. 2006). Two possible but not mutually exclusive explanations for this discrepancy are (1) that the V1/V2 loops of HIV and SIV are structurally different and (2) that the crystallization of the modified SIV gp120 resulted in a nonphysiologic location of V1/V2 (Liu et al. 2008). Further structural studies are needed to illuminate the detailed interactions that maintain and form stable trimers due to therapeutic and vaccine implications.

In addition, conflicting structural studies have described the native gp41 conformation as forming both a compact stalk and tripod-like structure (Liu et al. 2008; Zanetti et al. 2006; Zhu et al. 2008) (Fig. 10.3). Zhu et al. first reported a tripod-like stalk structure of a cytoplasmic tail-truncated SIV that had an increased number of Env spikes in the viral membrane (Zhu et al. 2006). They later reached a similar conclusion using native HIV trimers. However, Zanetti et al. and Liu et al. independently concluded that, like other class I viral fusion proteins studied to date, the viral stalk is compact and devoid of tripod-like legs (Liu et al. 2008; Zanetti et al. 2006). While it is possible that the discrepancy is due to inherent structural differences between the highly similar viral strains, it is more likely due to differential image collection and analysis. Specifically, electron tomography studies are limited by the "missing wedge" problem arising from the fact that the maximal angle between the microscope stage and image detector is typically 70°. This results in a "missing wedge," which the groups accounted for differently. In addition, the authors used different reference images to compensate for noise, which may result in downstream bias (Subramaniam 2006). Finally, all groups assumed threefold symmetry when solving the native Env structure, which may introduce a common bias.

10.4 The Entry Pathway

10.4.1 Attachment and CD4 Binding

HIV entry follows an intricate and sequential sequence of events whose primary mission is to deliver the viral payload. Our understanding of this complex pathway has been facilitated by the development of small molecule and protein-based inhibitors that target the key steps of the pathway. Entry

begins with virion binding to the cell surface. This is often, but not always, facilitated by noncovalent interactions between Env and cell attachment factors. While not required for virus infection, the presence of such attachment factors can enhance infection of virus particles by accelerating virus–cell interactions, typically the rate-limiting step of virus infection in vitro. Examples of such molecules include heparan sulfate and mannose-binding lectins such as DC-SIGN (Baribaud et al. 2001; Geijtenbeek et al. 2000; Mondor et al. 1998; Pohlmann et al. 2001a, b). The role of attachment factors likely differs between cell types and viral strains, and its significance in vivo where direct cell-to-cell spread via virological synapses can occur remains unclear.

While a single receptor is sufficient for most enveloped viruses, the sequential receptor-binding strategy utilized by HIV has several benefits. Initial binding of CD4 induces exposure and creation of the coreceptor-binding site and thus may serve to protect this conserved region from host immune recognition (Chen et al. 2005; Kwong et al. 1998; Lal et al. 2005). In addition, the requirement for CD4 binding may reduce inappropriate and irreversible triggering of the fusion machinery, thus maximizing the infectivity of each virion. Finally, the CD4 molecule protrudes substantially further from the cell surface than the chemokine receptors and thus may also serve to facilitate virus binding.

The CD4-binding site (CD4bs), located at the interface of the outer and inner domains of gp120, is depressed 20 Å from the apex of the trimer, surrounded by carbohydrate moieties, and is likely partially occluded by the flexible V1/V2 loops (Kwong et al. 1998). CD4 binding to this highly conserved site initiates a sequence of structural rearrangements in Env defined both biochemically (Myszka et al. 2000) and structurally (Chen et al. 2005; Kwong et al. 1998) that reveal and create the coreceptor-binding site.

