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REVIEW

Insights into the mechanism of HIV-1 envelope induced membrane fusion as revealed by its inhibitory peptides

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Abstract HIV-1 fusion with its target cells is mediated by the glycoprotein 41 (gp41) transmembrane subunit of the viral envelope glycoprotein (ENV). The current models propose that gp41 undergoes several conformational changes between the apposing viral and cell membranes to facilitate fusion. In this review we focus on the progress that has been made in revealing the dynamic role of the N-terminal heptad repeat (NHR) and the C-terminal heptad repeat (CHR) regions within gp41 to the fusion process. The involvement of these regions in the formation of the gp41 pre-hairpin and hairpin conformations during an ongoing fusion event was mainly discovered by their derived inhibitory peptides. For example, the core structure within the hairpin conformation in a dynamic fusion event is suggested to be larger than its high resolution structure and its minimal boundaries were determined in situ. Also, inhibitory peptides helped reveal the dual contribution of the NHR to the fusion process. Finally, we will also discuss several developments in peptide design that has led to a deeper understanding of the mechanism of viral membrane fusion.

Keywords Viral envelope protein · Membrane fusion · HIV-1 entry inhibitor · Peptide-membrane interaction

Membrane fusion is a fundamental event that is critical to many biological systems such as entry of enveloped viruses to their host cells, intracellular vesicle fusion, and cell to cell fusion in fertilization and development (Blumenthal et al. 2003; Eckert and Kim 2001b; Mohler et al. 2002; Rothman and Orci 1992; Weissenhorn et al. 1999). These processes are mediated by many transmembrane proteins that share remarkably similar motifs (Chernomordik and Kozlov 2003; Poranen et al. 2002; Sollner 2004; White 1992).

Membrane-active peptides: 455th WE-Heraeus-Seminar and AMP 2010 Workshop.

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General biophysical aspects of membrane fusion

The fusion of two stable biological membrane bilayers into one is a complex event that requires physical forces. In many biological systems, membrane merger can be divided into two steps: the formation of a hemifusion intermediate and the fusion pore (Gallo et al. 2003; White 1992). Both steps are governed by the membrane lipids and proteins. First, the contacting "cis" monolayer leaflets are merged, but the "trans" distal leaflets remain intact. The structures of these intermediates are often called the ''stalks'' that further progress to form a hemifusion diaphragm (Blumenthal et al. 2003). At this stage, lipid mixing occurs only between the cis monolayer leaflets with no content mixing. The hemifusion state is a key step because a productive fusion requires transformation of the hemifusion intermediate into an expanding fusion pore. Post-hemifusion is the rate-limiting step of the fusion reaction, and theoretical analysis confirms that fusion pore expansion is more energetically demanding than hemifusion (Chernomordik and Kozlov 2005). Overcoming the energy barriers is related to the membrane lipids and proteins that affect the tensions and stresses of the membranes by mechanisms that are not yet fully understood (Epand 2003; Reichert et al. 2007).

The HIV-1 ENV mediated membrane fusion

The fusion event of the human immunodeficiency virus (HIV-1) with its target cells is mediated by the envelope glycoprotein (ENV). Interestingly, HIV-1, like many other enveloped viruses, adopts trimeric organization for its ENV (Center et al. 2002; Harrison 2008). The ENV is synthesized as a precursor protein, gp160 (Freed et al. 1992), and undergoes cleavage by cellular proteases into two non-covalently associated subunits, gp120 and gp41 (Dedera et al. 1992; Okumura et al. 1999). Gp120 is the surface subunit that enables host tropism (Clapham and McKnight 2002), while gp41 is the transmembrane subunit responsible for the actual membrane merger (Finzi et al. 2010; Gallo et al. 2001; Kwong et al. 1998; Liu et al. 2007b; Moreno et al. 2004; Rizzuto et al. 1998).

