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Review

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HIV envelope: challenges and opportunities for development of entry inhibitors

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The HIV envelope proteins glycoprotein 120 (gp120) and glycoprotein 41 (gp41) play crucial roles in HIV entry, therefore they are of extreme interest in the development of novel therapeutics. Studies using diverse methods, including structural biology and mutagenesis, have resulted in a detailed model for envelope-mediated entry, which consists of multiple conformations, each a potential target for therapeutic intervention. In this review, the challenges, strategies and progress to date for developing novel entry inhibitors directed at disrupting HIV gp120 and gp41 function are discussed.

HIV and the role of envelope proteins

HIV is the causative agent of AIDS, a devastating disease that has resulted in over 25 million deaths and newly infects more than 5 million people each year [1]. In HIV, the envelope proteins glycoprotein 120 (gp120) and glycoprotein 41 (gp41) play crucial roles in the entry process, therefore, they have been the focus of intense study (for reviews, see [2,3]). gp120 and gp41 are synthesized as precursor gp160, which undergoes a number of maturation steps including the formation of disulfide bonds, extensive glycosylation, cleavage by cellular furin-like proteases, transport to the cell surface as a non-covalent membrane complex, and finally, incorporation into the budding virus [4]. As shown in Figure 1, gp120 is composed of five conserved domains (C1, C2, C3, C4 and C5) and five variable domains (V1, V2, V3, V4 and V5); gp41 is composed of seven domains: N-terminal fusion peptide, heptad repeat 1 (HR1), disulfide loop, heptad repeat 2 (HR2), membrane proximal ectodomain region (MPER), transmembrane, and cytoplasmic [5]. Based on the sequence homology of the envelope proteins, there are two types of HIV, namely HIV-1 and HIV-2. HIV-1 and HIV-2, as well as simian immunodeficiency virus (SIV), the homolog found in simians, exhibit approximately 30% sequence identity. HIV-1, which is the most prevalent, comprises 5 subtypes (or clades): A, B, C, D and E, with the primary differences being in the length and sequence of the gp120 variable loops [5]. Recently, significant progress has been made in understanding HIV envelope structure and function from structural and mutagenesis studies. In this review, I suggest that the envelope presents an abundance of target sites for therapeutic intervention. In addition, I summarize potential intervention strategies and discuss the progress to date.

HIV envelope protein structure

Owing to their critical role in HIV entry, the envelope proteins of HIV and the homologs found in SIV have been extensively studied by the structural techniques X-ray crystallography and NMR spectroscopy in the hope that structural knowledge may be exploited for the design of entry inhibitors and vaccines. However, due to technical difficulties, which include the insolubility of membrane-associated proteins, presence of metastable conformations, extensive glycosylation, and dynamic regions, structural studies to date have been limited to isolated domains. Structural information from X-ray and NMR studies include: the overall fold of the gp120 core [6,7] (Figure 2a) and the gp41 ectodomain trimer [8–10] (Figure 2b), the receptor binding sites on gp120 [6,11], structural changes of the gp120 core upon receptor binding [6,12], structure of the fusion peptide in a lipid environment [13], and the structure of the gp41 MPER, an important target for neutralizing antibodies [14–16]. Together, these structural studies suggest that the gp120–gp41 complex undergoes a series of conformational changes during the entry process. Nonetheless, many details are missing including high-resolution structural information about the gp120–gp41 interaction, in addition to structural information for numerous domains absent in all available structures (Figure 1). Indeed, the gp120–gp41 complex has been listed by the journal *Nature* as the fourth most desirable structure to obtain in biology [17] and many challenges remain in achieving this goal.

Mutational studies of the HIV envelope

As an alternative approach to structural biology, mutagenesis has been used to great effect to map function to specific regions of the envelope and give topological information about the gp120–gp41 complex. For example, site-specific mutagenesis studies have given insights into the functional roles of gp120-conserved domains and the gp41 fusion peptide, HR1, disulfide loop, and MPER domains [18–27]. Interestingly, many of the mutants are non-functional for entry, even with relatively conservative changes such as alanine substitution for threonine [23]. This observation is somewhat surprising given that the envelope mutates readily, presumably to avoid the immune system while

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0966-842X/$ – see front matter doi:10.1016/j.tim.2011.02.001 Trends in Microbiology, April 2011, Vol. 19, No. 4

