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From receptor recognition mechanisms to bioinspired mimetic antagonists in HIV-1/cell docking☆

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Abstract

Understanding the ways in which two or more proteins interact may give insight into underlying binding and activation mechanisms in biology, methods for protein separation and structure-based antagonism. This review describes ways in which protein recognition has been explored in our laboratory for the HIV-1/cell entry process. Initial contact between an HIV-1 virion particle and a human cell occurs between gp120 (an HIV-1 envelope protein) and CD4 (a human extracellular signaling protein). This interaction leads to a sequence of events which includes a conformational change in gp120, fusion of the HIV-1 and cellular membranes and eventual infection of the cell. Using an optical biosensor and a reporter antibody, we have been able to measure the conformational change in gp120 that occurs upon CD4 binding. We also have used this biosensor system to characterize CD4 mimetics, obtained by peptide synthesis in miniprotein scaffolds. Phage display techniques have been employed to identify novel miniprotein sequences. The combination of biosensor interaction kinetics analysis and phage display provides a useful approach for understanding the recognition mechanisms involved in the HIV/cell docking process. This approach may also be useful in investigating other protein complexes of importance in health and disease.

Keywords: Protein recognition; Peptide mimetic; HIV-1; Phage display; Optical biosensor

1. Introduction

Molecular recognition is a common theme in the function of proteins and other macromolecules in essentially all biological systems. Moreover, it is a key driving force in separation science, not only for affinity chromatography but also in structure-specific interactions important in chromatographic separations based on charge and hydrophobicity. Indeed, molecular recognition is a transcendent theme of the collection of papers in the special issue of the *Journal of Chromatography* devoted to the Nantes Symposium on Separation and Characterization of Biological and Synthetic Macromolecules.

We have become interested in receptor recognition mechanisms in the HIV-1/cell entry and infection processes. This review describes recent work on the recognition process of HIV-1 envelope gp120 with cell receptor CD4, our effort to understand the

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mechanism of recognition between these molecules and recent progress to design mimetic affinity ligands based on mechanism.

The life cycle of HIV begins by viral entry into a host cell. Fusion of the viral and host cell membranes, a process which is not completely understood, allows entry of the viral RNA, consequent integration of the viral genome into the host cell genome and the onset of viral protein production. These viral proteins are incorporated into new viral particles that bud off from the host cell surface, and the viral infection of more cells can ensue. Each step in the viral life cycle, from cell docking and membrane fusion to the budding process, involves recognition between two or more macromolecules, including protein–protein, protein–RNA, or protein–DNA. Also, because the virus uses cellular machinery during its life cycle, many of the recognition events that take place are between host cell molecules and viral counter ligands. The high resolution structures of many of the proteins involved in the HIV-1 life cycle have been determined [1]. A considerable amount of effort has been spent in understanding the underlying macromolecular recognition processes of proteins in the viral life cycle and to use this understanding to design structure- and mechanism-based antagonists.

The HIV-1/cell docking and entry process involves proteins from the viral envelope and host cell surface receptors. The two main proteins on the HIV envelope are glycoproteins gp120 and gp41 (named for their approximate size in kilodaltons). gp120 and gp41 are associated together non-covalently. Experimental evidence shows that three pairs of these glycoproteins are oriented to form a trimer [2]. The viral particle displays these trimers as spikes on its surface (Fig. 1, [3]). The first interaction between the HIV viral particle and the host cell involves gp120 and CD4, a surface receptor on the host cell. gp120 binds to the CD4 molecule with high affinity (K_D=4 n*M*) [4]. Recently, a crystal structure of gp120 and the D1–D2 portion of CD4 was solved using X-ray crystallography (Fig. 2A) [5]. The crystal structure shows that bound CD4 residues extend over 802 Å^2 on gp120. The most important contacts between CD4 and gp120 are located along an interface between a small gp120 cavity and a hairpin loop of CD4 (CD4 residues 37–47, CDR2 loop). Mutagenesis data have helped confirm that CD4 residues in/near the CDR2 loop are most influential on gp120 binding [6].