First, CD4 binding is thought to induce movement of the V1/V2 stem from the central axis of symmetry toward the lateral aspect of the trimer. This leads to V3 loop rearrangement and exposure of part of the coreceptor-binding site. Furthermore, the V3 stem shift toward the distal end of the trimer places it in direct apposition to the host target membrane, thus priming it for coreceptor engagement. Second, the bridging sheet, the second coreceptor-binding site, is formed by bringing together four antiparallel beta sheets, two each from the inner and outer domains of gp120. Third, the CD4-induced gp120 movement is thought to result in an outward rotation of each gp120 monomer that partially reveals the gp41 stalk. Finally, a recent model suggests that CD4–Env interactions cause CD4 to fold like a hinge between its second and third immunoglobulin-like domains. This folding brings the viral and host membranes into closer proximity and may serve to further limit antibody exposure of the conserved coreceptor-binding site (Liu et al. 2008).

In total, the aforementioned rearrangements culminate in coreceptor accessibility to its two binding sites, the V3 loop and the bridging sheet. The N-terminus of coreceptor engages the base of the V3 loop and the newly formed bridging sheet, while the ECLs, particularly ECL2, bind to the crown or tip of the V3 loop (Rizzuto and Sodroski 2000; Rizzuto et al. 1998). Most, but not all, Env strains depend upon both N-terminal and ECL2 interactions for entry.

10.4.1.1 Thermodynamics of CD4-Induced Changes

Thermodynamic studies complement the CD4-induced changes revealed by structural studies. Myszka et al. carried out a series of elegant experiments assessing the binding interactions between sCD4 and gp120 (Myszka et al. 2000). Both a full-length form of gp120 as well as a gp120 core devoid of N-linked glycans and containing truncations in the V1–3 loops, N-, and C-termini were compared. Both core and full-length gp120 had similar thermodynamic profiles suggesting that the CD4-induced changes between the two structures are conserved. Titration microcalorimetry revealed a surprisingly favorable CD4–gp120 binding enthalpy (−63 kcal/mol) suggesting that substantial, novel molecular interactions form, specifically van der Waals forces and hydrogen bonding. The

magnitude of enthalpy is significantly greater than other common protein–protein interactions such as antibody–antigen and T cell receptor–major histocompatibility complex binding. In addition, there is a dramatic and unfavorable change in entropy upon CD4 binding. This demonstrates a reduction in randomness and is further evidence of significant structural rearrangements. The profound magnitudes of both entropy and enthalpy suggest that nearly 100 amino acids in the full length gp120 change conformation upon CD4 binding – greater than nearly all other reported binary protein–protein interactions (Myszka et al. 2000).

10.4.2 Coreceptor Binding

CD4 binding is thought to induce movement of the V1/V2 loop to expose the V3 loop. This allows the tip, or crown, of V3 to interact with the ECL2 of coreceptor. A second key gp120–coreceptor interaction involves sulfated tyrosines present in the CCR5 N-terminal domain with the base of the V3 loop and the four-stranded bridging sheet, which is formed by CD4-induced rearrangement of a pair of spatially separated two-stranded beta sheets. Extracellular loops 1 and 3 (ECL1 and ECL3) may also interact with Env, although the strength and significance of such interactions are uncertain.

Evidence for the interactions between the tip of V3 and ECL2 comes from a V3-containing gp120 crystal structure revealing that the V3 tip protrudes 30 Å from the gp120 core and is directed toward the target cell membrane allowing V3 to act as a "molecular hook" (Huang et al. 2005). Additional evidence for this interaction comes from the mechanism in which small molecule coreceptor antagonists inhibit HIV entry. A number of compounds have been designed that bind to a hydrophobic pocket within the transmembrane helices of both CCR5 and CXCR4. These molecules inhibit coreceptor engagement by causing a stable conformational shift of the ECLs. This suggests that the orientation of the ECLs is critical for coreceptor function. In addition, antibodies directed at the ECL2 are sufficient to prevent entry of most HIV isolates (Trkola et al. 2001). These antibodies likely preclude Env binding by both steric hindrance and alteration of ECL2 conformation. Further, site-directed mutations in ECL2 can abrogate infection (Doranz et al. 1997; Quinonez et al. 2003). Finally, a laboratory-engineered virus containing a V3 loop truncation that preserves the V3 base and eliminates the crown results in broad resistance to a number of CCR5 inhibitors. This virus relies solely on the CCR5 N-terminus as opposed to both the ECL2 and N-terminus for entry (Laakso et al. 2007).

