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Biophysical evidence of two docking sites of the carboxyl heptad repeat region within the amino heptad repeat region of gp41 of human immunodeficiency virus type 1

Ding-Kwo Chang *, Chang-Sheng Hsu

Institute of Chemistry, Academia Sinica, Taipei 11529, Taiwan, Republic of China

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Abstract

Two HIV-1 gp41-derived peptide fusion inhibitors, T-20 and T-649, were synthesized and their binding profiles of the N-heptad repeat region (HR1) were compared to examine the molecular basis of the differential antiviral potency and viral resistance. Turbidity clearance experiments based on the overlapping 15-mer peptides derived from HR1 revealed a major binding site at the LLSGIV segment for both T-20 and T-649. Additionally, another docking site was found at the sequence encompassing the hydrophobic pocket of HR1 for T-649. Concordant results were observed from the surface plasmon resonance measurements. The binding affinity profile exhibited a major maximum around the LLSGIV motif for the two peptide fusion inhibitors while a less prominent docking region was located near the hydrophobic pocket for T-649. This bi-modal model deduced from T-20 and T-649 interaction with HR1 peptides could rationalize the failure of emergence of the fusion inhibitor-resistant virus with simultaneous mutations in each of the two binding regions, as well as the generally higher potency of T-649 against most viral strains.

Keywords: Heptad repeat region; Helix bundle; Fusion inhibitor; HIV-1; gp41 hydrophobic pocket; Turbidity clearance; Surface plasmon resonance; Bi-modal binding

1. Introduction

Despite the effective combination treatment on the HIV-1 infection targeting reverse transcription and viral protease in reducing the viral titer and the progress of the disease, the emergence of resistance strains necessitated the search for antiviral agents that interfere with other steps of viral life cycle. A recent addition to the anti-HIV-1 regimen is enfuvirtide (T-20), which exerts its action by blocking fusion between the virus and the target cell (Wild et al., 1994), the first step of the viral replication cycle. T-20, a peptide corresponding to the HR2 region (aa 638–673) of the transmembrane glycoprotein gp41, potently inhibits viral infectivity of a wide range of virus variants at the nanomolar level. Since then, several peptides thought to target membrane fusion (Jiang et al., 1993; Rimsky et al., 1998), including T-649 and T-1249, have been studied. T-649 is also a 36-mer peptide composed of aa 628–663 of gp41, with 10 amino acids shift to the N-terminal direction compared to T-20. In vitro and in vivo investigations revealed that in general T-649 is more potent (Heil et al., 2004) and development of resistance phenotype is more difficult than for T-20 (Chinnadurai et al., 2005; Kilby et al., 1998; Rimsky et al., 1998).

Understanding the mechanism underlying the action of the fusion inhibitors is crucial to not only the development of future generations of entry inhibitors but also the pathway and kinetics of the virus-mediated membrane fusion, in view of the increased use of fusion inhibitors as a new class of antiviral reagents. For example, T-20 has been shown to be effective when CD4 was incubated with the cells infected with the virus (Furuta et al., 1998), suggesting that the inhibitor acted on an intermediate gp41 structure exposed by the receptor binding to gp120. Similarly, T-20 and T-649 have been used to dissect the pathway of the virus-induced fusion reaction (Melikyan et al., 2006). The emergence of T-20 resistant HIV-1 strains in which the mutations have been mapped to the HR1 domain, which sometimes were accompanied by mutations in the HR2 region (Rimsky et al., 1998; Sista et al., 2004), afforded additional opportunity to unravel the interplay between the inhibitor, the viral envelope protein and the cellular receptors. The mechanism of the drug

* Corresponding author. Tel.: +886 2 27898594; fax: +886 2 27831237.
E-mail address: dkc@chem.sinica.edu.tw (D.-K. Chang).

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resistance is also important in the design of future generations of viral therapeutics.

The initial interpretation of T-20 action is the interference of the peptide with formation of the six-helix bundle in a dominant negative manner. T-20 and T-649 have been proposed to act at the exposure of HR1 domain in gp41 triggered by the Env-CD4 complex formation but before HR1 closure upon HR1–HR2 helix bundle formation (Derdeyn et al., 2001). The emergence of T-20 resistance isolates involving mutations in the GIV (aa 547–549) motif, which was subsequently expanded to the 547–556 region (Greenberg et al., 2002; Sista et al., 2004), suggests a role of the N-terminal portion of HR1 in the HR1–HR2 interaction and the mode of T-20 action. Moreover, the mutation in the GIV tripeptide has been proposed to maintain the conformation of the stem of the Rev responsive element and hence the replication fitness of the virus (Nameki et al., 2005). These investigations demonstrated that the mechanism of T-20 and its analogs that target the intermediate structures of fusion protein is multi-faceted and suggests that its elucidation may shed light on the fusion process.