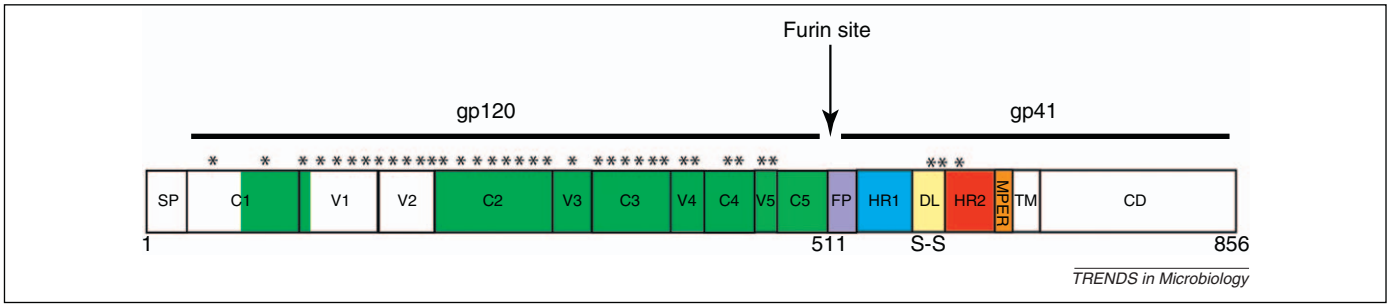

Figure 1. Organization of HIV envelope proteins. Numbering corresponds to that of strain HXB2 of HIV-1 [5]. Putative glycosylation sites are denoted by asterisks. Domain abbreviations: SP, signal peptide; C1–C5, conserved domains 1 to 5; V1–V5, variable domains 1 to 5; FP, fusion peptide; HR1, heptad repeat 1 (HR1 is sometimes referred to as N-Helix); DL, disulfide loop; HR2, heptad repeat 2 (HR2 is sometimes referred to as C-Helix); MPER, membrane proximal ectodomain region; TM, transmembrane domain; CD, cytoplasmic domain. High resolution structural information is available for gp120 and gp41 domains colored as in Figures 2 and 3.

maintaining its function. However, the sensitivity of the envelope to mutation might suggest that many sites are potential targets for therapeutic intervention. For example, a small molecule, peptide or protein that binds with relatively high affinity to a sensitive region could be expected to disrupt envelope function (i.e. HIV entry).

Mutagenesis, in combination with structural information, has given important insight into gp120–gp41 interaction for which structural information is absent. As summarized in Figure 3, mutations that result in dissociation of gp120 from the viral or cellular membrane (i.e. from gp41) suggest residues in direct intermolecular contact, or alternatively, residues that are proximal to the contact. Accordingly, C1 and C5 of gp120 are in putative contact with the C terminal region of HR1 and disulfide loop of gp41, an observation that is supported by recent biochemical studies [28]. Additional information about the gp120–gp41 association comes from the studies of Binley *et al.* [29] who have shown that gp120 C1 and C5 interact with the gp41 disulfide loop, as deduced from the presence of non-native intermolecular disulfides in a series of cysteine scanning mutants. Interestingly, the best characterized mutant, namely SOS (A501C/T605C), is not functional until the non-native disulfide bond is reduced, suggesting that conformational changes in the gp120–gp41 association, and perhaps dissociation of gp120 from gp41, are critical to function [24,30,31], which underscores the importance of conformational change to HIV entry. Finally, mutations of the envelope could also serve to stabilize constructs in a particular conformation to make them more amenable for structural studies and for use as vaccine candidates [32].