One residue in the CDR2 loop, Phe-43, is particularly important for CD4–gp120 binding. Mutation of Phe-43 to Ala in CD4 results in a 550-fold decrease in gp120 affinity [6]. The crystal structure of the gp120–CD4 complex clearly shows that the Phe-43 phenyl ring inserts into a hydrophobic cavity on gp120 (Fig. 2A). Here, Phe-43 makes contact with polar (e.g., Glu-370, Asp-368, Asn-328) and

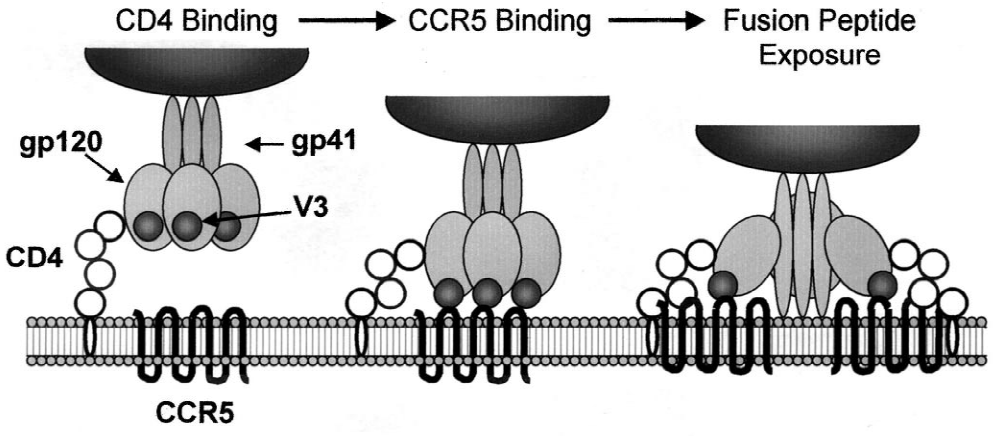

Fig. 1. Model of HIV binding to host cell. Glycoprotein gp120 binds to the host cell initially by contact with CD4. Subsequent chemokine receptor binding (for example, CCR5 binding) leads to exposure of the fusion peptide, gp41, from the HIV envelope. Figure adapted from Doms and Peiper [3].

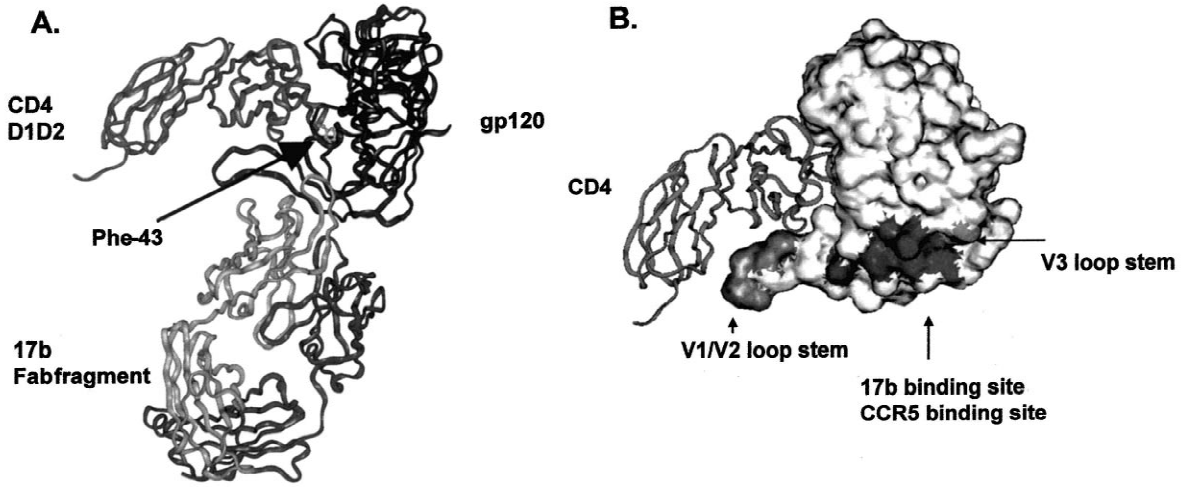

Fig. 2. (A) The crystal structure of CD4–gp120–17b. The interaction between CD4 and gp120 is largely mediated between Phe-43 of CD4 (indicated by the arrow) and a hydrophobic pocket on gp120. (B) The space-filling model of gp120 from the crystal structure indicates where the CCR5 and 17b binding sites overlap. The V1/V2 and V3 loop stems are also indicated. Figure adapted from Kwong et al. [5].

non-polar (e.g., Ile-371, Trp-427, Met-426) gp120 residues [5]. However, the relative energetic contribution of each type of interaction is not yet known.