To delineate the binding site of T-20 within HR1, we have carried out a turbidity clearance study using an array of overlapping 15-mer peptide encompassing residues 526–569 (Trivedi et al., 2003). It was deduced that the LLSGIV motif constituted a critical docking site of T-20. To better characterize the interaction of HR1 and HR2, we utilized an array of overlapping peptides covering the entire HR1 to map the docking sites of T-20 and T-649 by the turbidity clearance approach and by the surface plasmon resonance (SPR) technique. The measurements on these two HR2-derived peptides corroborated our previous results (Trivedi et al., 2003) and further disclosed an additional, minor binding site at the hydrophobic pocket of HR1 (Chan et al., 1997). The latter finding can account for, at least in part, the higher potency of T-649 in comparison to T-20 for a wide range of viral strains, as well as the differential sensitivity between the variants of the GIV motif of gp41 to T-20 and T-649 (Derdeyn et al., 2001). The multiple binding mode of the HR1/HR2 association probably allows the virus to escape the fusion inhibitors with lower fitness.

2. Materials and methods

2.1. Peptide synthesis

Synthesis of the HR1 peptides, T-20 (aa 638–673) and T-649 (aa 628–663) followed the protocols described previously (Chang et al., 1999). In brief, the peptides were synthesized in an automated mode by means of Fmoc chemistry with a solid phase synthesizer (model Ranin PS3, Protein Technologies (Tucson, AZ)). The synthesized peptides are N-capped by the acetyl group and C-capped with the amino group. They were cleaved from the resin and purified by HPLC on a reverse phase C18 column. The primary sequence of peptides was ascertained by electrospray or MALDI mass spectrometry. All reagents used in this study were of analytical grade. To increase the solubility of the HR1 peptides, two lysine residues were introduced to the N-terminus of the overlapping 15-mers of HR1, which span residues 526–590 (Fig. 1).