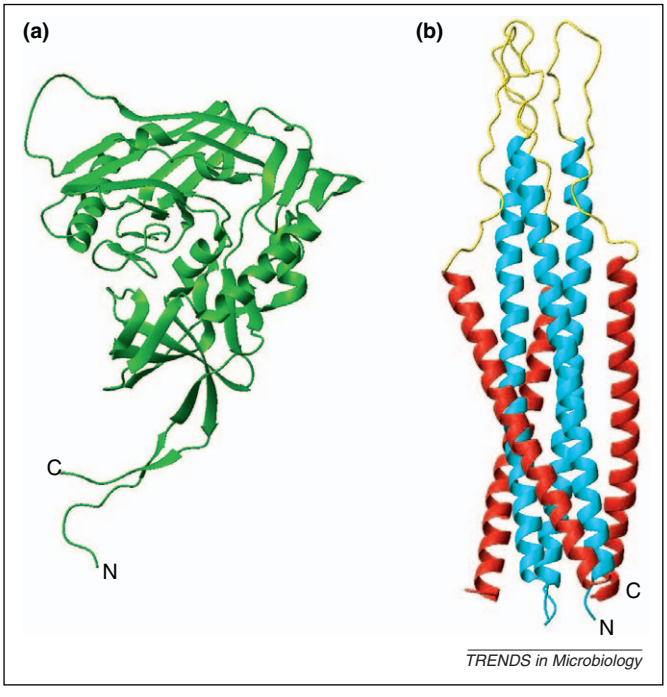

Figure 2. Structures of gp120 and gp41. **(a)** Ribbon diagram of the HIV gp120 core [7]. For clarity, the CD4 and antibody domains that are bound to the gp120 core are not shown. **(b)** Ribbon diagram of the SIV gp41 ectodomain [10]. In this structure, the HR1, disulfide loop and HR2 domains are colored cyan, yellow and red, respectively.

Model of envelope-mediated HIV entry

Based on structural and functional studies of HIV and SIV envelope constructs, in addition to structural studies of the

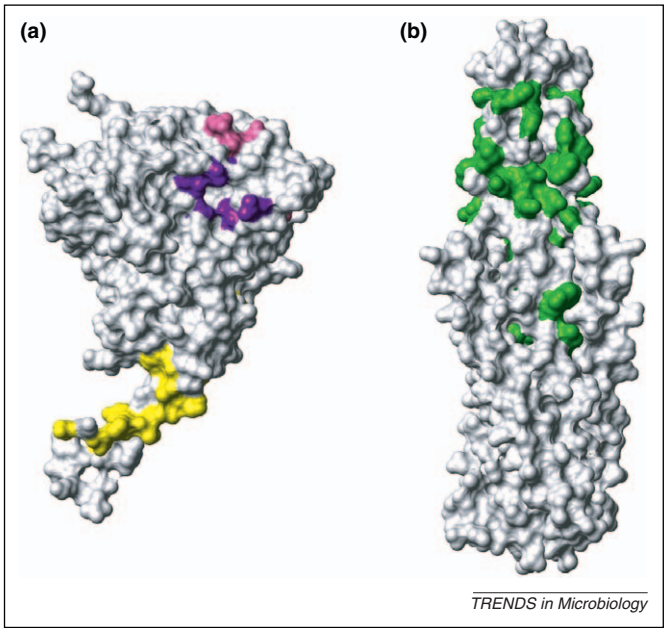

Figure 3. Intermolecular interactions of gp120 and gp41. **(a)** Space filling representation of the gp120 core. Gp120 residues that form interactions with CD4 (residues 112, 255, 257, 368, 370, 371, 427, 430 and 473, HIV-1 strain HXB2 numbering) and CR (residues 121, 419, 421 and 422, HIV-1 strain HXB2 numbering) are colored purple and pink, respectively, and based on the X-ray structure of the gp120 core in complex with CD4 domains and an antibody bound to the CR binding site [7]. gp120 residues that form putative interactions with the gp41 disulfide loop (residues 36, 40, 44, 45, 53, 491, 493, 494, 495, 496, 497, 498, 499 and 501, HIV-1 strain HXB2 numbering) are colored yellow and based on mutagenesis studies [24,25,29]. **(b)** Space filling representation of the gp41 ectodomain. Gp41 residues that form putative interactions with gp120 (residues 27, 30, 31, 33, 38, 42, 45, 47, 48, 49, 50, 51, 52, 53, 58, 67, 70, 71 and 72, HIV-1 strain HXB2 numbering) are colored green and based on the mutagenesis studies [23,26,29]. The orientation of gp120 and gp41 is identical to that shown in Figure 2.