The CCR5 N-terminus (residues 2–31) binds the base of V3 and the bridging sheet (Choe and Farzan 2009; Cormier et al. 2000; Huang et al. 2007). The CCR5 N-terminus contains four sulfotyrosine residues at positions 3, 10, 14, and 15; only that at position 15 is dispensible without reducing viral entry efficiency (Cormier et al. 2000; Farzan et al. 1998; Rabut et al. 1998). In addition, the N-terminus contains acidic residues which further facilitate binding to the negatively charged site in Env (Rizzuto and Sodroski 2000). Substantial evidence supports these interactions. First, sulfated N-terminal CCR5 peptides are sufficient to block entry, albeit at high concentrations (Cormier et al. 2000). Second, inhibition of tyrosine sulfation decreases the ability to engage CCR5 (Farzan et al. 1999). Third, several gp120 neutralizing antibodies have been reported that contain sulfated tyrosines at the site of antigen contact. These antibodies require sulfation for activity, are more effective in the presence of CD4, and compete with N-terminal-sulfated CCR5 peptides for gp120 binding (Choe et al. 2003). Recently, a crystal structure of one of these antibodies (412d) and CD4 bound to gp120 core containing V3 was solved (Huang et al. 2007). This structure demonstrated that sulfated tyrosine residues formed salt bridges and numerous hydrogen bonds with gp120. The molecular rearrangements in gp120 needed to form the tyrosine-binding domain for CCR5 Tyr14 and 412d Tyr100 result in the creation of a β-hairpin and stabilization of the previously flexible V3 loop.

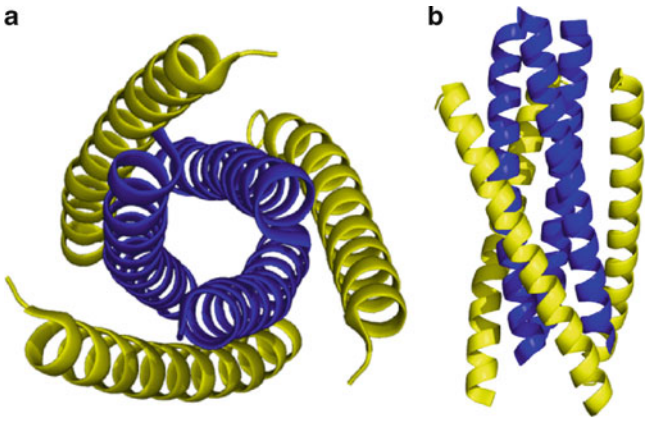

Fig. 10.4 Six-helix bundle structure (6HB). The three central HR-N domains are shown in *blue*, and the three HR-C domains are shown in *yellow*. The HR-C helices are antiparallel to the HR-N helices and pack into exterior grooves formed by the HR-N central coils. (**a**) Top view and (**b**) side view (PDB ID: 3F4Y) (Horne et al. 2009)

10.4.2.1 Env Determinants of CCR5 and CXCR4 Use

Elucidating the mechanistic differences governing CCR5 or CXCR4 use has significant clinical implications. The emergence of X4 viruses heralds progression to AIDS, and while causation has not been proven, it is known that X4 HIV is more pathogenic to CD4+ T cells in vitro. At present it is unclear whether the outgrowth of X4 HIV results from a depletion of CCR5-positive cells and thus exhaustion of the R5 HIV reservoir, or whether X4 viruses stochastically emerge and preferentially expand over R5 viruses. Being able to predict such a R5 to X4 coreceptor switch may enable clinicians to better predict a patient's disease progression and response to R5 antagonists.