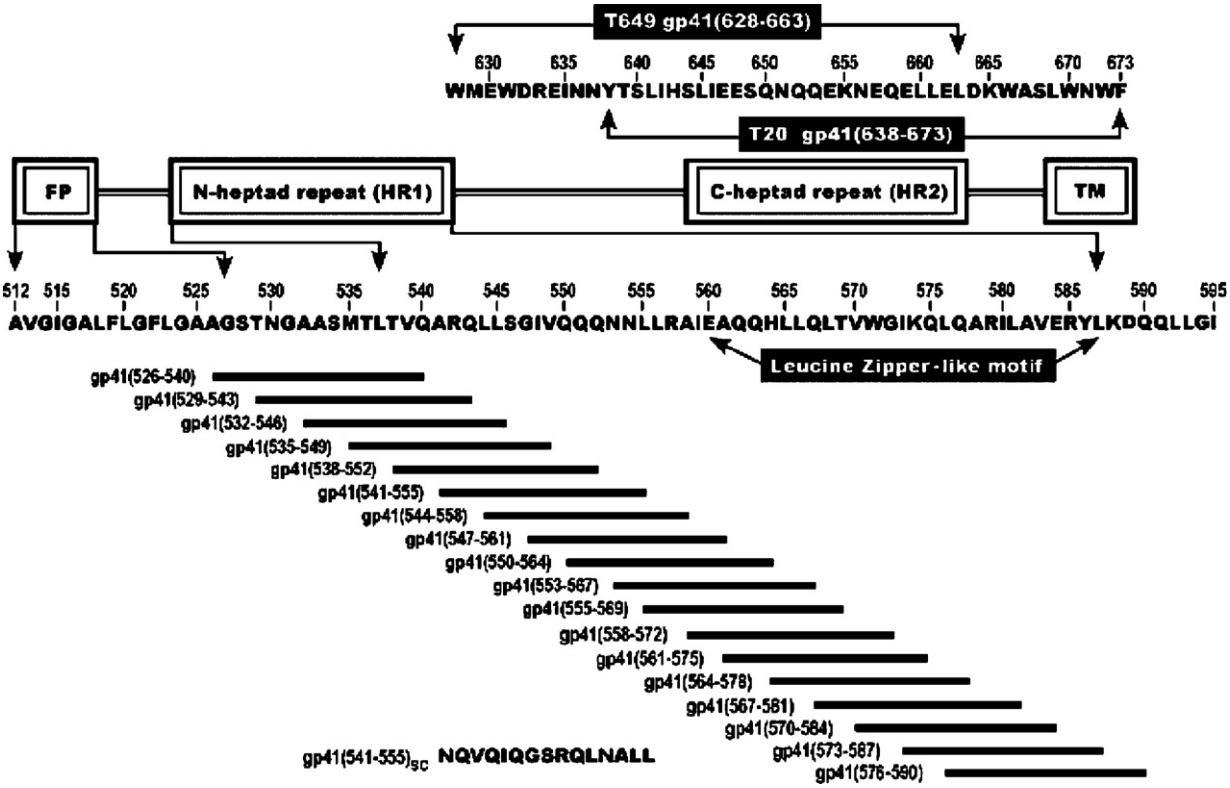

Fig. 1. The sequence of gp41 ectodomain and its functional domains. The overlapping HR1-derived peptides are indicated along with the scrambled gp41(541–555) peptide sequence. The residue numbering system is according to the HXB2 strain of HIV-1.

2.2. Turbidity clearance assay

Turbidity (or cloudiness) of the peptide solution is attributed to the scattering of light by large particles formed by suspension of aggregate of T-20 or T-649 molecules in the 10–100 μM range in selected solvents. The cloudy suspension is transformed into clear solution as the large T-20 or T-649 particles are segregated by specific interaction with HR1 peptides, resulting in a reduced optical density. T-20 turbidity clearance experiments were performed under the conditions described previously (Trivedi et al., 2003). For T-649, the measurements were carried out at 15 °C at pH 4.0. A stock 60 μM T-649 suspension was prepared by adding T-649 to 2% (v/v) DMSO in water. A 450 μl aliquot of the suspension was mixed with 50 μl of the stock HR-1-derived peptide dissolved in 2% (v/v) DMSO in water to give a final concentration of 54 μM. The turbidity reading of T-20 and T-649 was shown stable over a period of at least 3 h before used in the measurements whereas the HR1 peptide solutions were clear before mixing with the fusion inhibitor suspension. Corrections for dilution were made on the UV absorbance measured at 400 nm.

2.3. Surface plasmon resonance (SPR) measurements

To improve the sensitivity and to obtain the kinetic information of the interaction between the peptides tested, the SPR assay was undertaken. The measurement was based on the change in the solution refractive index (manifested as change in the RU value) upon association or dissociation of molecules tested. In the experiments, T-20 or T-649 was immobilized in a suitable sensor chip and each of HR1 peptides was dissolved in the solution to be eluted through a chamber containing the T-20 (or T-649)-embedded chip. SPR result, in addition to binding affinity, allows the evaluation of kinetics of complex formation between the HR1 peptides and the fusion inhibitors. The kinetic binding experiments were carried out on an optical biosensor BIAcore 3000 (Biacore AB) using CM5 sensor chips. The CM5 chip was first activated by the mixture of *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide (EDC) injected over the chip surface for 10 min at a flow rate of 5 μl/min according to the Manufacturer's manual. T-20 or T-649 was dissolved in 10 mM sodium acetate buffer, pH 4.0, filtered and injected over the activated sensor surface for 7–10 min at a flow rate of 5 μl/min until the RU level reached 500–1000. The remaining activated sites were blocked by 50 μl 1.0 M ethanolamine, pH 8.0. The analytes (HR1 peptides) were dissolved in 20 mM PBS buffer pH 7.4 and diluted to a final concentration range of 50–1.25 μM. The reaction time was 2–5 min. The sensor surface was regenerated using a 30 μl solution containing 0.5 M NaCl and 0.1 M NaOH.

3. Results

We have delineated a critical binding site LLSGIV motif of T-20 by turbidity clearance measurement in a previous study (Trivedi et al., 2003). To further probe the HR1 and HR2 association as a crucial step of the six-helix bundle formation and the difference in the HR1 binding affinity between T-20 and T-649, we expanded the overlapping gp41 HR1 15-mer coverage to residue 590, as shown in Fig. 1.

