Design of an engineered N-terminal HIV-1 gp41 trimer with enhanced stability and potency

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Abstract

HIV fusion is mediated by a conformational transition in which the C-terminal region (HR2) of gp41 interacts with the N-terminal region (HR1) to form a six-helix bundle. Peptides derived from the HR1 form a well-characterized, trimeric coiled-coil bundle in the presence of HR2 peptides, but there is little structural information on the isolated HR1 trimer. Using protein design, we have designed synthetic HR1 peptides that form soluble, thermostable HR1 trimers. In vitro binding of HR2 peptides to the engineered trimer suggests that the design strategy has not significantly impacted the ability to form the six-helix bundle. The peptides have enhanced antiviral activity compared to wild type, with up to 30-fold greater potency against certain viral isolates. In vitro passaging was used to generate HR1-resistant virus and the observed resistance mutations map to the HR2 region of gp41, demonstrating that the peptides block the fusion process by binding to the viral HR2 domain. Interestingly, the activity of the HR2 fusion inhibitor, enfuvirtide (ENF), against these resistant viruses is maintained or improved up to fivefold. The 1.5 Å crystal structure of one of these designs has been determined, and we show that the isolated HR1 is very similar to the conformation of the HR1 in the six-helix bundle. These results provide an initial model of the pre-fusogenic state, are attractive starting points for identifying novel fusion inhibitors, and offer new opportunities for developing HIV therapeutics based on HR1 peptides.

Keywords: fusion inhibitor; HIV; gp41; N-peptide; antiviral; coiled coil; protein design

Human immunodeficiency virus (HIV) fusion is mediated by a complex set of interactions involving cellular receptors (such as CD4, CCR5, and CXCR4) and viral glycoproteins (gp41/gp120) (Helseth et al. 1991; Weissenhorn et al. 1999). In the current model, a conformational change

in the gp41/gp120 oligomer after viral attachment allows the fusion peptide sequence, located on the amino (N) terminus of gp41, to insert into the membrane of the target cell (Chan and Kim 1998). The gp41 ectodomain is then thought to undergo a series of conformational changes to form the fusion-active state, which is believed to bring the viral and cellular membranes into closer proximity to facilitate membrane fusion (Chan and Kim 1998; Weissenhorn et al. 1999). Crystallographic studies of fragments of HR1 and HR2 provide support for a model where gp41 adopts a six-helix bundle structure that forms late in the fusion process (Lu et al. 1995; Chan et al. 1997; Weissenhorn et al. 1997). In peptide models of gp41, the heptad repeat 1 (HR1) region forms a trimeric inner coiled-coil core, which creates the

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binding interface for peptides from the HR2 region. A highly conserved portion of this groove, termed the “deep pocket,” has been previously shown to be an attractive target for blocking the viral fusion process (Wild et al. 1994; Judice et al. 1997; Chan et al. 1998; Eckert et al. 1999; Ferrer et al. 1999). The coiled-coil nature of the N- and C-terminal domains of HIV gp41 (Gallaher et al. 1989; Delwart et al. 1990) and other viruses (Chambers et al. 1990) had been predicted based on the identification of heptad repeat (HR) regions. The heptad repeat is characterized by a preference for hydrophobic amino acids at “a” and “d” positions of the heptad, and hydrophilic residues at the “b,” “c,” “e,” “f,” and “g” positions. This pattern places hydrophobic side chains along one face of the α -helix, which interact with other helices to form the coiled-coil structural motif. The HR1 region also has hydrophobic residues at “e” and “g” positions to facilitate binding with the viral HR2. Although the “a” and “d” positions have been demonstrated to affect oligomeric states (Bryson et al. 1995), the contributions by other positions in the heptad to the structural stability of the coiled coil are not well understood.

Despite extensive biophysical studies of gp41, it remains unclear what constitutes the pre-fusogenic state of the HR1 region. Although crystallographic evidence suggests a trimeric core, we have shown that in the absence of HR2 peptides, HR1 peptides self-associate into a tetrameric species (Lawless et al. 1996; Dwyer et al. 2003). Tetrameric oligomers have been observed in glycoproteins isolated from virus (Pinter et al. 1989; Earl et al. 1990; Thomas et al. 1991; Shugars et al. 1996), but there is no evidence that this oligomer plays a role in the fusion process. Recent work has also suggested that the HR1 region may not even form a coiled coil until after the virus has interacted with CD4 (Mische et al. 2005). Therefore, detailed structural information on the HR1 oligomer may provide insights into these early steps in viral fusion.

Isolated HR1 peptides have traditionally been difficult to study due to poor solubility; however, several strategies have been used to enhance the solution properties of these peptides. One approach that has been used successfully is to fuse a small portion of the HR1 to a designed trimeric coiled coil based on GCN4 (Eckert and Kim 2001), or to a thermostable core domain from gp41 (Louis et al. 2001). These constructs were found to be more soluble than the longer, isolated HR1 peptides, and were found to have enhanced antiviral activity. The activity of these chimeras was further improved by stabilizing the trimer via disulfide bonds (Louis et al. 2003; Bianchi et al. 2005). Another approach involved an expressed construct with one groove of the HR1 exposed (termed five-helix) (Root et al. 2001). Some of these constructs have also shown promise in developing neutralizing antibodies that target the HR1 domain of gp41 (Louis et al. 2003; Miller et al. 2005; Luftig et al. 2006).