In general, R5 viruses depend more upon the N-terminal interactions than X4 viruses which favor ECL2 interactions (Rizzuto and Sodroski 2000). The most significant Env contributor to coreceptor tropism is the V3 loop with the degree of charge playing a significant role in determining whether a virus uses CCR5 or CXCR4 (Hartley et al. 2005; Hwang et al. 1991). Most R5 viruses have a net V3 charge of +3 to +5, while most X4 viruses have a charge of +7 to +10, consistent with the increased negative charge on the CXCR4 N-terminus and ECLs compared to that of CCR5 (Jensen et al. 2003; Kwong et al. 2000). More specifically, positively charged residues at positions 11 and 24/25 in V3 are highly predictive of X4 tropism, while the absence of positive charges at these locations does not preclude CXCR4 use (Fouchier et al. 1992; Hartley et al. 2005).

10.4.3 Fusion: Formation and Opening of the Fusion Pore

Coreceptor binding triggers a "cast-and-fold" mechanism of membrane fusion (Melikyan 2008). The hydrophobic, N-terminal gp41 fusion peptide is exposed and likely projected toward the target cell membrane into which it inserts, tethering the viral and host membranes and destabilizing the host lipid bilayer (Tamm and Han 2000). The gp41 ectodomain contains two helical regions, both approximately 36 residues in length. The N-terminal helical regions (HR-N) from each gp41 subunit form a triple-stranded, coiled-coil structure. During the process of membrane fusion, the three C-terminal helical regions (HR-C) fold back and pack into exterior grooves formed at the external interface of the three HR-N domains in an antiparallel fashion. This results in a highly stable six-helix bundle (6HB) in which the fusion peptide and gp41 transmembrane domains, along with their respective membranes, are brought into close spatial proximity (Fig. 10.4) (Chan et al. 1997; Weissenhorn et al. 1997).

Several lines of evidence suggest that 6HB formation is critically important for membrane fusion. First, purified HR-N and HR-C peptides spontaneously fold into a 6HB in the absence of gp120.

Second, the 6HB is relatively thermostable compared to native gp41 suggesting it has adopted the lowest energy conformation. Third, both 6HB formation and fusion can be inhibited with peptides that mimic the 6HB N-terminal helices, even after CD4 binding. Fourth, the most robust amino acid conservation in gp41 between HIV and SIV is seen at the residues predicted to interact in the 6HB (Chan et al. 1997; Furuta et al. 1998; Weissenhorn et al. 1997). Finally, similar 6HB structures are seen in postfusion influenza hemagglutinin, Ebola glycoprotein, Moloney murine leukemia virus, and numerous other viral fusion machines suggesting this is a commonly utilized mechanism of membrane fusion (Bullough et al. 1994; Fass et al. 1996; Weissenhorn et al. 1997, 1998a, b).

Historically, it has been assumed that the highly conserved 6HB was responsible for the initiation of pore formation (Chan et al. 1997; Weissenhorn et al. 1997). However, recent evidence suggests that the prebundle complex actually initiates early pore formation and that the 6HB stabilizes and facilitates expansion of the nascent fusion pore (Markosyan et al. 2003). The prebundle state can be divided into an early prebundle, or temperature-arrested stage in which HR-N and HR-C are maximally separated, and a late prebundle that is present after hinge folding, but immediately before 6HB formation (Markosyan et al. 2003). The late prebundle can revert to the early prebundle in vitro by lowering the temperature to 4°C resulting in pore closure; however, the 6HB structure is the lowest energy state and cannot revert to the late prebundle, and thus it locks the pore open. Inhibitors of 6HB formation, such as the peptide enfuvirtide, allow pore formation, but prevent its expansion and stabilization.

10.5 Env Stoichiometry

While a tremendous amount has been learned about the molecular mechanisms of HIV entry and fusion, the overall stoichiometry of such entry events remains elusive. At present, it is still unclear how many Env trimers are required for entry and how many gp120 subunits per trimer are needed to productively engage CD4 and coreceptor. Resolving these questions may have implications for drug and vaccine design and may further reveal common principles of membrane fusion.