3.1. Turbidity clearance experiments on T-20 or T-649 suspension mixing with HR1-derived peptides revealed an additional HR2 interaction site near the hydrophobic pocket of HR1

Results of turbidity clearance for T-20 and T-649 reacting with the HR1 peptides are displayed in Fig. 2. When each of the HR1 peptides was introduced into the T-20 suspension, a prominent region of clearance activity around the LLSGIV stretch was deduced, consistent with the previous conclusion using HR1 15-mer peptides (Trivedi et al., 2003). The result demonstrates that addition of the two lysines to the N-terminus of the HR1 peptides has little effect on the interaction between the HR1 peptides and T-20 (see also Supplementary data S1). Analysis of the turbidity clearance data of the C-terminal end of HR1 reveals that a weak activity can be discerned for gp41(567–581) and gp41(570–584). Hence it is clear that the 15-residue sequences that contain residues 567–573 exhibit a weak reaction to T-20.

We also replaced leucine at 544 with serine and changed GIV motif by DIM or SIM in HR1 to confirm the importance of LLSGIV sequence to HR1–HR2 association. Indeed, as shown in Fig. 2, a substantial reduction in turbidity clearance activity is observed when the three variants of gp41(541–555) were mixed with either T-20 or T-649.

To exclude the possibility that the observed clearance is due to nonspecific association, a peptide with scrambled sequence of gp41(541–555) was synthesized. No activity was found for both fusion inhibitors.

Interestingly for data of the T-649 suspension summarized in Fig. 2, in addition to the major binding site of the LLSGIV segment, a moderate activity is observed for the gp41(567–581) and gp41(570–584) peptides and somewhat weaker response is found for gp41(573–587) and gp41(576–590). We inferred that the 567–573 stretch has a minor contribution to T-649 binding and that residues 580–584 may have yet a smaller contribution. This prompted us to employ surface plasmon resonance experiments to verify the result.

3.2. SPR data provide evidence that an additional docking site of HR2 is located in the hydrophobic pocket of HR1 C-terminal portion

To better pinpoint the binding site and quantitate the association constant of T-20 and T-649 with the HR1 peptides tested, we performed SPR measurements to directly evaluate the binding constant using the fusion inhibitor as a ligand and injecting incremental concentrations of the HR1 peptides. In Fig. 3, the semi-logarithmic plots of association constant versus HR1 peptides are shown for T-20 and T-649. Representative sensorgrams are displayed in Supplementary data.

Consistent with the turbidity clearance results, HR1 peptides containing the LLSGIV sequence displays higher affinity