The structure of gp41

The gp41 ectodomain is comprised of several regions (Fig. 1): the N-terminal fusion peptide (FP), fusion peptide proximal region (FPPR), N-terminal heptad repeat (NHR), loop, C-terminal heptad repeat (CHR), membrane proximal external region (MPER) and transmembrane domain (TMD). A crystal structure of the complex of N36 and C34 peptides (derived from the NHR and CHR regions, respectively) was solved and showed the formation of an internal trimeric NHR coiled-coil (Chan et al. 1997; Weissenhorn et al. 1997). The C-peptides bind in an anti-parallel manner into hydrophobic grooves created on the outer surface of the NHR coiled-coil structure. These grooves have a deep cavity termed "the pocket" (aa 565–581) that interacts with three conserved hydrophobic residues of the CHR region (Trp-628, Trp-631, and Ile-635) and is believed to be crucial for the complex stability (Chan et al. 1998; Dwyer et al. 2003; Gochin and Cai 2009; Ji et al. 1999). Thus, the CHR region contains a pocket-binding domain (PBD) (aa 628–635), and an NHR-binding domain (aa 628–666) demonstrating a heptad repeat with hydrophobic amino acids. The hydrophobic residues in the *a* and *d* positions of the CHR interact with the hydrophobic residues in the *e* and *g* positions of the NHR. The helices of the NHR self-interact (Bernstein et al. 1995) by its hydrophobic residues in the *a* and *d* positions (Bewley et al. 2002; Caffrey et al. 1998; Chan et al. 1997). This structure has been termed the six helix bundle (SHB) or the core structure. In recent years, accumulating functional evidence demonstrated that the core structure in the dynamic fusion event is larger than the complex of the N36 and C34 peptides (He et al. 2008a; Wexler-Cohen and Shai 2007). Recently, an additional crystal structure of the ectodomain of gp41 was reported with extensions from the SHB to the FPPR and the MPER regions (Buzon et al. 2010). This structure also supports a larger core complex.

Gp41 conformational changes during fusion

The prevailing models postulate that at least three conformational changes in the gp41 ectodomain are involved in apposing viral and cell membranes to facilitate fusion (Chan and Kim 1998; Eckert and Kim 2001b; Weissenhorn et al. 1999) (Fig. 2). The first conformational change is a native non-fusogenic state where gp120 subunits shield the hydrophobic gp41 ectodomains. The second conformational change is binding of gp120 to the receptor CD4 and a co-receptor such as CCR5 or CXCR4, which causes major conformational changes that drive the transition from the native state into the pre-hairpin intermediate state (Cocchi et al. 1996; Doranz et al. 1996; Finzi et al. 2010; Gallo et al. 2001; Kwong et al. 1998; Owman et al. 1998; Rizzuto et al. 1998). Gp41 is released in extended conformation and FP is inserted into the membrane. The FP is common to many enveloped viruses and is generally found at the amino terminus of viral fusion proteins. The FP utilizes several mechanisms to induce fusion: promotion of negative curvature, lowering the rupture tension of the lipid

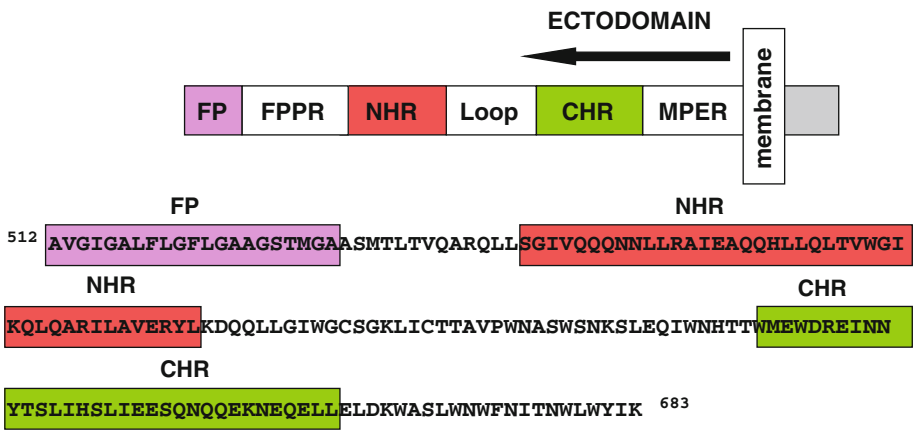

Fig. 1 Schematic representation of the regions within the ectodomain of gp41. Starting from the N-terminus is the FP, FPPR, NHR, loop, CHR and the MPER. The sequence is from HIV-1_{HXB2} and the residue numbers correspond to its gp160. Note that the boundaries for each region are estimated