It has been reported that most wild-type HIV virions contain 10–15 randomly distributed Env spikes per virion (Liu et al. 2008; Yuste et al. 2004; Zhu et al. 2006), placing an upper limit on the number of spikes needed for membrane fusion. The trimeric Env spikes are likely mobile within the plane of the viral membrane, since an electron tomography study analyzing HIV and CD4+ T cells revealed between five and seven electron dense structures consistent in size and shape with HIV spikes at the point of virus–cell contact (Sougrat et al. 2007). Such structures were not detected in the presence of a small molecule CCR5 inhibitor or fusion inhibitor, consistent with the densities playing a role in entry. However, the presence of five to seven Env trimers at the site of virus–cell contact does not necessarily mean that all are involved in the membrane fusion process.

A variety of modeling approaches have been taken to estimate the number of Env trimers needed for membrane fusion, though these have yielded different answers, sometimes even from the same datasets. Yang et al. expressed in cells different ratios of wild-type Env proteins along with very similar Envs that, due to one or more amino acid changes, could not be neutralized by a specific monoclonal antibody (Yang et al. 2005). Since coexpression of similar Envs in the same cell results in the production of mixed trimers, varying the ratio of wild-type to mutant Env would in turn vary the proportion of wild-type to mixed heterotrimers. Using this approach, and assessing the efficiency with which virus particles could be neutralized, Yang et al. concluded that a single Env spike is sufficient for HIV entry. However, several other groups analyzed the data generated by Yang et al. and reached a different conclusion with $T>1$. Klasse concluded that virions containing nine spikes require a T of five (Klasse 2007). Later, Magnus et al. generated several different mathematical models to determine a range for T between 2 and 19. The discrepancy between the analyses results from the different assumptions including how to account for imperfect transfections, nonrandom

heterotrimer formation among the mutant and wild-type Env subunits, and the distribution of spike number among different virions (Magnus et al. 2009). Furthermore, Kuhmann et al. (2000) created stable cell lines expressing wild-type, N-terminal, and ECL2 mutants of CCR5 at various levels and then determined the relative infectivity of each cell population. Employing mathematical modeling, they determined that entry requires four to six coreceptor–gp120 interactions suggesting that two to six spikes are required per virion.

In addition to the uncertainty regarding the number of spikes needed for entry, it is at present uncertain how many CD4 and coreceptor molecules are needed to activate an Env trimer. In vivo, CCR5 expression is likely limiting on most target cells, compared to both CD4 and CXCR4. As a result, viruses bearing Env proteins that can be activated by one or two CCR5-binding events rather than three might be better able to infect cells expressing low levels of CCR5. In fact, it is commonly observed that some Envs are more "fusogenic" than others even when expressed at similar levels, though the mechanism(s) that accounts for these functional differences is not known. Conceivably more fusogenic Envs could be triggered by a lower number of receptor-binding events, though they could also undergo the conformational changes needed for membrane fusion with a higher degree of fidelity. A fuller understanding of how receptor and Env cooperativity govern membrane fusion will likely provide greater insight into viral tropism, and why some virus strains are not able to infect some primary cell types even though the relevant viral receptors are expressed (Salazar-Gonzalez et al. 2009).

10.6 Inhibitors of HIV Entry

While studying HIV entry has informed us about basic principles of virology and cell biology, the overarching goal should be to develop novel and effective therapeutics to limit the morbidity and mortality of the HIV/AIDS pandemic. Currently, two entry inhibitors, the CCR5 inhibitor maraviroc and the fusion inhibitor enfuvirtide, are FDA approved for the treatment of HIV infection. A number of other compounds that have targeted nearly every step of the entry pathway have also been tested in the clinic. For purposes of this discussion on therapeutics, HIV entry will be divided into three components: (1) attachment and CD4 binding, (2) coreceptor binding, and (3) membrane fusion (Table 10.1).