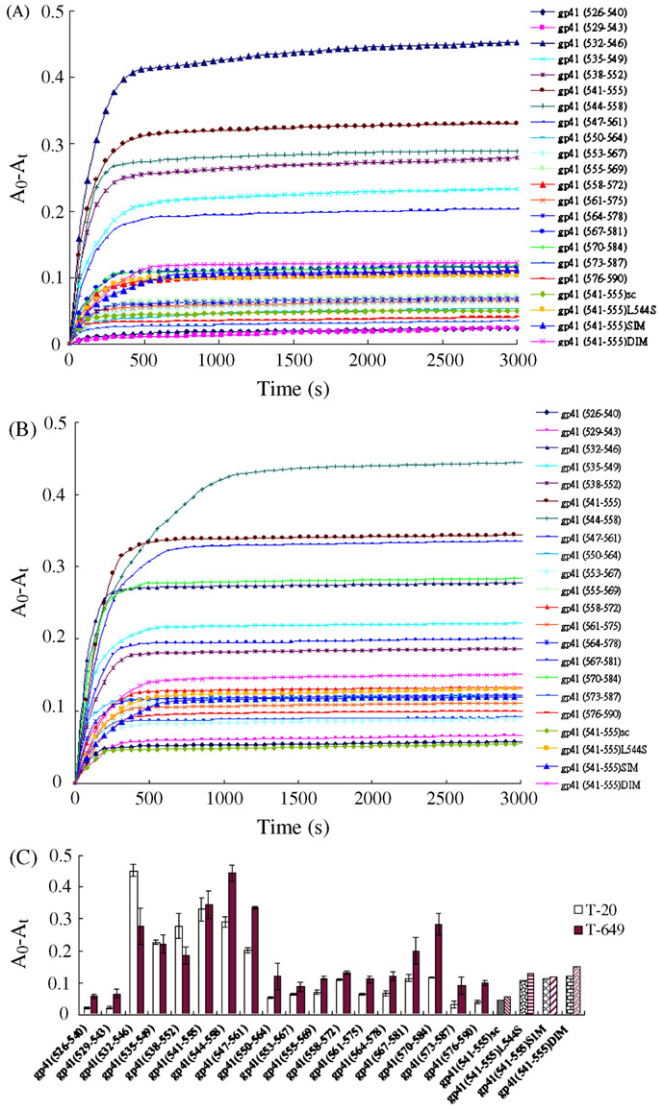

Fig. 2. The time course of turbidity clearance of T-20 (A) and T-649 (B) suspensions by HR1 peptides. A_0 and A_t denote the absorbances of the suspensions before and at time t after introduction of the HR1 peptides, respectively. (C) Turbidity clearance profile of T-20 and T-649 suspensions by the HR1 peptides. A prominent activity is observed for peptides containing the LLSGIV motif for T-20 and T-649, whereas an additional active region QLTVWGI can be identified for T-649. The data with T-20 confirm the previous results using 15-mers derived from the HR1 domain. Specificity of the turbidity clearance is demonstrated by a scrambled sequence of gp41(541–555) showing little activity to T-20 or T-649 suspension. Note the decrease in the turbidity change of greater than 0.2 O.D. for the three gp41(541–555) variants upon mixing with both T-20 and T-649 suspensions suggests a major role of the LLSGIV motif in the HR1–HR2 association.

to T-20, noting that gp41(526–540) exhibits undetectable association with T-20 and a small $K_A(=1/K_D)$ for gp41(529–543). Similarly, no significant binding to T-20 is observed for the stretch 550–564. The downward trend in K_A toward the HR1 C-terminus is slightly reversed around gp41(567–581) and gp41(570–584), while gp41(573–587) and gp41(576–590) exhibit little activity.

For T-649, the predominant binding is contributed by the N-terminal region of the HR1 encompassing LLSGIV motif, as in the case of T-20. However, the hydrophobic pocket region (aa 567–573) is seen to have a moderate activity as deduced from the observation that gp41(567–581) and gp41(570–584) have elevated binding affinity to T-649 compared to other HR1 sequences such as gp41(573–587) and gp41(576–590). We note that such a difference is not found for T-20 affinity (Figs. 2C and 3B). In short, SPR data yielded that two separate docking sites can be discerned in the HR1–HR2 interaction, which is therefore bi-modal.

Consistent with the turbidity clearance data (Fig. 2), binding affinity to T-20 or T-649 decreased by more than an order of magnitude for the L544S, DIM and SIM mutants compared to gp41(541–555). Similarly, the specificity of HR1 peptides/T-20 or T-649 interaction is demonstrated by the small binding constant obtained with a scrambled gp41(541–555).

4. Discussion

4.1. Turbidity clearance and SPR results consistently reveal two HR2 interaction sites near the HR1 N- and C-terminal regions

The development of entry inhibitor was a response to the urgent need following the virologic failure of anti-reverse transcriptase and anti-protease treatments. The attachment inhibitors targeting gp120 interaction with CD4 and coreceptors suffered from high gp120 sequence variability and glycan protection. T-20 (Enfuvirtide, Fuzeon) is a first generation of the fusion inhibitors based on the HR2 sequence of gp41. Despite its derivation from the LAI strain of HIV-1, T-20 is effective against a wide range of HIV-1 strains (Wild et al., 1994). However, T-20 resistance viruses emerged with residues in or near the GIV motif (aa 547–549) mutated (Rimsky et al., 1998; Matthews et al., 2004; Wei et al., 2002).

Both the turbidity clearance and SPR results exhibited a low activity of gp41(526–540) to both T-20 and T-649 suggesting that the flexible stretch between FP and HR1 (Chang et al., 1999) is not critical in the HR1–HR2 interaction. The segment may provide a non-rigid hinge between the fusion peptide and the coiled coil region of HR1 to facilitate fusion process. The abrupt increase in activity to both fusion inhibitors is seen for the HR1 peptides containing residues 544–550, showing the LLSGIV motif as a major binding site for HR2. It is of interest that L544S mutation (Armand-Ugón et al., 2003a) was discovered to confer resistance to the inhibitor C34, corresponding to gp41(628–661), suggesting that this residue is critical in blocking HR1–HR2 association. It is noteworthy that the C34-resistant variant also reduced the susceptibility to T-20 to a similar extent (Armand-Ugón et al., 2003b).