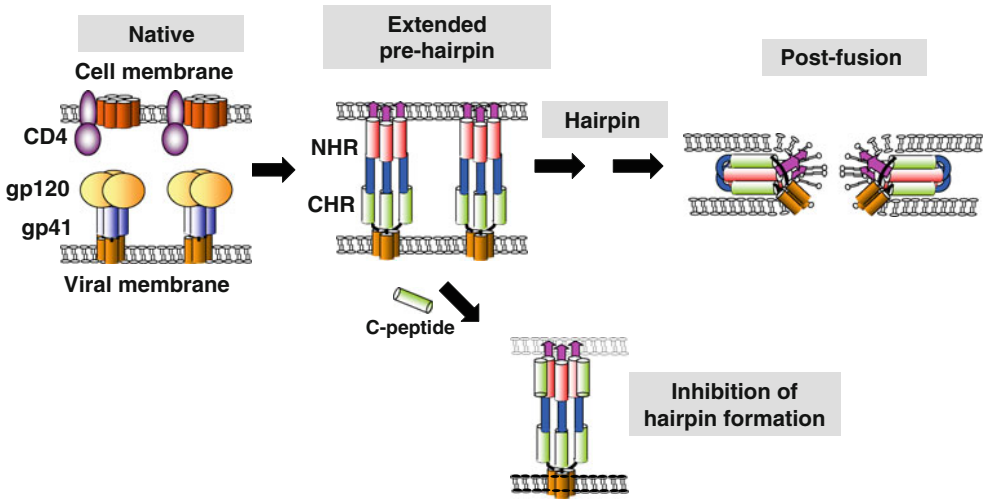

Fig. 2 Mechanism of gp41 induced fusion and its inhibition. In the native non-fusogenic state, gp120 subunits shield the transmembrane gp41. Binding of gp120 to the receptor CD4 and a co-receptor causes conformational changes releasing gp41 to form an extended pre-hairpin intermediate, characterized by exposed NHR and CHR with the FP inserted into the target membrane. This intermediate is sensitive to the action of fusion inhibitors. In the absence of the inhibitors, gp41 folds into a hairpin conformation followed by hemifusion and formation of a fusion pore (post-fusion). C34 fusion inhibitor, for example, can bind to the gp41 NHR region in an anti-parallel manner and competitively block SHB formation, thus preventing the formation of gp41 hairpin conformation

monolayer, and acting as an anchor to join the fusion membranes. Peptides derived from the FP inhibit fusion suggesting an additional role for this region in the assembly of the trimeric protein (Curtain et al. 1999; Durell et al. 1997; Epand 2003; Gallaher 1987; Gerber et al. 2004; Kliger et al. 1997; Lau et al. 2004; Nieva et al. 1994; Rafalski et al. 1990; Sackett and Shai 2003). The third conformational change facilitating fusion is folding of gp41 into a low energy trimeric hairpin conformation which comprises the core or SHB structure (Chan et al. 1997; Furuta et al. 1998; He et al. 2008b; Melikyan et al. 2000; Tan et al. 1997; Weissenhorn et al. 1997). The core structure is involved in the fusion mechanism of many viruses, as well as in intracellular vesicle fusion (Colman and Lawrence 2003; Poranen et al. 2002; Rapaport et al. 1995; Skehel and Wiley 1998).