In the present work, we have used several protein engineering strategies to design synthetic HR1 peptides that form highly soluble, thermostable trimers which have up to 10-fold enhanced antiviral activity. The engineered peptides are in some cases as potent as ENF, and in vitro passaging using one of these peptides resulted in a virus resistant to HR1 peptides. The resistance changes are localized in the HR2 region of gp41, demonstrating that the mechanism of action is direct binding to the HR2 and not the formation of hetero-oligomers with the viral HR1. Finally, the 1.5 Å X-ray structure of a 49-mer HR1 trimer was determined, which provides a detailed analysis of the engineered peptides. This is the first crystal structure of an HR1 peptide that forms a trimer without the need for artificial fusion proteins (Eckert and Kim 2001; Louis et al. 2003; Bianchi et al. 2005), and is the only extended HR1 sequence crystallized in the absence of HR2 peptides. The side chain placement and local geometry of the hydrophobic pocket is strikingly similar to that observed in the wild-type HR1/HR2 peptide complex (Chan et al. 1997), and this structure could form a preliminary structural model of the pre-fusogenic HR1. In addition to their potential use as HR2-directed fusion inhibitors, these designs are also attractive for use in screening small molecule libraries for novel fusion inhibitors and may be useful for HIV vaccine strategies targeting the HR1 region of gp41.

Results

Engineered HR1 peptides form trimers

Table 1 shows the biophysical and antiviral data for the wild-type 51-mer HR1 peptide, T865, as well as shorter wild-type peptides (denoted by Δ and the number of residues truncated from the N or C terminus). Our initial attempts to direct trimer formation were focused on changes to the “a” and “d” positions in the coiled coil. Since isoleucine has been shown previously to direct trimer formation, we placed isoleucine residues at each core position. This led to significant reduction in solubility, such as in N13 Δ Ile. Using information from alanine scanning of the HR1 and a computational algorithm that predicts coiled-coil propensity (Lupas et al. 1991; Wolf et al. 1997), several hydrophobic sites were selected to be replaced with hydrophilic amino acids. These substitutions increased coiled-coil propensity and reduced local concentrations of hydrophobicity in the peptide. The resulting peptide, N13 Δ Trimer, had reasonable solubility, and was found by circular dichroism to be nearly 100% helical and very thermostable (Table 1). Antiviral activity of the peptide was found to be enhanced relative to N13 Δ T865 for some primary isolates (see below) but was similar to N13 Δ T865 against many primary isolates (not shown) and IIB (Table 1).

Table 1. Sequence, biophysical properties, and antiviral activity of designed peptides

Peptide	Design	Sequence ^a	T _m (C) 10 μ M	% helicity 10 μ M	MW 100 μ M	N ^c	HR1/HR2 T _m (C) ^b	IC50 nM
T865	WT 51-mer	QARQLLSGIVQQNNLLRAIEAQHLLQLTVWGKQLQARILAVERYLKDQ	82 ^f	74 ^f	22857 ^f	3.9	91	536
T865 Δ	WT 49-mer	QARQLLSGIVQQNNLLRAIEAQHLLQLTVWGKQLQARILAVERYLK	83	83	agg ^d	--	93	930
N10 Δ T865	WT 41-mer	QQQNNLLRAIEAQHLLQLTVWGKQLQARILAVERYLKDQ	76	78	18583	3.8	63/75	387
N13 Δ T865	WT 38-mer	NNLLRAIEAQHLLQLTVWGKQLQARILAVERYLKDQ	75	83	16210	3.6	66	397
N13 Δ leu		NNILRAIEAIQHLIQLVWGKIQARILAIERYIKDI	--	--	--	agg	--	--
N13 Δ Trimer		NNILRAIEKIQLIQLVIRGKIQERILAIERYIKDI	>97	99	--	--	--	204
IQN17 ²³		RMKQIEDKIEEIESKQKKEIENIARIKLLQLTVWGKQLQARILAVERYLKDQ	>97					364
T865AA	51-mer + Ala subs	QARQLLSGIVQQNNLLRAIEAQHALQATVWGKQLQARILAVERYLKDQ	71	100	19323	3.2	81	32
T865 Δ AA	49-mer + Ala subs	QARQLLSGIVQQNNLLRAIEAQHALQATVWGKQLQARILAVERYLK	69	87	17560	3.1	80	105
N10 Δ T865AA	41-mer + Ala subs	QQQNNLLRAIEAQHALQATVWGKQLQARILAVERYLKDQ	50	76	14476	3.0	49	604
T1772	RSV	AVSKVLHLEGEVNVNKKISALLSTNKAVVSLNGSVLTSKVLDLKNYIDKQ	42 ^e	83 ^e	12897 ^e	2.4	--	>5000
T865RSV	RSV	QARQLVSGLVQQNNILRALEATQHL VQLLVWGVKQLQARVLALERYIK	>97	73	agg ^d	--	84/>97 ^h	686
T865RSV_AA	Core RSV	QARQLVSGLVQQNNILRALEATQHAVQALVWGVKQLQARVLALERYIK	92	78	18143	3.2	73/>97 ^h	25
	Core + Ala subs							
T649	HR2 peptide	WMEWDREINNYTSLIHSLEESQNQQEKNEQELLEL	<10	25	4525	1.0	--	1.3

^aThe N- and C-terminal of each peptide was acetylated and amidated, respectively.^b10 μ M HR1 + 10 μ M T649.^cN is equal to MW_{observed}/MW_{monomer}.^dAggregated.^eData from Lawless-Delmedico et al. (2000).^fMolecular weights and CD data for WT HR1 peptides from Dwyer et al. (2003).^hTwo transitions are observed (Dwyer et al. 2003).