Box 1. Envelope proteins

Viruses possessing an outer lipid membrane, derived from the host, are named enveloped viruses; viruses possessing a protein coat are named non-enveloped. Enveloped viruses, including HIV and SIV, contain proteins that mediate entry: a surface unit (SU) that binds to receptor(s) and a transmembrane unit (TM) that enables membrane fusion. In HIV and SIV, the SU and TM proteins are gp120 and gp41, respectively. In Ebola virus, influenza virus, paramyxovirus and SARS coronavirus, the SU proteins are named GP1, HA1, P1 and S1, respectively; the TM proteins are GP2, HA2, P2 and S2, respectively. Importantly, high resolution structural information is available for domains of each protein [3,86–88].

analogous envelope proteins from Ebola virus, influenza virus, paramyxovirus and SARS coronavirus (Box 1), an evolving model for HIV entry arises [2,3]. As shown in Figure 4, this model depicts three steps for envelope-mediated entry: (i) binding of virus to the target receptors; (ii) tethering of the virus to the target membrane; and (iii) fusion of the viral and target membranes. During the initial step, gp120 associates with the CD4 receptor to form the CD4-bound state and subsequently associates with a chemokine receptor (CR) that is specific to cell type (CCR5 or CXCR4 [33]) to form the receptor-bound state. At this stage, structural rearrangements occur in the gp120–gp41 complex (and possibly dissociation of gp120 from gp41) that allow for exposure of gp41 heptad repeats, namely HR1 and HR2, and insertion of the N-terminal region of gp41, a hydrophobic region named the fusion peptide, into the target membrane to form the tethered state, which is an extended metastable form of gp41 that is relatively short-lived. In the final step, gp41 undergoes a conformational change that brings the HR1 and HR2 together to form the fusion state, thereby bringing the plasma and viral membranes in close proximity to promote fusion (there is recent evidence that fusion might actually occur with the endosomal membrane [34]). For simplicity, gp41 is shown as a monomer in Figure 4 but gp41 is generally thought to be trimeric during the three steps of entry; however, there is *in vitro* and *in vivo* evidence that gp41 exists in a monomer–trimer equilibrium [35–38], and therefore the infection process could be significantly more complex. Importantly, electron tomography studies, which enable the study of envelope structure *in situ* (i.e. in the context of the viral membrane), have confirmed the trimeric nature of the envelope, in addition to giving novel insights into the interaction of the envelope with the CD4 receptor and the viral membrane [39–41]. Note that the evidence for the extended conformation of gp41 (i.e. the tethered state depicted in Figure 4) comes from analogies with the influenza hemagglutinin (HA) protein, for which there are structures of extended and compact forms [3]. Furthermore, peptides corresponding to the HR1 and HR2 of gp41 are potent antivirals, which presumably act by binding to the extended and exposed conformation of gp41 and thereby block formation of the fusion state [42–44]. In summary, the three steps of envelope-mediated entry require that the gp120–gp41 complex exists in at least four distinct conformations: free, receptor bound, tethered and fusion.

Challenges for discovery of entry inhibitors

The HIV envelope presents a number of challenges for the development of entry inhibitors as well as vaccines. As noted above, structural details, which are prerequisites for structure-based strategies, are missing for many parts of the HIV entry model (Figures 1 and 4). Furthermore, HIV has evolved to be relatively intractable to the human immune system by using a number of strategies, which are also relevant to the design of entry inhibitors. First, HIV reverse transcriptase is error prone and therefore mutations readily occur in HIV proteins [45]. However, discounting the variable domains of gp120, approximately 50% of all envelope residues are very highly conserved between HIV-1, HIV-2 and SIV [5], suggesting that these