Gp41 derived peptides as powerful tools for exploring the dynamics of gp41-mediated fusion

The current model for the gp41 induced fusion is based on several lines of evidence: (i) the crystal structure, (ii) differential binding of gp41 antibodies during the fusion process, (iii) mutagenesis studies, and most importantly (iv) the inhibitory effect of synthetic gp41 derived peptides on the fusion process (Bar and Alizon 2004; Chan and Kim 1998; Furuta et al. 1998; Jiang et al. 1993; Kliger et al. 1997; Sanchez-Martinez et al. 2006; Zolla-Pazner 2004). These peptides usually bind gp41 at the intermediate steps and disrupt fusion. The most prominent ones are the N- and C-peptides (derived from the NHR and CHR regions, respectively). They prevent the progression of gp41 into the core structure through a dominant-negative mechanism leading to fusion arrest (Eckert and Kim 2001b; LaBranche et al. 2001). Although both peptides were discovered simultaneously, the C-peptides are well-known because they are powerful fusion inhibitors, making them attractive for studying gp41 dynamic conformations as well as templates for drug design.

C-peptides fusion inhibitors

The most familiar peptides derived from the gp41 CHR region are C34 (aa 628–661), and T-20 (enfuvirtide, brand name Fuzeon) (aa 638–673). T-20 is the first clinically approved HIV entry inhibitor (Cooper and Lange 2004). Both C34 and T-20 contain the NHR-binding domain, which interact with the NHR region, comprising the GIV (547–549) motif (Fig. 3). Mutations in this motif are associated with viral resistance to T-20 and C34 (Briz et al. 2006; Eggink et al. 2009; Rimsky et al. 1998). These CHR peptides bind to the gp41 NHR region to competitively block SHB formation, resulting in the inhibition of HIV fusion (Fig. 2) (Kliger and Shai 2000; Miyauchi et al. 2009). Although the peptide sequences are largely overlapping, C34 contains the PBD at its N-terminal region, while T-20 lacks those residues. Instead, it has a part of the MPER with a tryptophan-rich domain (aa 666–673) in the C-terminus, which is important for its fusion inhibitory potency (Biron et al. 2005; Liu et al. 2005; Salzwedel et al. 1999).

Fig. 3 The folded hairpin conformation of gp41 that comprises the FPPR, NHR, CHR and MPER. These regions were used to determine the high resolution structure of the core (Buzon et al. 2010; Chan et al. 1997). The sequence is from HIV-1_{HXB2} and the residue numbers correspond to its gp160. The sequence and designation of the central peptides discussed in this review are indicated. The *central motifs* that are shown in this review to stabilize the core structure are *highlighted* and *underlined*: at the N-terminus are indicated the RQLL, GIV and the pocket sequences; at the C- terminus the QIWNHTT and the PBD sequences are indicated

Accumulating evidence suggests that the C-terminal portion of T-20 has a membrane binding property: T-20 has two suggested mechanisms of inhibition. The first one is prior to the hemifusion state and involves binding to the NHR thus preventing gp41 from folding into the core structure. In the second one, following hemifusion, it is suggested that the C-terminal portion of T-20 inhibits the recruitment of several gp41-membrane complexes, thus inhibiting fusion pore formation (Kliger et al. 2001). Additionally, peptides derived from the membrane-spanning domain in gp41 significantly abrogate the antiviral activity of T-20 (Liu et al. 2005). Substitution for d-amino acids in the C-terminal of T-20 did not interfere with the ability of the peptide to inhibit fusion and did not affect its membrane binding ability, suggesting a membrane-anchoring property to this domain (Apellaniz et al. 2009; Sal-Man et al. 2004; Wexler-Cohen et al. 2006). A recent crystal structure of gp41 core supports these functional studies and provides structural evidence that part of MPER will be membrane inserted within trimeric gp41 (Buzon et al. 2010).

N-peptide fusion inhibitors

N-peptides have unique characteristics in comparison to C-peptides. Since the NHR region creates an internal trimeric coiled-coil (Chan et al. 1997; Weissenhorn et al. 1997), N-peptides have two binding sites, and therefore two fusion inhibitory modes. They can bind the CHR region or they can bind the endogenous homotrimeric NHR region (Bewley et al. 2002). Since gp41 exists in monomer-trimer equilibrium (Caffrey et al. 1999), the N-peptides can shift the equilibrium into the monomeric/dimeric forms. In both modes the result is prevention of gp41 progression into the hairpin conformation and fusion arrest. The well known N-peptides are DP107 (aa 553–590) and N36 (aa 546–581). Both peptides comprise the crucial pocket sequence, but DP107 is partially overlapped with the NHR and has an extended N-terminus (Wexler-Cohen and Shai 2009; Wild et al. 1992).