Trimer formation in 51-mer HR1 peptides

Although the strategy of isoleucine substitution led to poor solubility in longer HR1 peptides, we felt that improved potency could be most easily achieved by increasing the peptide length. While investigating the oligomeric behavior of 51-mer HR1 peptides with alanine substitutions, several trimers were observed (J.J. Dwyer, K.L. Wilson, A. Hasan, J.E. Seedorff, and M.K. Delmedico, unpubl.). T865AA and T865 Δ AA are peptides with substitutions L565A and L568A, located at position “g” and “c,” respectively, in the heptad repeat. These residues are part of the network of amino acids that constitutes the deep pocket. This highly conserved pocket has been shown to be important for HR2 binding in the six-helix bundle structure (Chan et al. 1998), and we have previously identified this region as being critical for HR1 oligomerization (Dwyer et al. 2003). The two alanine substitutions result in a drop in thermal stability of 11°C–14°C (Table 1; Fig. 1), suggesting that the leucine residues are influencing HR1 stability, either by direct contacts or by altering oligomeric specificity. T865AA and T865 Δ AA were analyzed by analytical ultracentrifugation and were found to have molecular weights within 10% of a trimer. Figure 2 shows that T865 Δ AA

remains trimeric from 10 μ M to 500 μ M, while T865 is tetrameric up to 200 μ M, after which it begins to aggregate. Therefore, the Leu to Ala changes are promoting the formation of a soluble trimer. The loss of stability and the formation of trimers were unexpected, since there is no evidence from the crystal structure of the HR1/HR2 peptide complex (Chan et al. 1997) that these leucines

Figure 1. Thermal unfolding transition of HR1 oligomers as measured by circular dichroism. Measurements were made in phosphate buffer at a peptide concentration of 10 μ M for T865 Δ (●), T865 Δ AA (■), T865RSV (○), and T865RSV_AA (□).

Figure 2. Apparent molecular weight determined as a function of loading concentration for T865 (◆), T865ΔAA (■), and T865RSV_AA (□). Molecular weights were determined by fitting the data at each concentration to a single ideal species model and averaging the results from different rotor speeds and wavelengths. Errors in molecular weight determinations are <10%.

make contact with other amino acids on the trimeric HR1. These changes were introduced into N10ΔT865 (Table 1) and trimers were also observed, suggesting that the switch from tetramers to trimers is not specific to the longer peptides. However, N10ΔT865AA was 25°C less stable than N10ΔT865, suggesting that in the context of the shorter peptide, the oligomeric switch is more deleterious to stability.

RSV HR1 core residues do not direct trimer formation

An alternative strategy to design HR1 trimers is to replace the “a–d” core residues of T865Δ with the corresponding residues from the analogous HR1 peptide T1772 from respiratory syncytial virus (RSV) (Morrison 1988; Lawless-Delmedico et al. 2000), which has been shown previously to exist as a trimer (Lawless-Delmedico et al. 2000). Since the core residues are thought to be involved in directing oligomer formation in coiled coils, we speculated that HIV HR1 trimers could be designed by this strategy. The resulting peptide, T865RSV, exhibited greatly enhanced stability ($T_m > 97^\circ\text{C}$, Fig. 1) but was somewhat aggregated in the sedimentation analysis, much like the parent peptide, T865Δ (Table 1). Although a definitive molecular weight could not be obtained, the mass observed with the best fits at low concentrations were consistent with a tetrameric oligomer (data not shown). The increase in stability was unexpected since the RSV HR1, T1772, has a T_m of 42°C (Lawless-Delmedico et al. 2000), which is well below the T_m of T865 (82°C) and T865RSV (>97°C).

Engineering a stable trimer

The enhanced stability of T865RSV and the trimers observed with T865AA suggested that a combination of these two designs (T865RSV_AA) might produce a stable, trimeric HR1 oligomer. T865RSV_AA was found to be trimeric from 10 μM to 500 μM (Table 1; Fig. 2) and also preserved much of the enhanced stability of T865RSV (T_m of 92°C; Fig. 1). Although the oligomer is less stable than

T865RSV, some loss of stability was expected based on the 11°C difference observed between T865 and T865AA.