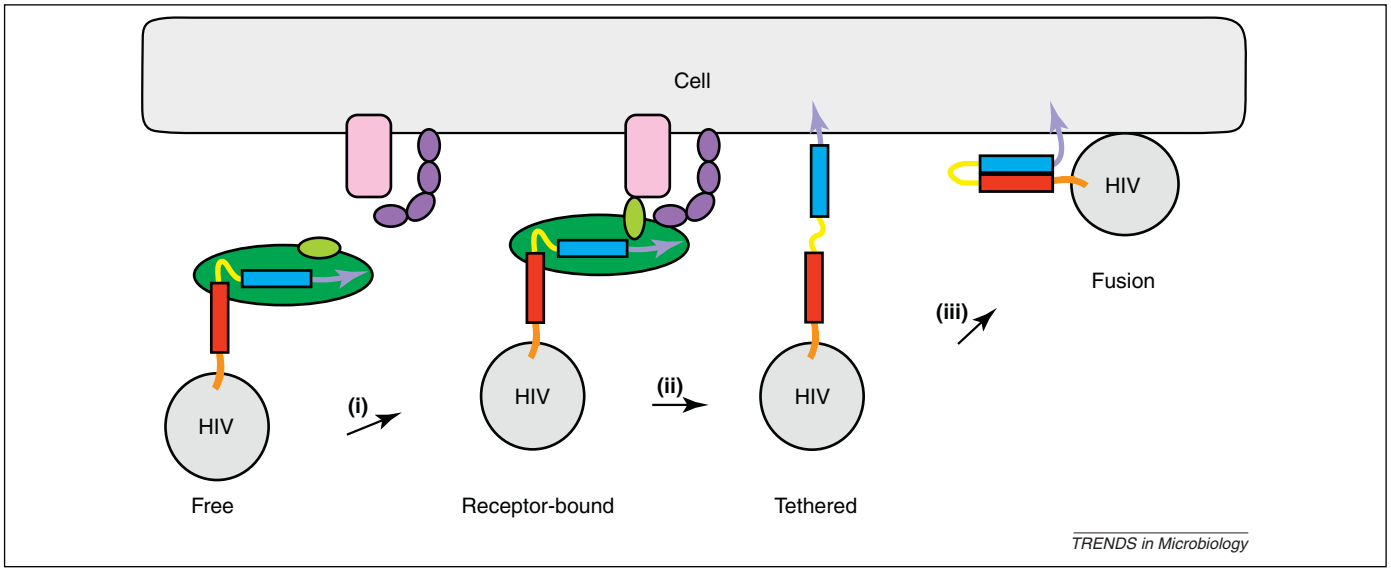

Figure 4. Role of envelope proteins in HIV entry. The CD4 and CR of the target cell are colored purple and pink, respectively. gp120 is colored green with the V3 loop colored light green. The gp41 domains shown are the fusion peptide (light purple), HR1 (cyan), disulfide loop (yellow), HR2 (red), and MPER (orange). (i) In the first step, the free state of gp120 binds to the CD4 receptor and subsequently the V3 loop of gp120 engages CR to form the receptor-bound state. (ii) In the next step, gp120 dissociates from gp41, gp41 undergoes a large conformational change, and the newly exposed fusion peptide inserts into the target cell membrane to form the tethered state. (iii) In the final step, the HR1 and HR2 interact, thereby bringing the target and virus membranes in close proximity to allow for membrane fusion and virus entry.

regions are not able to mutate without significantly affecting envelope function, a notion clearly supported by the mutagenesis studies mentioned above [18–27]. Second, many of the conserved envelope regions might not be accessible due to high levels of glycosylation in the variable loops and relatively high mobility, which is sometimes referred to as the 'glycan shield' [6,46,47]. Counter intuitively, the monoclonal antibodies 2G12, PG9 and PG16, which neutralize HIV-1 from multiple clades, bind to glycosyl moieties or V2 and V3 of gp120 [48,49]. Nonetheless, the sequence variability, glycosylation and mobility make the envelope a moving target, which complicates the search for molecules that bind with high specificity. Despite this, highly conserved regions that are transiently exposed would be expected to be attractive sites for therapeutic intervention. I would suggest the medieval armored knight as an analogy. Before battle, the knight (HIV envelope) is relatively impervious to attack (e.g. binding of an entry inhibitor). During a sword thrust (binding to receptors and target membranes), the knight's underarm is briefly exposed, which in the context of the envelope could present a conserved site with potential for therapeutic intervention. Note that such transient exposure of target sites could also be due to 'breathing' of the envelope (i.e. fluctuations of conformation from the lowest energy state), as well as the transient states present during the entry process (e.g. the tethered state shown in Figure 4). Importantly, strategies to exploit transient states have been extremely important to date and will be discussed in further detail below.