Other CD4 residues near the CDR2 loop play a role in binding to gp120. The interactions of these residues seem to be less important than those of Phe-43. Arg-59 and Lys-29 make contacts with gp120 [5]. Mutations of Arg-59 or Lys-29 to alanine, however, result in only nine- and sevenfold decreases in binding, respectively, which are significantly less than the 550-fold decrease seen with Phe-43 [6].

Binding of CD4 to gp120 causes a conformational change, within gp120 [7], which uncovers a binding epitope used to bind to a chemokine receptor, another host cell receptor co-localized with CD4. This co-receptor binding to gp120 appears to induce further conformational changes in the HIV-1 envelope that lead to exposure of a second HIV envelope protein, gp41, which participates in membrane fusion between the virus and the cell (Fig. 1) [8]. Some forms of viral envelope can bind to the co-receptor independently of CD4 [9].

Because chemokine receptors are transmembrane proteins, crystallographic and biophysical analyses of their interactions have been limited. However, antibody 17b binds to a site on gp120 that overlaps with the chemokine receptor binding site [10]. Hence, binding properties of 17b provide a possible window into the mechanism of gp120–viral co-receptor binding. The 17b antibody was used in the CD4–gp120–17b crystal structure and, therefore, a picture of how these three moieties interact has emerged (Fig. 2A and B).

We have initiated work to use the crystal structure of the CD4–gp120–17b complex as a starting point to investigate the mechanism of cooperativity between CD4 and co-receptor binding site interactions and to use mechanistic insights to design CD4 mimetic peptides that bind to gp120 in a CD4-competitive manner. CD4 peptide mimetics with two sets of activities are possible. Mimetics that bind to gp120 and cause the conformational change associated with CD4 binding which leads to upregulated gp120 binding of chemokine receptors/17b could be considered CD4 agonists. These molecules may be particularly useful tools in the development of antibodies and vaccines against gp120 [11] or for gp120 separation. Conversely, mimetics that bind to gp120 and do not cause the CD4-like conformational change (i.e., CD4 antagonists) may be useful in the development of molecules that inhibit the action of gp120. Both kinds of molecules would give insight into the recognition mechanism of gp120 for CD4

and may lead to an understanding of the structural basis behind gp120's conformational change and subsequent chemokine receptor/17b binding.

Peptide mimetics can be used to determine key components in the CD4–gp120 binding interaction by evaluating their efficacy to mimic the interaction properties of CD4 that play roles in binding gp120. Effects of changes in the mimetic's structure can give insight into the structural requirements for gp120 binding, as well as for the cooperative binding of chemokine receptors/17b. Previous work has examined the ability of linear and cyclic peptides to mimic the CDR2 loop [12–15]. The gp120 binding activity of these molecules (unmeasurable to m*M* range) has dampened their further consideration as antagonist leads. Conversely, CD4 mimetics involving miniprotein scaffolds have resulted in molecules with higher affinities (μM) [16,17] and are, therefore, potentially more promising CD4 starting points in developing antagonists.

Two recent CD4 mimetic peptides are based on the structures of particular scorpion toxins. Scorpion toxins are small (29–39 residues), rigid proteins which lend themselves to be used as miniprotein scaffolds for mimetics design. These toxins are characterized by a double stranded, antiparallel β-sheet and a short α-helix held in a conformationally stabilized structure by three disulfide bridges [18]. Scorpion toxins have a hairpin loop that resembles the CDR2 loop of CD4 (Fig. 3). Charybdotoxin and scyllatoxin, members of the scorpion toxin family, have been used as molecular scaffolds on which CD4-like activity has been built.

TXM1, a peptide mimetic based on charybdotoxin, was designed to mimic the CDR2 loop of CD4 (Fig. 3) [16]. The CDR2 loop residues in CD4 are Gln–Gly–Ser–Phe (i.e., QGSF). The residues of charybdotoxin in the corresponding positions were designed to match the CD4 QGSF sequence. In this way, Phe-27 of TXM1 mimics the position and functionality of Phe-43 in CD4. In addition, the crystal structure of CD4 shows that the charged side chain of Arg-59 lies in close proximity to the phenyl ring of Phe-43. In order to retain a positive charge near the phenyl ring of Phe-27, the N-terminal region of charybdotoxin was truncated by several residues, leaving an N-terminal glycine. The α-amino group of this residue was intended to provide a mimic for the charged Arg-59 residue side chain.