HR2 binding is reduced in the stabilized trimer

The alanine substitutions at positions 565 and 568 are located within the hydrophobic groove of the HR1 trimer, a location known to be important for HR2 binding. To determine the effect of these substitutions on the binding of an HR2 peptide, the thermal stability of the designed HR1 trimers in complex with the HR2 peptide T649 was determined at equal, 10 μM concentrations (Fig. 3). The stability of T865 and T865Δ was enhanced by 10°C upon addition of T649, indicating the formation of a stable bundle, and these bundles had similar thermal transitions (91°C and 93°C, respectively). T865AA/T649 was found to have a lower thermal stability than T865/T649, but was still 10°C more stable than the T865AA trimer, suggesting that the HR2 interaction is not significantly perturbed in the designed HR1 peptides. The thermal melt for T865RSV_AA/T649 exhibited two distinct transitions (Fig. 3) that can be revealed by analysis of the first derivative of the thermal unfolding transition. Previously, we have attributed one transition to the HR2 peptide dissociating from the HR1, and the other to the unfolding of the HR1 oligomer (Dwyer et al. 2003). The T865RSV_AA/T649 complex has an initial transition around 73°C and a second transition near the T_m of T865RSV_AA by itself (95°C). Although it is more difficult to quantify HR2 interactions with the stable oligomers, the observed T_m of 73°C is less than the 80°C–90°C seen in the T865/T649 and T865AA/T649 oligomers, and suggests that the HR2 peptides are binding less well.

To further assess the interaction between T649 and the designed trimers, kinetic measurements were made using surface plasmon resonance (SPR). Figure 4 shows the binding sensorgrams of T649 binding to immobilized T865ΔAA

Figure 3. Thermal unfolding transition of HR1/T649 complexes as measured by circular dichroism. Measurements were made in phosphate buffer at a peptide concentration of 10 μM plus 10 μM T649 for T865Δ (●), T865ΔAA (■), T865RSV (○), and T865RSV_AA (□).

Figure 4. Binding of HR2 peptide T649 to the trimeric T865 Δ AA as determined by surface plasmon resonance at 25°C in HBS-P buffer on a Biacore 3000 instrument. Biotinylated T865AA was immobilized onto the surface of a streptavidin-coated sensorchip (Sensorchip SA) at a level of $\sim$ 500 response units (RUs), and T649 was injected across the surface at a rate of 20 μ L/min at concentrations of 250 nM to 7.8 nM (*top* panel). The *bottom* panel shows the result of a global fit to the 7.8 nM data assuming a 1:1 (Langmuir) binding model. The measured data are denoted by the diamonds (for clarity, not every point is shown) and the fit is represented by the line. The results of the global fit agree well with the average of the individual fits at low concentrations but global analysis failed at higher concentrations, presumably due to mass transport limitations. Kinetic parameters are listed in Table 2.

and the kinetic data is summarized in Table 2. T649 binding to T865 Δ AA was less than twofold worse relative to T865, suggesting the alanine substitutions do not have a significant impact on HR2 binding. In contrast, T865RSV_AA (Table 2) has a sixfold reduction in T649 affinity compared to T865AA, suggesting that the stabilization afforded by the RSV core is somewhat more detrimental to HR2 binding than the alanine substitutions alone. These experiments were repeated at a flow rate of 100 μ L/min and the observed on-rates and K_d values were within threefold of those reported in Table 2 (not shown). Therefore, the SPR data generally supports the trend observed in the thermal unfolding transitions of the HR1/HR2 complexes.

Trimeric HR1 peptides have enhanced potency

Peptides from the HR1 and HR2 regions of gp41 have been previously shown to possess antiviral activity (Wild et al. 1993; Judice et al. 1997; Eckert and Kim 2001; Louis et al. 2001; Bewley et al. 2002), with HR2 peptides typically being more potent (Wild et al. 1992; Jiang et al.

1993). Figure 5 shows the antiviral activity of the engineered trimers compared to wild-type HR1 peptides. The antiviral activity of the (tetrameric) peptide T865 is 536 nM and other tetrameric peptides of different lengths and stabilities are also in this range (Table 1). The trimeric T865 Δ AA is ninefold more potent than T865 Δ despite the fact that the oligomer is 14°C less stable and T649 is shown to have a threefold lower affinity to this HR1 (Fig. 4; Table 2). T865AA has an IC_{50} of 32 nM, over 15-fold better than T865, despite an 11°C drop in stability.

Replacement of the core “a” and “d” residues of T865 Δ with those from RSV has little influence on activity (686 nM) despite the enhanced stability. In contrast, the potency was found to improve nearly 30-fold (to 25 nM) when the leucine to alanine changes are included, resulting in the trimeric T865RSV_AA. The CD data and SPR results indicate that T649 binds less well to T865RSV_AA than to wild-type HR1 peptides; therefore, the enhancement in potency is likely related to the formation of the trimeric oligomer.