Table 10.1 HIV entry inhibitors

Class	Compound	Development status	Reference
Attachment inhibitor	PRO 2000	Phase III trial showed no efficacy	Rusconi et al. (1996)
Attachment inhibitor	Carraguard	Phase III trial showed no efficacy	Skoler-Karpoff et al. (2008)
Attachment inhibitor	Cellulose sulfate	Phase III trial showed no efficacy	Halpern et al. (2008) and Van Damme et al. (2008)
CD4-binding site inhibitor	BMS-378806	Preclinical development	Lin et al. (2003)
CD4 mimetic	Soluble CD4	Phase I study showed no efficacy	Daar et al. (1990) and Schacker et al. (1994)
CD4 downmodulators	CADA derivatives	Preclinical development	Vermeire et al. (2002, 2007, 2008)
CCR5 antagonist	Maraviroc	FDA approved	(2009)
CCR5 antibody	PRO 140	Phase II trials	Jacobson et al. (2008)
CXCR4 antagonist	Plerixafor	FDA approved for hematopoietic stem cell mobilization	Flomenberg et al. (2010)
Fusion inhibitor	Enfuvirtide	FDA approved	Wild et al. (1992, 1993)
Fusion inhibitor	D-peptides	Preclinical development	Welch et al. (2007)

10.6.1 Attachment and CD4-Binding Inhibitors

Attachment and CD4-binding inhibitors include relatively nonspecific anionic polymers, CD4bs inhibitors, sCD4 mimetics, and CD4 downmodulators. Anionic polymers, which act by preventing the favorable electrostatic interactions between the positively charged Env and negatively charged cell surface, have been predominantly studied for use in vaginal microbicides. PRO 2000, a naphthalene sulfonate polymer, inhibits soluble gp120 and CD4 binding in vitro (Rusconi et al. 1996). However, in a phase III clinical microbicide trial, PRO 2000 demonstrated no efficacy (CONRAD 2009, 14 December). Other anionic polymers including cellulose sulfate and Carraguard, derived from seaweed, demonstrated no efficacy, and cellulose sulfate may have actually increased the risk of HIV transmission (Halpern et al. 2008; Skoler-Karpoff et al. 2008; Van Damme et al. 2008).

Another approach involves targeting the CD4bs on gp120. Several small molecules that bind to gp120, such as BMS-378806 and BMS-488043, have antiviral activity in vitro and prevent CD4–gp120 binding (Ho et al. 2006; Lin et al. 2003). However, for at least BMS-378806, HIV quickly adapts resistance limiting its therapeutic potential (Lin et al. 2003). Furthermore, sCD4 demonstrated anti-HIV activity in vitro, but early-stage clinical trials were unable to demonstrate antiviral activity, most likely due to insufficient circulating concentrations of sCD4 (Daar et al. 1990). An additional class of compounds inhibits Env–CD4 interactions by downregulating CD4. These drugs, derivatives of cyclotriazadisulfonamide (CADA) (Vermeire et al. 2002), reduce CD4 expression by an unknown mechanism. However, they do not alter CD4 mRNA levels suggesting they exert their function in a posttranscriptional manner (Vermeire et al. 2007).