In both types of experiment performed in this study, T-649 exhibited a moderate activity with HR1 peptides containing residues near 570 that form hydrophobic grooves in the crystal structure of the six-helix bundle (Chan et al., 1997; Weissenhorn et al., 1997). However, these peptides have considerably less reactivity to T-20. It is noteworthy that the sequence of the 36-mer T-649 is shifted by 10 amino acids toward the N-

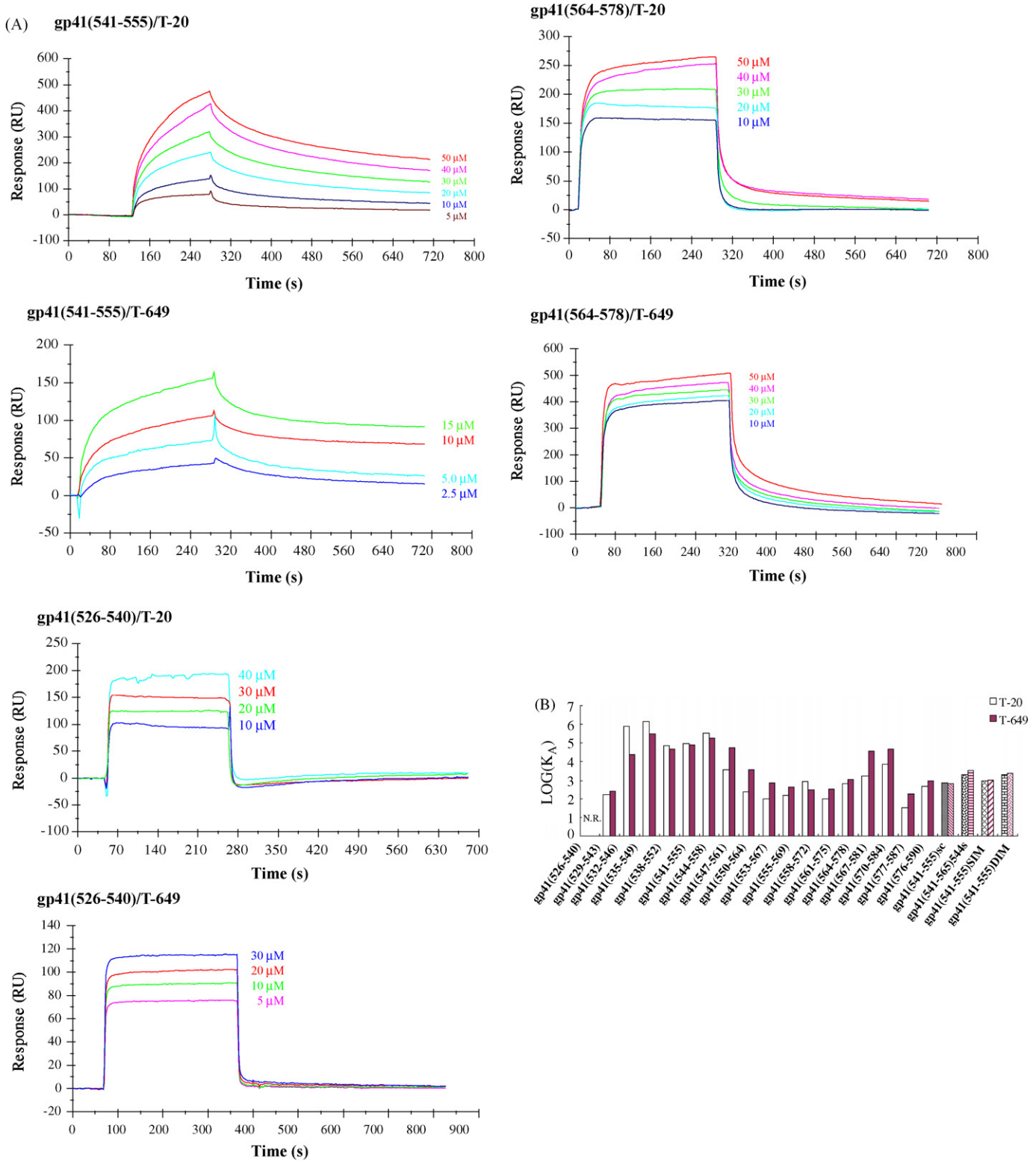

Fig. 3. Representative sensorgrams (A) and logarithmic plots (B) of the binding affinity of HR1 peptides to T-20 or T-649 to a Biocore sensor chip. To both fusion inhibitors, the HR1 peptides with the highest binding constant are those encompassing the LLSGIV sequence. A minor reactivity to T-649 can be attributed to the hydrophobic pocket near the C-terminus of HR1. Bi-modal binding profile between HR1 and HR2 of gp41 can therefore be deduced. Specificity of the binding affinity measurements is confirmed by a small K_A for T-20 or T-649 dispersion obtained with a scrambled gp41(541–555) sequence. Importance of the LLSGIV motif is highlighted by a more than 10-fold reduction of K_A for the three gp41(541–555) mutants in association with T-20 and T-649.

terminus with respect to that of T-20 and these 10 residues are in close contact with the hydrophobic groove formed by the coiled coil of HR1. Taken together, our results suggest that the gp41 HR1–HR2 association is bi-modal with the aa 544–549 and 567–573 segments being the two docking sites in HR1 for HR2. Note that residues of T-20 do not contain the full sequence required for the interaction with the hydrophobic 567–573 region and T-20 would thus have lower affinity to HR1 than T-649. The model can in part account for the lower IC_{50} and higher susceptibility to T-649 and T-1249 than T-20 for a wide range of HIV-1 isolates because the two docking sites are included in T-649 and presumably T-1249 (Rimsky et al., 1998; Heil et al., 2004; Derdeyn et al., 2001).

Examination of Figs. 2 and 3 on the HR1 peptides derived from the N-terminal portion of HR1 revealed that gp41(532–546) containing the LLS sequence had a high activity to T-20 and T-649, in sharp contrast to gp41(529–543) which lacks the tri-peptide. On the other hand, we did not observe the dramatic difference in the T-20/HR1 peptide activity for HR1 peptides with and without the GIV triad. The result implies that LLS triad exerts more influence than the GIV triad on the HR1/HR2 interaction.