In vitro passaging generates HR1-resistant virus

To further characterize the mechanism of action of the stabilized HR1 trimers, we created HR1-resistant virus through *in vitro* passaging. Resistant virus were selected by co-culturing the virus in the presence of escalating concentrations of peptide. We used N13 Δ Trimer for these experiments since it possessed better activity against 060 and 098 than T865RSV_AA. After 6–10 passages, the virus was able to replicate in the presence of 50 μ g/mL of N13 Δ Trimer, suggesting that the virus was resistant to the peptide. The virus was harvested after each dose escalation, and several of these time points were sequenced through gp41 to look for changes associated with the development of resistance. Table 3 shows the sequencing results from viruses harvested after the 50 μ g/mL passage. In contrast to ENF, in which resistance mutations tend to occur in a localized region of HR1 (Greenberg and Cammack 2004), for N13 Δ Trimer the mutations occur in the HR2 region. In IIIB, a single H643Y change conferred over 10-fold resistance to N13 Δ Trimer. For the primary isolates 060 and 098, more than one mutation was required to alter susceptibility by more than 10-fold. We also tested these HR1-resistant viruses for their susceptibility to ENF (Table 3). The potency of ENF against the IIIB-N13 Δ Trimer resistant virus was reduced by about threefold. Interestingly,

Table 2. Binding of HR2 peptides to trimeric HR1 peptides

Peptide ^a	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (nM)
T865	12.0×10^5	1.2×10^{-3}	2.1 (0.81)
T865 Δ AA	6.1×10^5	1.9×10^{-3}	3.2 (1.5)
T865RSV_AA	14.8×10^5	0.13×10^{-3}	12.3 (0.21)

Figure 5. HIV antiviral activity of HR1 peptides determined using a MAGI assay. Percent inhibition is determined as a function of peptide concentration for T865 (◆), T865AA (◇), T865Δ (●), T865ΔAA (■), T865RSV_AA (□), and T649 (×).

the potency of ENF against the HR1-resistant primary isolates was enhanced three- to fivefold. Additional mutagenesis will be required to confirm that these mutations uniquely confer HR1 resistance.

Structure determination of the engineered trimer

To examine the structural aspects of the engineered trimer, we determined the crystal structure of the 49-residue peptide, T865RSV_AA. Extensive efforts to crystallize T865AA and T865ΔAA have yet to yield suitable crystals. Details of the data collection and refinement are given in Table 4. The final model consists of residues Val 545 through Lys 588 (Fig. 6) and the helix begins at residue Leu 548. The observed disorder in the first eight residues of the peptide is consistent with our earlier conclusion that the amino-terminal end of isolated HR1 peptides does not form a coiled coil (Dwyer et al. 2003). The most conspicuous feature of the T865RSV_AA structure is the bend observed in the middle of the α -helix (Fig. 7). The $\sim 10^\circ$ increase in the bend of the helix causes the main chain on the N-terminal end of the helix to differ significantly from that of the trimer in the HR1/HR2 complex (Chan et al. 1997). It is unclear whether this change is a consequence of the enhanced stability of this oligomer or a result of crystal packing; however, bends in other coiled-coil structures have been hypothe-

sized to be important for function (Brown et al. 2001). The distortion of the HR1 groove may also explain why HR2 peptides bind less well to T865RSV_AA.

The hydrophobic pocket is similar in bound and unbound HR1. Figure 7 shows the superposition of the N-terminal end and the hydrophobic pocket of T865RSV_AA with the corresponding regions of the HR1 trimer from the six-helix bundle (Chan et al. 1997). Local alignment using Trp-571 suggests that the presentation of side chains and the geometry of the contact region around the hydrophobic pocket remains remarkably similar in the absence of HR2 peptides. This is consistent with the CD and SPR data, and suggests that HR2 binding to this region of the trimer is likely achieved by a similar set of interactions as wild-type peptides.

In contrast to the pocket region, the backbone on the amino-terminal end of the HR1 is somewhat different in the present structure compared to the HR1/HR2 complex. The difficulty in superposition is due to the bend observed in the α -helix; however, Figure 7 also shows that there are differences in side chain geometry as well. This suggests that the N-terminal end of the peptide may undergo some structural rearrangement upon HR2 binding. This notion is supported by the lack of sensitivity to the truncation of the N-terminal side of HR1 peptides (Dwyer et al. 2003), which suggests that coiled-coil formation in this region is not favored in the absence of HR2 peptides. The dissimilarity in the structure of the N-terminal end of T865RSV_AA may arise from forcing this region into a nonoptimal coiled-coil conformation upon stabilization by the RSV core.

The structure of N13ΔTrimer was also determined to better than 2 Å resolution (not shown) and confirmed the formation of a trimer. The deep pocket region is very similar to that observed with T865RSV_AA; however, the absence of the amino-terminal end of the peptide does not permit comparison with WT or T865RSV_AA.

Discussion

The difference in potency between peptides from the two HR domains is thought to be due to the presence of a

Table 3. Sequence changes observed during *in vitro* passaging with N13ΔTrimer

Virus	HR2 sequence in virus	IC ₅₀ (μg/mL)	
		N13ΔTrimer	ENF
IIIB	WMEWDREINNYTSLIHSLIEESQNQQEKNEQELLEL	0.859	0.006
IIIB-N13ΔTri	Y	8.724	0.020
060	WDNMTWMQWEREIDNYTGIIYNLIEESQNQQEKNELELLEL	0.020	0.021
060-N13ΔTri	N E K	0.325	0.006
098	WDNMTWMQWEREIDNYTGIIYTLIEESQIQEKNEKELLEL	0.052	0.050
098-N13ΔTri	N E/K D/G	10.69	0.011