Opportunities for discovery of entry inhibitors

The HIV envelope is an attractive target for therapeutic and immunological intervention of HIV entry as discussed above. Such intervention has the advantage that it occurs before the virus enters the cell as opposed to more traditional anti-HIV therapies that inhibit the downstream viral processes of reverse transcriptase, integration and proteolytic processing (i.e. reverse transcriptase, integrase and protease inhibitors), which abrogate the necessity for therapeutics to cross the cellular membrane. Importantly, entry inhibitors are attractive candidates for microbicides designed to prevent sexual transmission of HIV [50]. In general, entry inhibitors, which could include small drug-like molecules, peptides, proteins or antibodies (Box 2), could be directed at viral targets (i.e. gp120 or gp41) or cellular targets (i.e. CD4 or CR). The disadvantage of targeting human proteins is that normal cell functions could be disrupted, resulting in undesirable side effects. However, resistance due to mutation is less problematic for cellular targets than for viral targets, which mutate readily. Therapeutic intervention could occur for any of the three steps shown in Figure 4, as well as formation of the free state. Importantly, mutagenesis studies suggest that binding of therapeutics or antibodies to many sites of gp120 or gp41 would severely disrupt HIV entry, perhaps in an allosteric manner. HIV entry inhibitors discovered to date can be divided into three classes: (i) those that target formation of the free state (i.e. interfere with envelope maturation); (ii) those that target formation of the receptor-bound state (i.e. disrupt binding of gp120 to CD4 and/or CR); (iii) those that target formation of the fusion state (i.e. bind to the tethered state of gp41). In the following sections, examples of each class of inhibitor are given.

Box 2. Discovery of entry inhibitors

Entry inhibitors have been discovered by a number of different strategies: (i) monoclonal antibodies; (ii) phage display; (iii) small molecule screening; (iv) structure-based drug design. Monoclonal antibodies that bind specifically to the HIV envelope (and block entry) can be found in patients exposed to HIV [48]. Phage display is performed by exposing a library of phage expressing short peptide regions (there are similar techniques that employ bacteria or yeast libraries) to the HIV envelope or CD4 and CR [89]. Small molecule screening is performed by using an *in vitro* binding or cell-based assay [90]. Finally, rational drug design can be employed to target the envelope in cases where high-resolution structure is known using *in silico* methods [79]. Note that an interesting aspect of all of these discovery techniques is that they can potentially be directed to target transient conformations of the envelope as shown in Figure 4.

Targeting formation of the free state

Proteolytic cleavage of gp160 by furin or furin-like proteases has been shown to be crucial for HIV envelope function [51] and therefore inhibition of the cellular furin is a possible strategy for therapeutic intervention with the aforementioned advantages and disadvantages of targeting cellular targets. Interestingly, furin inhibitors are also of therapeutic interest for their role in cancer [52]. Examples of furin inhibitors of HIV envelope processing include peptides containing the HIV cleavage sequence (the amino acid sequence of REKR) [53,54]. Examples of protein inhibitors of furin include alpha-2 macroglobulin engineered to contain the furin cleavage site as bait [55] and alpha-1-antitrypsin, a potent inhibitor of serine proteases [56,57]. A notable advantage of furin inhibitors is that they could also be effective against numerous other enveloped viruses including Ebola virus, influenza virus, and SARS coronavirus. More recently, a small drug-like molecule, UK-201844, has been suggested to disrupt gp160 processing by binding directly to the envelope [58], which is consistent with studies showing that envelope processing is sensitive to mutations at proximal and distant residues with respect to the cleavage site [24,59]. Finally, an alternative strategy for targeting formation of the free state of the envelope would be to enhance premature dissociation (shedding) of gp120 from gp41. The gp120–gp41 association is relatively weak based on the presence of soluble gp120 in the supernatant of cells expressing envelope and the sensitivity of the association to mutation [18,22–27]. However, design of such strategies is impeded by the absence of structural details for the gp120–gp41 interaction (Figure 3) and therefore, to my knowledge, agents designed to disrupt the gp120–gp41 association have not been exploited to date.

Targeting formation of the receptor binding state

An attractive strategy for entry inhibition is to target step 1 of the entry process (Figure 4) by the binding of a therapeutic molecule to the CD4 or CR binding sites on gp120 or alternatively to the gp120 binding sites on CD4 or CR. Note that the CR site of gp120 is only present after CD4 engagement and therefore it is transient in nature [60]. As a consequence, gp120–CD4 site antagonists target