N-peptides that aggregate in solution are less potent fusion inhibitors than the C-peptides (e.g. micromolar versus nanomolar concentrations, respectively) (Eckert and Kim 2001a). Several approaches were utilized to investigate this assumption and improve their fusion inhibitory properties. Chimeric variants of an N-peptide were constructed in which soluble trimeric coiled coils are fused to portions of the gp41 N-peptide. Such coiled coils are also covalently stabilized within the peptide by using interchain disulfide bonds (Bianchi et al. 2005). These molecules, which present the N-peptide in a trimeric coiled-coil conformation, are remarkably more potent inhibitors than the N-peptides themselves and likely target the CHR region of gp41 (Bianchi et al. 2005; Eckert and Kim 2001a). An N36 mutant was utilized in which the sites of interaction with the C-helices have been mutated, but the sites of intermolecular contacts between the N-helices have been preserved. This peptide was significantly more potent than the native N36 peptide suggesting that the homotrimeric coiled-coil of N-helices in the pre-hairpin state can be disrupted (Bewley et al. 2002).

Conjugation of lipophilic moiety to a peptide: a powerful tool to study gp41 conformational changes during fusion

Synthetic peptides derived from gp41 have the potential to act as potent fusion inhibitors (e.g., to inhibit fusion at nanomolar concentrations). It is not sufficient for a peptide to comprise a sequence that corresponds to one of the active domains of gp41, and it should have additional properties: the peptide needs to be targeted to the cell membrane and to act on the fusion site, and it needs to bind the active domain of gp41 in a particular orientation and with high affinity. Several studies have shown that the binding of potent C-peptide fusion inhibitors to gp41 is

reversible and could be eliminated by performing washing steps (Jacobs et al. 2007; Markosyan et al. 2003). N36 is not a good fusion inhibitor, although it comprises the critical pocket sequence. This is mainly due to the tendency of the peptide to aggregate (Eckert and Kim 2001a). Therefore, shifting the equilibrium from oligomeric forms to monomeric forms may improve the peptide's inhibitory potency.

Conjugation of a hydrophobic moiety (e.g. fatty acid) to gp41 derived peptides has overcome the above mentioned obstacles and has restored or amplified their activity (Ingallinella et al. 2009; Wexler-Cohen and Shai 2009). Several similar effects were observed also by conjugation of a fatty acid to antimicrobial peptides, suggesting common functional mechanisms in diverse biological systems (Makovitzki et al. 2006; Malina and Shai 2005). A fatty acid is conjugated to a peptide via a simple chemical ligation between the carboxylic group of the fatty acid and the free amine in the N-terminus of the peptide. This chemical ligation yields an N-terminal conjugated lipopeptide. In addition, fatty acids can also be conjugated to the C-terminal side of the peptide by including an appropriately protected lysine at the C-terminus during peptide synthesis and ligating the fatty acid via the side chain of the lysine.

Functional and biophysical studies have revealed interesting information regarding the fusion inhibitory properties of several lipopeptides. Firstly, the conjugation of a fatty acid to a C- or N-peptide conferred an inhibitory activity that was dependent on the length of the fatty acid. By elongating the carbon chain from C8 to C16 the inhibitory potency was improved. The fatty acid anchors the peptide to the cell membrane, thus increasing its local concentration at the fusion site (Wexler-Cohen et al. 2010). Secondly, several lipopeptides that were conjugated at different termini showed dramatic differences in their fusion inhibitory activity, which implies an orientation dependent inhibition towards the membrane (Wexler-Cohen et al. 2010; Wexler-Cohen and Shai 2007). For example, conjugation of a fatty acid to the N-terminus of a peptide directs the lipopeptide to bind the viral and the cell membrane through its N-terminus, and may dictate the binding of the peptide to the endogenous protein in a parallel or anti-parallel manner (Fig. 4). C-peptides bind the NHR region in an anti-parallel manner (Liu et al. 2007a), thus location of the fatty acid within the peptide sequence becomes an important factor in the design of a potent inhibitor. The activity information of differently designed inhibitors may give hints about the mode of action of the peptide during dynamic gp41 conformational changes. Thirdly, the fatty acid can amplify the secondary structure of the peptide (Avrahami and Shai 2002; Peisajovich et al. 2003). This factor can be important for the inhibitory potency when the binding of the peptide to its target region on gp41 is driven by helix-helix interactions