Table 4. Crystallographic data and refinement statistics

Crystallographic data		
Space group	<i>R</i> 3	
Unit cell (Å)	<i>a</i> = 103.291, <i>c</i> = 44.494	<i>a</i> = 103.569, <i>c</i> = 44.895
Data set	Native	LiBr soak
Resolution (Å)	100.0–1.5	20.0–1.96
Observations used in scaling	134,374	141,790
Unique Reflections	27,085	24,637
Mean (<i>I</i> / σ <i>I</i>) overall	43.97	48.3
Highest ~ 0.1 Å shell	1.54	5.7
Completeness (%)	95.5	99.6
Highest ~ 0.1 Å shell	68.6	97.2
Multiplicity	5.0	5.8
Mosaicity (°) Wilson B factor (Å ²)	0.56 24.9	0.30 23.9
<i>R</i> _{sym}	0.046	0.071
Highest resolution shell	0.312	0.306
Refinement statistics		
Average B factor of model (Å ²)		
Protein (# of atoms)		24.5 (1046)
Main chain		21.4 (528)
Side chains		29.6 (518)
Waters		40.7 (185)
<u>RMS deviations of the model</u>		
Bonds (Å)		0.015
Angles (°)		0.9
Torsional angles (°)		
Planar		2.4
Staggered		13.6
Transverse		18.0
Planarity (Å)		
Peptide		0.013
Aromatic		0.013
<i>B</i> factors (Å ²)		
Main chain		3.1
Side chain		5.5
ESU of atom position based on ML (Å)		0.06
<i>R</i> _{factor}	20.0–2.0 Å	0.221
	2.04–2.00 Å (9021 reflections)	0.537
<i>R</i> _{free} (5%)	20.0–2.0 Å	0.266
	2.04–2.00 Å (454 reflections)	0.522

pre-formed HR1 oligomer on the virus, which becomes the target for HR2 peptides (Chen et al. 1995; Lu et al. 1995; Rimsky et al. 1998). Since the HR2 region is thought to adopt a helical conformation in the transition to the fusion-active state, antiviral activity of HR1 peptides could be limited by the requirement for the formation of an oligomer. Therefore, increasing the stability of the oligomer might be expected to enhance the potency of the HR1 peptides (Eckert and Kim 2001; Louis et al. 2003; Bianchi et al. 2005). However, we have found that HR1 potency is not strictly dependent on oligomer stability, but can be improved through the formation of a trimer. The 11°C–14°C loss of stability in the trimeric HR1 oligomers may simply reflect that the trimer is intrinsically less stable than the tetramer; however, it does not support the notion that a stabilized HR1 oligomer is an absolute requirement for improved activity. Instead, it

appears that the presentation of an HR1 trimer can also contribute to enhanced activity. Since a trimeric HR1 is observed in the six-helix bundle, the HR2 interaction with this oligomer would not require a “switch” from the tetrameric state and so it makes intuitive sense that this oligomer might have better activity. However, the observed decrease in oligomer stability and modest reduction in HR2 affinity makes the improved activity of these peptides remarkable. Efforts to optimize T649 binding with these constructs are currently underway to more clearly define the limits of potency for HR1 peptides.

Eckert and Kim (2001) presented an elegant study on the stability and activity of HR1 peptides fused to GCN4 variants. In that work, stabilization of short HR1 peptides by trimeric GCN4 variants improved activity by more than 100-fold. Since we have shown previously that isolated HR1 peptides are tetramers (Lawless et al.

Figure 6. Ribbon diagram of the T865RSV_AA trimer. Residues 540–545 are not visible, and residues 546–549 are not part of the helical structure.

1996; Dwyer et al. 2003), the improvement in activity may be related to the trimeric specificity afforded by GCN4 rather than by stabilization, per se. In the three trimers with L565A and L568A substitutions, decreasing HR1 stability does result in less antiviral activity; however, the stabilized T865RSV_AA trimer was no more potent. T865RSV was the most stable oligomer, yet the activity was nearly identical to T865. Since the peptides described in the present work do not require a trimeric fusion protein, further studies with these designs may help to clarify the relationship between activity, stability, and oligomeric specificity of HR1 peptides.

HR1 peptides have been proposed to act by either binding to the viral HR2 region (Chan and Kim 1998) or by interfering with the oligomeric HR1 domain (Weng and Weiss 1998; Bewley et al. 2002). In vitro passaging of the thermostable HR1 peptides presented here result in resistant virus with mutations appearing in the HR2 domain that are associated with decreased antiviral potency. This data supports the notion that the designed peptides are blocking fusion by binding to the HR2 region. It is likely that any potential contribution to activity by interactions with the viral HR1 is eliminated by the high thermostability of the HR1 trimer, which would preclude the formation of hetero-oligomers between monomeric HR1 peptides and the viral HR1 domain.