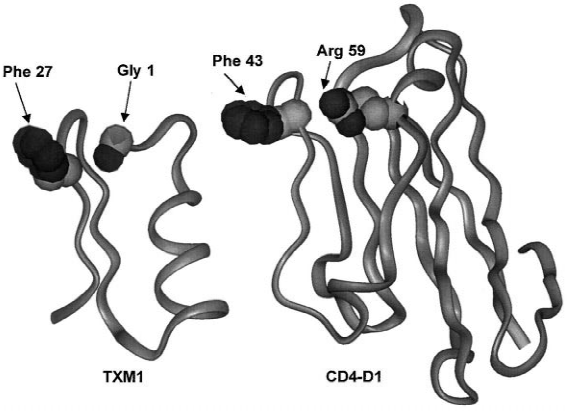

Fig. 3. Comparison of TXM1 and CD4 modeled structures. The phenyl ring of Phe-43 and side chain of Arg-59 in CD4 lie in close proximity to one another in the crystal structure (right). Phe-27 in TXM1 (left) is meant to mimic Phe-43 of CD4. The N-terminal glycine residue in TXM1 (Gly-1) is thought to mimic the positively charged Arg-59 side chain in CD4. Figure adapted from Zhang et al. [16].

For comparison purposes, a peptide with the loop sequence QGFS was made and called TXM0. The positions of the last two loop residues of TXM0 (i.e., –FS–) were reversed compared with TXM1 and CD4 (i.e., –SF–). Molecular modeling studies indicated that, while the phenyl ring of TXM1 (Phe-27) closely mimics that of CD4's Phe-43, the phenyl ring of TXM0 does not [16].

This review describes a combination of techniques used in the development of CD4 mimetic peptides. We have used a surface plasmon resonance (SPR) assay to observe the conformational change in gp120 upon binding certain ligands. As evidenced by TXM1, this assay can be used to compare the gp120-activation effects of CD4 mimetics. Lastly, we describe here preliminary data on the incorporation of phage display for the development of novel gp120-binding sequences.

2. Materials and methods

2.1. Isolation of recombinant proteins and synthetic peptides

HIV envelope protein, gp120 (JR-FL) was expressed in *Drosophila Schneider* 2 (S2) cells as previously described [19]. The recombinant protein

was purified using affinity chromatography. Briefly, F105, a high affinity gp120 monoclonal antibody was immobilized onto protein A-coated, fast-flow Sepharose. Cell supernatant was passed over the F105 column to capture the expressed gp120. The column was washed with phosphate-buffered saline (PBS) to remove undesired supernatant components, and purified gp120 was eluted using 0.1 *M* glycine (pH 2.8). CD4 was expressed and purified as previously described [20,21].

TXM1 was synthesized using solid-phase peptide synthesis on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA, USA). Fmoc protected amino acids were coupled after activation with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotrizaole (HOBt). Side chain protecting groups were trityl (Asn, Cys, Gln, His), *tert*-butyloxycarbonyl (Lys, Trp), *tert*-butyl (Glu, Ser) and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Arg). The peptide was cleaved from the resin using a mixture of 81.5% trifluoroacetic acid (TFA), 5% thioanisole, 5% phenol, 2.5% ethanedithiol, 5% water and 1% triisopropylsilane. The peptide was oxidized and purified as described previously [16]. Briefly, the reduced peptide was subjected to oxidative conditions (5 m*M* reduced glutathione, 0.5 m*M* oxidized glutathione, 1 *M* Tris–HCl, 0.2 *M* NaCl, pH 7.8). These conditions led to the formation of the required three disulfide bonds. The oxidized peptide was purified using reversed-phase high-performance liquid chromatography (RP-HPLC) (Vydac C_{18} column, linear acetonitrile gradient, 10–22% buffer B over 45 min. Buffer A=5% acetonitrile, 0.1% TFA; buffer B=90% acetonitrile, 0.1% TFA). Purified peptide was lyophilized, verified by mass spectrometry and dissolved in PBST (PBS+0.005% Tween 20) prior to use.