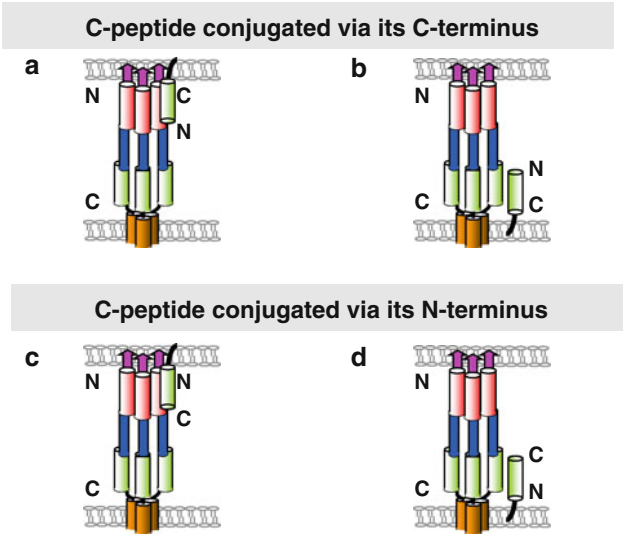

Fig. 4 Conjugation of a fatty acid to the C- terminus or the N-terminus of a peptide can determine its fusion inhibitory activity. For peptides derived from T-20, C-terminal conjugates were drastically more active than the N-terminal conjugates (Peisajovich et al. 2003; Wexler-Cohen and Shai 2007). A mode of action involving orientation dependent inhibition is suggested for such lipopeptides. Theoretically, there are several options for anchoring the peptides to the membrane. If the conjugation is via the C-terminus of the peptide, it can be: **a** anchored to the viral membrane, and thus located in an *anti-parallel* manner towards the endogenous NHR, and **b** anchored to the cell membrane, and thus located in a *parallel* manner towards the endogenous CHR. If the conjugation is via the N-terminus of the peptide, it can be: **c** anchored to the viral membrane, and thus located in a *parallel* manner towards the endogenous NHR, and **d** anchored to the cell membrane, and thus located in an *anti-parallel* manner towards the endogenous CHR. Option (**a**), as expected, is likely the suitable one for the lipopeptides derived from T-20 (Peisajovich et al. 2003)

(Judice et al. 1997). Finally, conjugation of a fatty acid may alter the peptide's ability to oligomerize. For example, fatty acids with increasing lengths that were conjugated to the trimeric N36 peptide shifted its oligomeric state towards a monomer and improved its ability to inhibit cell fusion (Wexler-Cohen and Shai 2009). Therefore, gp41 derived lipopeptides can serve as a powerful tool to study gp41 conformational changes and stability during a dynamic membrane fusion.

New elements that contribute to the stabilization of the core structure were revealed using gp41 derived peptides and lipopeptides

Domains in the C-terminal region of gp41 that stabilize the core structure

It was well established that the PBD is crucial for the binding of the C-helices to the coiled-coil created by the

N-helices (Chan et al. 1998). Recent studies suggest that other C-terminal domains are involved in the stabilization of the core structure. Peptides comprising: (i) the motif QIWNNMT (aa 621–627) which is upstream to the CHR (Fig. 3); (ii) the PBD, and (iii) the partial CHR sequence but lacking the GIV (aa 547–549) motif-binding sequence that could interact with the NHR peptides to form extremely stable SHB. These peptides exhibit highly potent antiviral activity against HIV-1 strains, especially those strains that are resistant to C-peptides (He et al. 2008a). Note that the QIWNNMT sequence is from the HIV-1_{IIIB} strain. Its corresponding sequence in HIV-1_{HXB2} is QIWNHTT.