The effect of the N13ΔTrimer resistance mutations on ENF activity in the 060 and 098 isolates is intriguing. The observed resistance mutations will need to be engineered back into a fixed genetic background to further clarify the impact on HR2 activity. However, we speculate that the HR1 resistance mutations may be altering the accessibility of the HR1 region in gp41 or changing the kinetics of viral fusion to permit better activity of ENF. It will be of great interest to study how HR1 resistance mutations affect the ability of HR2 resistant virus to overcome fusion inhibition. It may be that passaging experiments done in the presence of suitably engineered HR1 and HR2 peptides (so as not to form a six-helix bundle) will show that the virus has great difficulty overcoming both fusion

inhibitors simultaneously. Moreover, if the HR1 resistance mutations are altering the accessibility or kinetics of gp41, there could also be a significant impact on the activity of neutralizing antibodies, such as 2F5 or 4E10.

T865RSV_AA has a T_m that is greater than either T865Δ or T1772. The non-additivity observed in the thermal stability of the engineered oligomer suggests that both HIV and RSV HR1 regions have structural determinants that modulate their stability. Polar residues located in the “a” and “d” positions of coiled coils have been speculated to play a functional role in modulating stability, oligomeric specificity, or both (Liu et al. 2001; Lu et al. 2001). The increase in stability upon substitution of core residues from an oligomer with a T_m of 42°C was therefore unexpected. It is possible that destabilizing interactions that are present in the HIV or RSV oligomer are relieved in the designed trimer. Studies are underway to determine if the increase in stability is due to differences in core packing or if there are contributions from noncore residues.

We have speculated that the N-terminal side of the HR1 region in gp41 may be unstructured in the virus and only adopts a helical structure upon binding of the HR2 region, while the hydrophobic pocket may be more important for HR1 oligomerization (Dwyer et al. 2003). The similarity of side chain geometry in the hydrophobic pocket suggests that there is no large structural rearrangement in this area upon HR2 binding. Therefore, the reduction in HR2 binding to T865RSV_AA and the bend observed in the helix backbone may be highlighting that HR2-induced coiled-coil formation is distinct from artificially stabilized HR1 oligomers. In the context of the virus, interactions between the HR1 region and gp120, the cellular membrane, or other proteins may influence the conformation of the

Figure 7. Superposition of T865RSV_AA and N34 from the structure of the HR1/HR2 complex (Chan et al. 1997). Arg-557 was used for the alignment of the N-terminal end of the peptides and Trp-571 was used to align the deep pocket region. For clarity, not all side chains are shown. The $\sim 10^\circ$ bend observed in the helix of T865RSV_AA is also highlighted. The angular bend is an estimate based on the angle between line segments through the helical axis of the N- and C-terminal halves of the peptide. The alignment and the figure were done with the program PyMOL (DeLano Scientific).

HR1 prior to bundle formation. However, the general similarity in side chain geometry, particularly in the deep pocket region, suggests that the trimer may represent a good target for high-throughput screening, antibody development, or computational design of small molecule fusion inhibitors. The designs reported here also exhibit excellent antiviral activity and further optimization of HR1 peptides could lead to the development of novel fusion inhibitors.

Materials and Methods

Preparation of peptide samples

Peptides were synthesized as previously reported (Lawless et al. 1996). Lyophilized peptide samples were resuspended in dH₂O to an initial concentration of 1–2 mg/mL. Concentrations of stock solutions were determined using the method of Edelhoch (1967).

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were obtained using an AVIV Associates 62DS spectrometer as described (Dwyer et al. 2003). The reported T_m value is the temperature corresponding to the maximum value of the first derivative of the thermal transition (Dwyer et al. 2003). Helicity and T_m values were made at a sample concentration of 10 μ M (individual) or 10 + 10 μ M (HR1/HR2 mixtures) in 50 mM KH₂PO₄, 100 mM KCl pH 7.0.

Analytical ultracentrifugation

The sedimentation equilibrium experiments were performed on a Beckman Optima XL-A analytical ultracentrifuge at 4°C as described (Dwyer et al. 2003). For reported molecular weights, there was no significant decrease in absorbance due to the sedimentation of very high molecular weight aggregates. Peptide samples were diluted using 50 mM KH₂PO₄, 100 mM KCl pH 7.0 to final concentrations ranging from 1 μ M to 500 μ M. Data were collected at 18,000 and 22,000 rpm at wavelengths of 235 nm, 240 nm, and 280 nm.

Weight-averaged molecular weights were obtained by fitting each data file individually using a single ideal species model in the Beckman-Origin software (version 3.78 for Windows). The solvent density of the phosphate buffer ($\rho = 1.00894$) and partial specific volumes of peptides were calculated using the program SEDNTERP (Laue et al. 1992). The reported molecular weight is the value obtained for a 100- μ M sample. Poor fits to the single ideal species model (systematic residuals) were considered a diagnostic for aggregation, and no attempt was made to deconvolute multiple species.

Surface plasmon resonance

Kinetic analysis of HR2 binding to engineered HR1 targets was done on a Biacore 3000 instrument at 25°C (Karlsson and Falt 1997). Biotinylated HR1 peptides were diluted to ~50 μ g/mL and injected onto a streptavidin-coated carboxymethyl dextran sensor chip (Sensor Chip SA, Biacore, Inc.) to a surface density of about 500 RU. Surface densities significantly lower than 500 RU reduced signal quality to unacceptable levels. This may

reflect the need for a critical surface concentration of HR1 to allow oligomerization in the presence of HR2 peptides. In addition, it is presumed that monomeric HR1 peptides are immobilized using this procedure and so the oligomeric state of the HR1 on the surface is unclear. T649 samples were diluted to 250 nM in HEPES-buffered saline with 0.005% surfactant P20 added (HBS-P, Biacore, Inc.) and serially diluted (1:2) to 7.8 nM. Samples were injected at a flow rate of 20 μ L/min or 100 μ L/min for 180 sec followed by a 360-sec dissociation phase.