10.6.2 Coreceptor-Binding Inhibitors

Discovery of the *ccr5Δ32* mutation demonstrated that CCR5 is not essential for normal human growth and development, suggesting that it could be safely targeted by small molecule inhibitors (Dean et al. 1996; Liu et al. 1996; Samson et al. 1996). Several small molecule CCR5 antagonists have been developed and shown to have antiviral activity in vivo, including maraviroc, which received FDA approval in 2007 for use in treating HIV-infected individuals. Most small molecule CCR5 inhibitors, including maraviroc, function by binding to a hydrophobic pocket within the transmembrane domains of the protein – a region of the receptor not thought to directly interact with the viral Env protein (Baba et al. 1999; Tilton et al. 2010; Tilton and Doms 2010). As a result, CCR5 antagonists likely function by an allosteric mechanism, inducing conformational changes in the ECL domains of the receptor that subsequently prevent Env binding. Viral resistance to such compounds occurs by one of two pathways. In vivo, it appears that the most common resistance pathway is outgrowth of CXCR4-using viruses, even when present below the limit of detection in standard assays at the initiation of therapy. A second, less common pathway results from mutations in Env that enable it to utilize the drug-bound conformation of the receptor (Berro et al. 2009). In at least some cases, it appears that enhanced utilization of the CCR5 N-terminal domain is associated with this phenotype. In addition to traditional small molecule inhibitors, CCR5 blocking antibodies are being explored for therapeutic purposes. One such antibody, PRO 140, blocks HIV utilization of CCR5 while preserving CCR5 ligand function. PRO 140 is currently in phase II clinical trials (Jacobson et al. 2008; Tilton and Doms 2010).

Unlike for CCR5, inhibiting CXCR4 has been met with limited success primarily due to the concerns of systemic toxicity. Several CXCR4 inhibitors advanced to early-stage clinical trials, but none are currently ongoing for the treatment of HIV. One CXCR4 inhibitor, plerixafor, was recently FDA approved to mobilize hematopoietic stem cells to the peripheral blood for harvesting prior to bone marrow transplantation (Flomenberg et al. 2010).

10.6.3 Fusion Inhibitors

Membrane fusion is the net result of Env–receptor interactions and is the target of the first entry inhibitor ever approved, enfuvirtide. Enfuvirtide, previously known as T20, is a 36-amino-acid mimetic of the HR-C domain. The peptide binds the central coiled coils comprised of three HR-N molecules and inhibits 6HB formation, thus preventing fusion (Wild et al. 1992, 1993). Despite the in vivo efficacy of enfuvirtide, resistance mutations in a ten-amino-acid region of HR-N that prevent enfuvirtide binding have been well documented (Greenberg and Cammack 2004). Importantly though, resistance to enfuvirtide does not confer cross-resistance to other classes of entry inhibitors (Reeves et al. 2005). Novel fusion peptide-based inhibitors have been designed to combat enfuvirtide-resistant viruses, and some of these molecules have synergistic effects with enfuvirtide (Dwyer et al. 2007; Pan et al. 2009a, b).

While these peptide-based fusion inhibitors exhibit efficacy, they are limited by the fact that they are not orally bioavailable and therefore must be injected, a significant hindrance in maintaining patient adherence. One potential solution is the development of orally bioavailable small molecules that recapitulate enfuvirtide's mechanism of action by blocking the hydrophobic "knob-into-hole" interactions. The knobs are hydrophobic HR-C residues, specifically tryptophans and isoleucines, that pack into large hydrophobic holes present in the HR-N central coil (Chan et al. 1997). D-peptide inhibitors of gp120 represent one such exciting new class of compounds. They have potent in vitro activity and are not degraded by intestinal proteases and thus have the potential to be orally bioavailable (Welch et al. 2007).

10.7 Conclusion

Work on the mechanisms of HIV entry has led to the discovery of human mutations affecting HIV susceptibility and disease progression as well as the development of new antiviral agents, such as enfuvirtide and maraviroc. In addition to the continued development of entry inhibitors, a critical future challenge is translating our molecular understanding of HIV entry into therapeutically useful information. Will our knowledge of HIV entry make it possible to predict which patients stand most to benefit from the use of entry inhibitors? More generally, the discovery of the HIV coreceptors revealed a new mechanism for triggering conformational changes in viral membrane fusion proteins – that of sequential receptor engagement. In addition, the ability of attachment factors such as DC-SIGN to enhance HIV-1 infection has led to similar discoveries for other viruses. Finally, the ability of HIV-1 to enter particular T-cell subsets is still not fully understood. As our understanding of HIV entry becomes more complete, so too should our understanding of HIV tropism and pathogenesis.

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