2.2. Optical biosensor interaction analysis evaluation

The surface plasmon resonance assays were conducted on a Bia3000 optical biosensor (Biacore, Uppsala, Sweden) as described previously [16]. In this assay, a protein ligand is immobilized on the surface of a carboxymethyldextran sensor chip (CM5) via an amine capture procedure according to the manufacturer's specifications. A solution containing the analyte is passed over the sensor chip surface, and the change in mass due to binding is recorded optically. In these experiments, antibody fragment 17b Fab was immobilized to the sensor chip. A solution of gp120 with or without added CD4/CD4 mimetic was passed over the chip as the analyte and binding was measured.

2.3. Phage display of charybdotoxin scaffold

As a means of randomizing the QGSF loop sequence found in TXM1, phage display was employed. Phage were constructed to contain a charybdotoxin insert with the loop sequence of QGSF. Using phage detection by the anti-M13 antibody, phage-bound QGSF peptide was shown to effectively bind to gp120. This binding to plate-immobilized gp120 was competed by both soluble gp120 and sCD4. The loop sequence of the phage was randomized as previously described [22] to give a series of selectants with affinity for gp120. These selectants are currently being characterized for sCD4 competition.

3. Results and discussion

3.1. Biosensor analysis of CD4-induced cooperative effects

CD4 mimetic peptides can be examined for CD4 competition and gp120 activation using an optical biosensor. Optical biosensors provide a means of looking at the interaction between macromolecules as it occurs in real time. Thus, the kinetics of the interaction, in addition to the affinity, can be measured. Changes in the relationship between CD4 competition and activation of gp120 binding to 17b (due to structural changes, for example) can be easily seen as changes in association and dissociation of the interacting molecules.

An optical biosensor experiment, based on the relationship between CD4–gp120–17b, was designed to assess the behavior of the CD4 mimetic peptides (Fig. 4A). With 17b immobilized on the sensor surface, binding of gp120 to 17b is greatly enhanced in the presence of sCD4 (soluble CD4) versus in its absence (Fig. 4B). This result mimics that seen for binding between gp120 and chemokine