The minimal T-20 inhibitory sequence that enables the creation of a stable, functional core in situ was recently determined (Wexler-Cohen and Shai 2007). A truncated variant peptide, named DP (aa 638–662) (Fig. 3), which lacks the whole C-terminal region of T-20, was conjugated to a fatty acid and regained fusion inhibitory activity similarly to that of the wild type peptide. Therefore, the C-terminal of T-20 appears to serve as a hydrophobic membrane anchor. Since further attempts to truncate the C- or N-terminal regions of the peptide resulted in abolished activity, the DP sequence seems to be the minimal sequence able to create a functional core with the endogenous gp41 protein in situ. As expected, the C-terminal fatty acid conjugated DP was more active than its N-conjugates. In another study, a fatty acid (C8) was conjugated to the simian immunodeficiency virus inactive T-20 mutant peptide; only conjugation to the C-terminal of the peptide rescued its activity (Peisajovich et al. 2003). An orientation dependent inhibition is likely the mode of action for the lipopeptides derived from T-20 (Fig. 4). These lipopeptides can bind the NHR region of the extended gp41 pre-hairpin conformation in an anti-parallel manner and inhibit its folding into the hairpin conformation.

Domains in the N-terminal region of gp41 that stabilize the core structure

The pocket domain, within the N-terminal region of gp41, was established as a crucial determinant to the fusion process. Thus, most of the peptides that were synthesized from this region comprise the pocket sequence (Bianchi et al. 2005; Eckert and Kim 2001a). Recently, a short N-peptide, named N27 (aa 543–568) (Fig. 3), was studied (Wexler-Cohen et al. 2010): this peptide lacks the pocket sequence but comprises an extended N-terminus of N36 peptide (RQLL sequence). N27 is the corresponding sequence to DP in the hairpin conformation (Wexler-Cohen and Shai 2007). N27 alone was poorly active but dramatically increased its fusion inhibitory potency when a fatty acid was conjugated to its N-terminus but not to its C-terminus. This fusion inhibitory effect was abolished by either deleting or mutating the RQLL sequence, demonstrating that the extended N-terminus of the NHR plays a role in stabilizing the core structure, in addition to the pocket domain (Fig. 3). Additionally, it is suggested that the sequence of N27 comprises the N-terminal edge of the endogenous core structure in situ and hence is complementary to the C-terminal edge of the core structure (Wexler-Cohen and Shai 2007).

Concluding remarks

HIV-1 entry into its host cells has been under intensive studies for the last two decades. The most significant contributions were: (i) deciphering the high-resolution structure of the gp41 core, (ii) differential binding of gp41 antibodies during the fusion, (iii) functional studies using inhibitory peptides derived from gp41, and (iv) biological computations. Taken together, these data assist in determining models for membrane fusion induced by gp41 comprising at least three conformational changes in its ectodomain during viral fusion: (i) a native state where gp120 subunits shield the gp41 ectodomains; (ii) an extended pre-hairpin intermediate state in which the FP is inserted into the target membrane exposing the NHR and the CHR regions, and (iii) a trimeric hairpin state that comprises the core structure in which the CHR regions bind the NHR coiled-coil complex in an anti-parallel manner.

In this review we focused on the progress that has been made thus far in revealing the dynamic role of the NHR and CHR regions within gp41 to the fusion process by utilizing fusion inhibitory peptides and lipopeptides from these regions. The unique inhibitory properties of the N-peptides assisted in revealing the dual role of the NHR region in gp41 assembly and folding into the hairpin state. In addition, determination of the mode of action of lipopeptides derived from truncated C- or N-peptides revealed the minimal functional domains that are required to stabilize the core structure within the gp41 hairpin state in situ. However, gp41 comprises other regions, besides the CHR and the NHR, that are involved in membrane fusion. High resolution structures for these regions, together with functional studies, will assist in refining a more comprehensive and revised model for the fusion mechanism induced by the HIV-1 envelope protein.

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