The idea that the N-terminal region of HR1 encompassing the LLSGIV motif is more important than the hydrophobic pocket in binding to HR2 is corroborated by the finding that a single amino acid substitution L544S in HIV-1_{NL4-3} is sufficient to confer resistance to T-20 and T-649 (Lohrengel et al., 2005), possibly by disrupting the interaction between the fusion inhibitor and HR1 aside from other factors. Another support comes from the report that variation of a residue in the aa 657–662 region has larger effect than that in the aa 628–636 region on the inhibitory function of C34 (Seo et al., 2005)—possibly because the former region binds to LLSGIV motif while the latter binds to the hydrophobic pocket. A recent study (Heil et al., 2004) of the determinants of HIV-1 susceptibility to T-20 and T-649 demonstrated that the GIV motif was not involved in the viral insensitivity to the drugs. Furthermore, the difference in sensitivity to T-20 and, to a less degree, T-649 between X10 and R21 strains may be attributed to the residues outside the GIV motif in the N-terminal region of HR1 that are involved in the helix initiation. Our results on the reduced activity of L544S, DIM and SIM variants of gp41(541–555) toward both T-20 and T-649 also lend support to the concept of a major role of LLSGIV motif in HR1–HR2 binding.

It is of interest that the cells expressing membrane-anchored T-20 are not protected against the viral strains selected by the inhibitor, whereas the membrane-anchored peptide containing expanded sequence that spans both T-20 and T-649 sequences effectively inhibits infection of the cells by the virus (Lohrengel et al., 2005). While higher surface expression of the latter may account for the different inhibitory efficacy, higher binding affinity to HR1 due to the longer peptide sequence, according to our bi-modal mode, may also contribute to the differential inhibition. This explanation also reaffirms that the interaction involving the HR1 hydrophobic pocket is significant to the overall HR1–HR2 association.