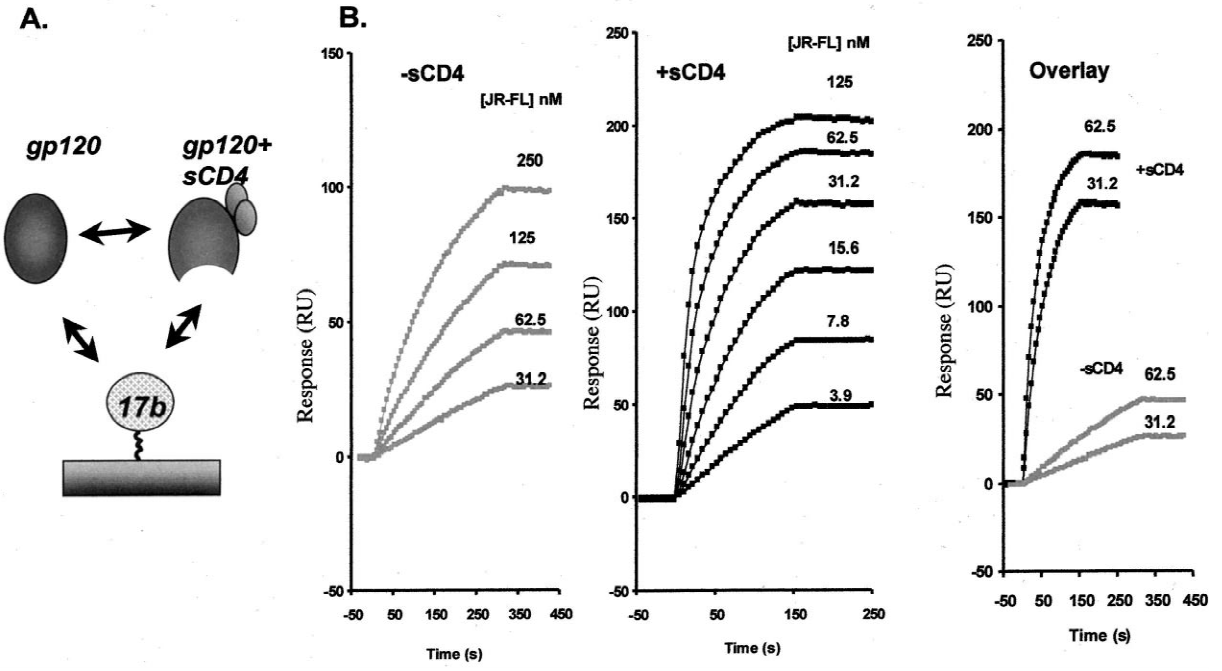

Fig. 4. Binding of gp120 to 17b in the presence versus absence of sCD4. (A) The optical biosensor configuration involves 17b Fab immobilized while a solution of gp120 (JR-FL, ±sCD4) is passed over the sensor chip surface. (B) The resulting sensorgrams of gp120 alone, gp120 in the presence of sCD4 and a comparison of the overlay at two concentrations of gp120 (left to right, respectively) indicate that sCD4 greatly enhances the binding of gp120 to 17b. Figure adapted from Zhang et al. [16].

receptors: binding between gp120 and these receptors is increased in the presence of CD4 [23].

TXM1 was examined in a series of optical biosensor assays to determine its influence on gp120's ability to bind CD4 and 17b (Fig. 5). With sCD4 immobilized, a mixture of gp120 (JR-FL) and TXM1 was passed over the sensor chip surface. This binding result was compared with gp120 binding to the sCD4 surface in the *absence* of TXM1 (Fig. 5A). In the *presence* of TXM1, binding between gp120 and sCD4 is significantly reduced. This result indicates that TXM1 competes with sCD4 for gp120 binding.

With the antibody 17b immobilized, a mixture of gp120 (JR-FL) and TXM1 was passed over the sensor chip surface. The binding of gp120 to 17b was enhanced in the *presence* of TXM1 versus in the *absence* of TXM1 (Fig. 5B). In this way, TXM1 activates gp120 toward 17b binding in a similar manner as sCD4. TXM0 was also examined in this series of optical biosensor assays. Neither competition of sCD4 binding nor enhancement of 17b binding by gp120 were observed in the presence of TXM0 (data not shown).

From TXM1, we learned that it is possible to mimic the structural and functional attributes of a very large protein with a much smaller peptide. While the affinity of TXM1 for gp120 (~60 μM) is modest compared with that of the CD4–gp120 interaction, an influence of this small peptide can be seen. The 17b binding effect that TXM1 exerts on gp120 is similar to that seen when gp120 is combined with sCD4. In addition, TXM1 seems to be competing with sCD4 in binding to gp120. These two pieces of evidence, as measured by optical biosensor, indicate that TXM1 is mimicking the action of sCD4 at gp120. Also recently, CD4M9, a CD4-mimetic based on the scyllatoxin scaffold with a AGSF loop sequence, was synthesized by Vita et al. [17] and found to compete with CD4 for gp120 binding with an increased affinity versus TXM1. We have re-made the CD4M9 peptide and found that, as