Kinetic association and dissociation rate constants were determined separately by fitting the Langmuir 1:1 binding model to the family of curves and averaging the obtained rates (Karlsson and Falt 1997). The dissociation rates were obtained by fitting the first 100 sec of the dissociation phase and the association rates were fit using approximately the first 50 sec of the association phase. Global fits at lower HR2 concentrations produced similar rates. Errors in the association rates were as high as 25% due to higher sample concentrations and differed by two- to threefold at higher flow rates. Errors in dissociation rates were <10%.

Crystallization and data collection

T865RSV_AA was resuspended in dH₂O to a concentration of 18 mg/mL and crystallized at room temperature using Crystal Screen Lite from Hampton Research. Diffraction quality crystals were grown directly and reproducibly out of condition #40 (10% v/v iso-propanol, 0.1 M Na Citrate pH 5.6, 10% w/v PEG 4000). Crystals were flash-frozen with 17% ethylene glycol as cryoprotectant. Diffraction data were collected from a native T865RSV_AA crystal with Cu K α radiation using an R-AXIS IV++ detector mounted on a Rigaku RU-H3R rotating anode X-ray generator. All data were integrated and reduced to structure factors with HKL2000 (Otwinowski and Minor 1997) and Truncate (French and Wilson 1978). The crystals were determined to be in space group R3, $a = 103.29$ Å, $c = 44.90$ Å. Attempts to solve the structure by molecular replacement were unsuccessful, so native crystals were soaked in cryoprotectant solution containing LiBr (Dauter et al. 2000) (500 mM LiBr for 12 sec), and phases were determined from single-wavelength anomalous dispersion differences. Diffraction data from bromide-derivatized crystals were collected at the COM-CAT beamline (Advanced Photon Source, Argonne National Laboratory) at an incident radiation wavelength of 0.92 Å (K absorption edge of bromine) to maximize the bromide anomalous signal. Anomalous difference Patterson maps revealed Harker peaks for at least five highly occupied bromide ions in the asymmetric unit. Coordinates for potential bromide sites were located by direct methods with RANTAN (Yao 1981) and refined to 1.96 Å with MLPhaRe (Collaborative Computational Project, Number 4 1994). Six highly occupied sites, chosen based upon a sharp discontinuity in the distribution of B factors and occupancies, were then used to calculate improved phases by direct methods with OASIS (Hao et al. 2000). These phases were used as the starting point for automated model building and refinement against the 1.5 Å native data using ARP/warp (Perrakis et al. 1999) that resulted in a nearly complete model. The final coordinates were produced with multiple rounds of model building using XtalView (McRee 1999) and refinement against the native T865RSV_AA data set using REFMAC (Murshudov et al. 1997).

Antiviral activity

Antiviral activity was measured using a cMAGI infectivity assay (Kimpton and Emerman 1992; Chackerian et al. 1997; Hu et al.

2000). Peptides were resuspended at ~ 1 mg/mL in dH₂O and actual concentrations were determined by the method of Edelhoch (Edelhoch 1967). The day prior to infection, 2×10^4 cells were plated in 0.2 mL of experimental media (DMEM, 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL Streptomycin, 300 μ g/mL L-glutamine, and 20 μ g/mL DEAE-Dextran) into 48-well plates. Serial dilutions of peptides were made in experimental media to final concentrations used in the assay and added to the plates along with a predetermined virus dilution that would yield 1000 to 2000 infectious centers. To ensure that secondary rounds of infection by progeny virus do not complicate the analysis of inhibitors of virus entry, ENF was added at high concentrations to all experimental wells 18–24 h post-primary infection. Seventy-two hours post-infection, cells were fixed with formaldehyde and glutaraldehyde and stained with X-gal (5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside). Infected nuclei were counted using a charge-coupled device imager. Wells containing virus and no inhibitor represent 100% infection. One-half these values represent the cutoff for estimating the concentration of peptide, which reduces infectious virus titer by 50% (IC₅₀). Errors in IC₅₀ determination are typically within twofold.

In vitro passaging

CEM4 or MT2 cells were infected with the laboratory isolate IIIB and clinical isolates 060 and 098 and cultured in the presence of increasing concentrations of HR1 peptide (beginning at approximately twice the IC₅₀) to select for resistance. When in vitro selections reached 50 μ g/mL, virus stocks were expanded in the presence of 50 μ g/mL of peptide and peptide-free harvests were collected. Virus stocks were characterized for gp41 genotypic changes by dideoxy sequencing chemistries using a Beckman Coulter CEQ 2000XL system and DNASTar software. Phenotypic susceptibility was determined using a cMAGI infectivity assay (Hu et al. 2000).

Data deposition

The coordinates of the T865RSV_AA have been deposited in the Protein Data Bank (accession no. 2ZFC).

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