the free conformation and gp120–CR site antagonists would target the receptor-bound conformation (Figure 4). There are numerous examples of therapeutics that target the receptor binding sites of gp120. For example, BMS-806 is a small molecule that is thought to bind to the gp120 CD4 site and thereby inhibit HIV entry [61]; however, the mechanism of action is currently under debate [62]. Similar efforts have led to the discovery of other small molecules with broad efficacy [63,64]. An example of a monoclonal antibody that binds to the gp120 CD4 site and thereby inhibits entry is b12 [65]. The lectins, which are carbohydrate binding proteins, inhibit receptor interactions by binding to the mannose sugars of gp120 in a relatively nonspecific manner, but nonetheless with high efficacy (e.g. the lectin cyanoviron exhibits an $EC_{50}<1$ nM for most strains of HIV [66,67]). In a similar manner, silver nanoparticles have been recently shown to bind to gp120 and inhibit binding to CD4, presumably in a relatively nonspecific manner [68]. Note that nonspecific binding of molecules such as lectins and silver nanoparticles might be advantageous in the case of HIV because they present the possibility of targeting multiple HIV strains and perhaps multiple enveloped viruses. Betulinic acid derivatives are examples of small molecules that target the gp120 V3 loop (i.e. the CR binding site) [69]. Interestingly, the peptide 12p1 and its derivatives bind to gp120 and are examples of allosteric inhibitors that simultaneously disrupt binding to both CD4 and CR [70,71]. Finally, there are several examples of entry inhibitors that target the gp120 binding site of cellular targets CD4 and CR. For example, the drug maraviroc (brand-name Selzentry or Celestri), which is a US FDA approved therapy for treatment of HIV infection, targets CCR5 [72]. Moreover, the monoclonal antibodies PRO140 and Ibalizumab, which target CCR5 and CD4, respectively, are currently in clinical trials [73,74]. In summary, numerous strategies for targeting the receptor binding step of HIV entry have exhibited promise.

Targeting the tethered conformation

To date, there has been surprising success in targeting the tethered conformation of gp41, which is transient in nature with a half-life estimated to be >15 min [75]. The rationale behind such therapies is to block formation of the thermodynamically favored fusion state (Figure 4). As noted above, a peptide corresponding to part of the gp41 HR2, namely DP-178, T-20, Fuzeon or enfuvirtide, is a US FDA approved therapy in humans [42–44,76]. The success of enfuvirtide is important in two aspects. First, it is an efficient therapy for which resistance is observed but relatively rare. Second, enfuvirtide provides proof of principle that transient metastable targets of the envelope are feasible in HIV. More recent strategies have exploited peptides composed of unnatural amino acids to increase resistance to proteolysis and efficacy against multiple HIV strains [77,78]. Based on the entry model presented in Figure 4, interesting alternative strategies employ the use of three or five helix bundle versions of the envelope fusion state, which are missing three or one HR2, respectively, and provide binding sites for virion bound HR2 in the tethered state [79,80]. ADS-J1 is an example of a small molecule that that was designed to inhibit HIV entry by binding to an exposed hydrophobic pocket of the gp41 HR1, thereby disrupting formation of the fusion state [81]; however, the mechanism of action could be significantly more complex [82]. Finally, the rationale of targeting the tethered state of gp41 is further supported by the neutralizing abilities of antibodies 2F5, 4E10 and Z13, which bind to the gp41 MPER, an epitope that is also only transiently exposed at a late stage in the entry mechanism [83–85]. Importantly, based on similarities between the entry mechanism of HIV and other enveloped viruses [3], transient metastable states are presumably viable targets in general.

Conclusions

In this review, I have considered the challenges and opportunities for developing entry inhibitors that target the HIV envelope. Envelope-mediated HIV entry is a well orchestrated event consisting of multiple conformations, some of which are metastable. Structural biology and mutagenesis studies have given important insights into envelope function; however, many of the details, which would be crucial in structure-based therapeutics, are missing. Mutagenesis studies have suggested that the envelope is a target-rich environment with numerous potential sites for therapeutic intervention. I have given examples of small molecules, peptides, proteins and antibodies that are HIV entry inhibitors. Importantly, there are US FDA approved therapies that target viral and cellular targets, as well as numerous other therapies in clinical trials or at early stages in development. Consequently, in the near future I anticipate the arrival of multiple new entry inhibitors to HIV, in particular, as well as to other enveloped viruses such as Ebola virus, influenza virus and SARS coronavirus.

Acknowledgments

This work was partially supported by the Chicago D-CFAR (P30 AI082151).

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