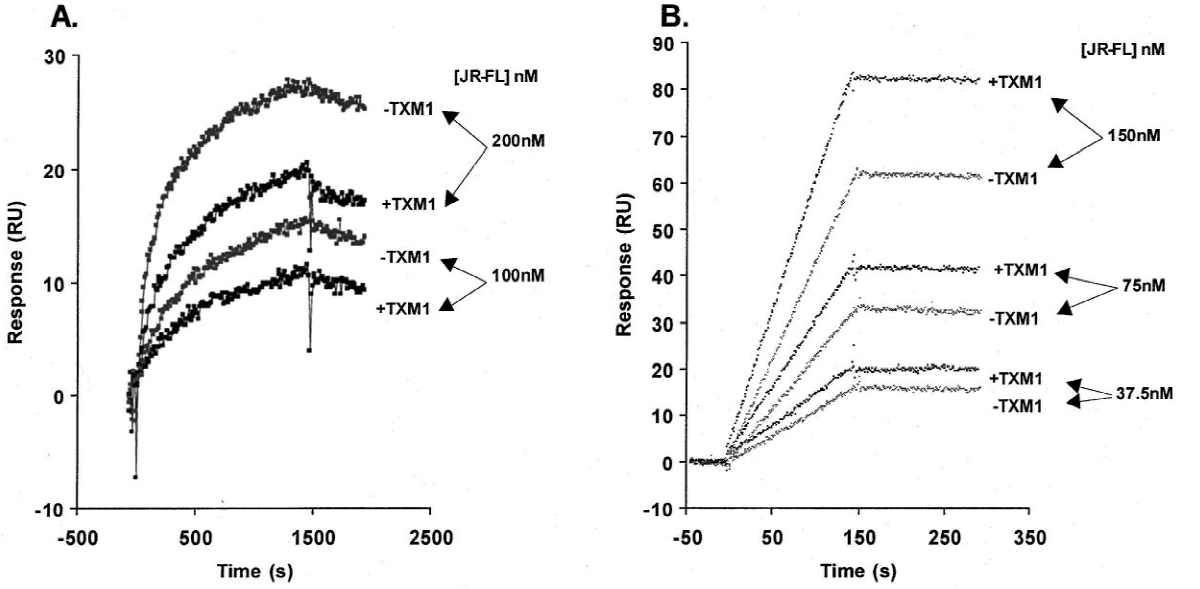

Fig. 5. (A) Blank-subtracted sensorgrams of TXM1 CD4 mimetic peptide. With sCD4 immobilized, addition of TXM1 reduces the binding of gp120 (JR-FL) to sCD4. (B) In the presence of TXM1, binding of gp120 to immobilized 17b is increased. Figure adapted from Zhang et al. [16].

TXM1, this mimetic activates gp120 binding to 17b (unpublished results).

3.2. Phage display of TXM1 and epitope randomization

The data obtained to date with scorpion toxin-based mimetics of CD4 have demonstrated the efficacy of the toxin scaffold to form CD4-mimicking molecules that bind gp120 in a manner that, as CD4, induces enhanced 17b binding. Since the binding sites on gp120 for 17b and co-receptor overlap, we expect that the scorpion toxin mimetics made so far would upregulate co-receptor binding as they do 17b binding. Importantly, however, this effect may not be desirable for a true antagonist, since increasing co-receptor binding might induce, not antagonize, viral entry. Hence, we are investigating the possibility to identify mimetics which bind in the CD4 binding site of gp120 but do not induce an increase in 17b binding.

In order to examine alternative mimetic sequences, phage display was employed as a means of randomizing the TXM1 QGSF loop sequence. As a prelude to randomization, we successfully expressed TXM1 on the surface of phage fused to the gene III protein (Fig. 6A). We have found that phage-bound TXM1 can bind to gp120 in a manner that is competitive with sCD4 (Fig. 6B).

Based on this result, we recently have made a charybdotoxin mimetic library (Fig. 6A, manuscript in preparation). The four residues of what we believe to be the key recognition sequence QGSF of TXM1 (and indeed CD4 itself) were randomized, while the remaining portion of the TXM1 structure was held constant. We currently are evaluating peptides, selected from this library by gp120 panning, that are competitive with CD4 (unpublished results).

4. Concluding remarks

Overall, we believe that understanding the CD4–gp120–17b interaction system can help both to determine cooperativity mechanisms involved in the HIV-cell infection process that leads to AIDS and to design mechanism-based mimics of the interaction components that disrupt the infection process. The availability of a crystal structure provides a key starting point by enabling visualization of the inter-