4.2. Biochemical and functional ramifications of the bi-modal binding model of HR1/HR2 complex

We have identified two HR2 binding sites within HR1 of gp41 by comparison of the HR1 interaction of the two fusion inhibitors T-20 and T-649 of HR2 using the overlapping HR1 peptide library. The data presented herein should be useful in understanding the molecular mechanism of the drugs as well as formation of the gp41 core structure. Analysis of the HR1 sequences of T-20 resistant HIV-1 isolates revealed an important finding: there are no drug-resistant viral variants with simultaneous mutations in each of the LLSGIV motif and the hydrophobic pocket or their immediate neighbors (Chinnadurai et al., 2005; Carmona et al., 2005). This can be rationalized by the double docking model proposed in the present study. Thus with mutation in only one combining site, the effect on stability of the fusion-active six-helix bundle of gp41 is not detrimental, allowing the virus to replicate – albeit with lower efficiency – and develop drug resistance by weaker association with T-20. However with mutations occurring in both sites, the gp41 core structure becomes too unstable to support the membrane fusion, thus leading to a non-viable virus. Accordingly, the binding affinity and the kinetic measurements on the double mutant in these two segments would be valuable to test our model. In addition, the kinetic study of the refolding on the gp41 mutants involving residue 627 will be performed in comparison to the wild type gp41 ectodomain.

In agreement with our assertion on the importance of N-terminal region of HR1 in the helix bundle stability, Dwyer et al. (2003) have reported that truncation of the aa 540–549 fragment resulted in a dramatic reduction in the stability of HR1/T-649 complex but little change in the self-assembly of HR1 peptide; the reverse is true for the effect of truncation at the C-terminal side of HR1.

The two HR2 docking sites within HR1 deduced in the present work are in line with the X-ray crystal and NMR solution structures of HIV-1 and SIV gp41 ectodomain polypeptides, in which proximity of L544–L660 and G547–N656, and W571–W628 and W571–W631 (V570–W631) pairs can be found (Weissenhorn et al., 1997; Chan et al., 1997; Caffrey et al., 1998; Yang et al., 1999). In particular, our SPR results provide measurements on binding affinity and therefore the free energy of combining overlapping HR1 peptides with HR2 peptides, enabling a direct evaluation of contribution to the thermodynamic stability of HR1–HR2 complex. On the other hand, unlike the point mutation approach that substitutes a single amino acid residue, the determination of binding site using three-residue shift on the neighboring peptides does not distinguish individual residue's effect on the binding affinity.

4.3. Fusion inhibitor resistance phenotype and the relation to amino acid variation in the LLSGIV and its C-terminally adjoining regions

Recently, the fusion inhibitors, e.g., T-20 and T-1249 have emerged as a new addition to the arsenal of anti-HIV therapy. The treatment prompted selection of the drug-resistant variants

with substitution primarily in the 547–556 region (Menzo et al., 2004), and occasionally in the 544–546 motif, along with changes in the N-terminal portion of HR2 domain. Since the 547–556 sequence has been shown to participate in the gp41 association with gp120 (Cao et al., 1993), mutations in this region would alter the stability of gp120/gp41 complex and therefore affect the window of opportunity of exposure to the fusion inhibitor of the prehairpin intermediate. However the GIV motif also contributes to the HR1–HR2 interaction and the folding dynamics of gp41 core. The mechanism of drug-resistance due to the mutation in this region can thus be attributed to these two aspects. In contrast, the L544S mutation reduces the viral susceptibility to the fusion inhibitors by disrupting the association of HR1 and HR2.

The study conducted by Reeves et al. (2005) clearly showed slower fusion kinetics of the mutant virus – namely, the gp41(541–555)-DIM mutant – and its correlation with the T-20 resistance. The result is in line with our data on the double mutant HR1 peptide in that the DIM-mutant peptide has a weaker association with T-20 or T-649 HR2 peptide and possibly slower folding for HR1–HR2 helix hairpin containing the double mutation. Another mechanism of resistance to T-20 by HIV-1 virus was proposed by Nameki et al. (2005) who stipulated that the nucleotides encoding aa 541–549 region are involved in the maintenance of the secondary structure of the Rev response element (RRE) and therefore affecting the viral replicating fitness. Thus the resistance phenotype may reside at the transcriptional level.

The above discussion suggests that the mechanism of inhibition of T-20 and T-649 on the viral replication is multi-faceted (Yuan et al., 2004). Hence it is likely that, in terms of the HR1 binding constants, the disparity of T-20 and T-649 reflects only one aspect of the differential inhibitory efficacy between the two peptides.

In conclusion, our finding points to the need to take into consideration both the N-terminal LLSGIV motif and the hydrophobic pocket of HR1 in the study of membrane fusion mechanism and the design of gp41-targeting drugs.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2006.12.006.

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