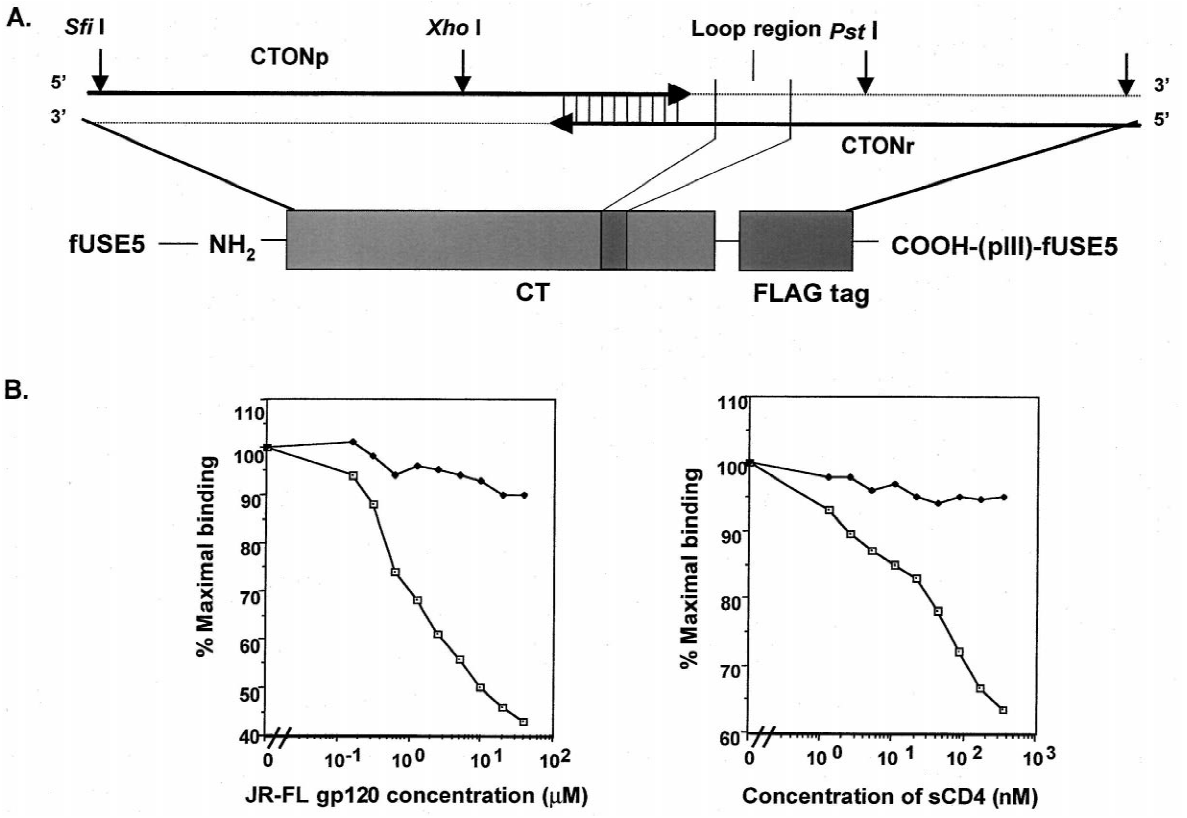

Fig. 6. (A) Construction of phage-displayed, charybdotoxin-based CD4 mimetic TXM1. The mimetic displayed contains QGSF in the loop region of residues 24–27. This region, as well as other structural elements believed important for gp120 binding, are candidates for phage epitope randomization. (B) Phage-enzyme-linked immunoassay (ELISA) results from TXM1 (open squares) displayed on phage versus wild type fUSE5 phage itself (closed diamonds). In the left panel, TXM1-phage is mixed with increasing amounts of gp120 (JR-FL) and added to a gp120-immobilized enzyme-linked immunoassay (ELISA) plate. Anti-M13 antibody (anti-phage, HRP-conjugated) is used to detect bound phage. Decreased phage levels indicate that TXM1-phage is binding to gp120. In the right panel, TXM1-phage is mixed with increasing amounts of sCD4 and added to a gp120-immobilized ELISA plate. Using the same anti-phage antibody, decreasing levels of phage indicate that TXM1-phage competes with sCD4 for gp120 binding.

actions, albeit static, of these three proteins. The water solubility of these molecules has allowed their investigation by such methods as optical biosensor and titration calorimetry assays. These techniques hold promise in measuring the effects of structural variation of gp120 ligands on both affinity and conformational change. Miniprotein mimetics such as scorpion toxin scaffolds can be prepared by automated synthesis and therefore can be evaluated in the same activity assays. Finally, these small peptide mimetics can be displayed on filamentous phage. Phage-based epitope randomization offers the chance to examine a breadth of structural diversity and hence may yield novel gp120 binding sequences that may not be apparent when viewing only the crystal structure.

By combining information from these methodologies, it is hoped to deduce the structural elements that govern gp120–sCD4 binding versus CD4-induced gp120 activation toward chemokine receptor/17b binding. This may help to design peptide mimetics which bind without activation and which, hence, may be advantageous as lead molecules in the area of AIDS therapeutics.

Acknowledgements

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