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Critical Role of the N-Loop and β 1-Strand Hydrophobic Clusters of RANTES-Derived Peptides in Anti-HIV Activity

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Abstract

HIV initiates its infectious cycle by docking to CD4 and a chemokine receptor, most commonly CCR5. RANTES, a natural CCR5 ligand, is a potent inhibitor of HIV-1. Despite the lack of structural information on the RANTES-CCR5 complex, determinants of HIV blockade were previously identified within the RANTES N-loop and β 1-strand regions. A prototype N-loop/ β 1-strand peptide, named R11-29, contains two terminal hydrophobic stretches separated by a central hydrophilic region. Here, the role of the terminal hydrophobic clusters was investigated by means of amino acid substitutions or deletions. Most hydrophobic residues in these clusters were shown to be fundamental for the anti-HIV activity. However, increasing the hydrophobicity of the two clusters using non-natural amino acids did not significantly improve the potency of the peptides. These results may provide instrumental knowledge for the rational design of RANTES-derivative molecules with increased anti-HIV activity.

Keywords

RANTES; HIV; CCR5; biologically active peptides

Introduction

Human immunodeficiency virus (HIV) enters target cells following the binding of its envelope to a cell-surface receptor complex *via* a sequence of molecular events involving stepwise conformational changes on both membrane sides [1]. HIV-1 gp120 trimers dock to the N-terminal domain of the primary viral receptor, CD4, undergoing a profound conformational change whose details have been evinced from the structure of CD4-bound HIV-1 gp120 and unbound SIV gp120 [2,3]. Consequently, gp120 exposes the binding site for the coreceptor, CCR5 or CXCR4 [1]. Finally, virus entry information is transmitted through the virus envelope protein to the gp41 trimer moiety that undergoes a series of conformational changes eventually leading to type I virus-cell membrane fusion [4]. Considerable interest is focused on this

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intricate series of molecular events in an attempt to develop novel therapeutic and preventive measures. A prototype HIV-entry inhibitor, T20, a synthetic peptide mimicking a portion of gp41, has shown promising results in clinical studies [5]. Strikingly, the case of T20 has also provided the strongest proof-of-principle that peptide drugs may be powerful tools in antiviral therapy.

Presently, a wealth of research is aimed at the development of inhibitors capable of blocking HIV entry by targeting the coreceptor CCR5 [6,7]. Despite their excellent anti-HIV activity as CCR5 antagonists, several small-molecule compounds revealed deleterious side effects and have been prematurely withdrawn from clinical trials [1]. RANTES, a CC-chemokine that binds CCR5, is a potent natural anti-HIV factor [8] and therefore may be an ideal candidate to derive anti-HIV peptides [1]. Biologically active peptides have indeed been obtained from the RANTES N-loop and β 1-strand regions [9]. Remarkably, these peptides showed a dramatic increase in activity upon homodimerization, suggesting a favorable effect related to the adoption of a more stable conformation or, alternatively, to avidity increase [9]. To set the basis for further improvement of RANTES-derived peptide design, we investigated the importance of the hydrophobic residues clustering at the N and C termini of peptides encompassing RANTES N-loop and β 1-strand regions.

Materials and Methods

Peptide synthesis

Peptides were synthesized by standard solid-phase Fmoc chemistry [10] using an automated peptide synthesizer ABI433 (Applied Biosystems). Peptides N and C termini were acetylated and amidated, respectively. After assembly, peptides were deprotected using reagent K [11], purified by RP-HPLC to >95% purity and freeze dried. For stable dimerization, the peptides were incubated overnight in 50% DMSO (Sigma) aqueous solution. Dimerized peptides were purified to homogeneity by RP-HPLC (>95% dimers). The Ellman [12] test for free sulphydryl groups was negative. Peptide masses were confirmed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry on a Voyager-RP Biospectrometry Workstation (PE Biosystems).

Circular dichroism analysis

CD spectra were recorded at 22 °C on a Jasco J-810 spectropolarimeter (Jasco) using a 1 mm path-length quartz cuvette at a peptide concentration of 0.1 mg/ml. Final spectra resulted from averaging 3 scans and were corrected by subtracting the corresponding base line spectra obtained under identical conditions. Results were expressed as mean residue ellipticity ($[\Theta]$) at a given wavelength.

HIV-1 Env-mediated cell fusion assay

The cell fusion assay was performed as described [13] using as effectors PM1 cells chronically infected with HIV-1 BaL or HeLa cells infected with a recombinant vaccinia virus encoding the homologous Env. As targets, NIH3T3 mouse fibroblast cells stably expressing human CCR5 and CD4 were used. Twelve hours before the test, effector cells were infected with a vaccinia vector expressing bacteriophage T7 RNA polymerase, while target cells were infected with a vaccinia vector expressing the *lacZ* reporter gene under the control of the T7 promoter [13]. All vaccinia virus infections were performed in DMEM medium supplemented with 2.5% FBS. The cells were then washed with DMEM 2.5% FBS. Target cells were pre-incubated with the peptides for 20 minutes at 37 °C and subsequently mixed for 2 hours with effector cells in presence or absence of inhibitors. Cell fusion was determined by measurement of β -galactosidase activity in nonionic detergent cell lysates as described [13].

Results

Structural features of the prototypic peptide R11-29

Analysis of the 3D structure of RANTES provides relevant information for the family of RANTES-derived peptides spanning the N-loop and β 1-strand. In the prototypic peptide R11-29 there are two clusters of hydrophobic residues, many of which aromatic, positioned at the peptide termini (residues 11–15 and 27–29, according to full-length RANTES numbering). These clusters are connected by a stretch of hydrophilic residues (Fig. 1). Within the folded full-length RANTES dimer, the side chains of the hydrophobic residues are solvent-exposed, forming two hydrophobic patches (Fig. 1).

To evaluate the secondary structure content of R11-29, we performed circular dichroism (CD) analysis. The disulphide-bonded R11-29 dimer reveals a random coil conformation in aqueous solution at physiological pH (Fig. 2, spectrum 1). However, in the presence of 2 mM SDS there is an increase in β content (spectrum 2). Moreover, after raising the SDS concentration to 10 and 25 mM (over the SDS critical micellar concentration), the peptide presents mixed α - β features (spectra 3 and 4).

Role of the N-terminal hydrophobic patch in the anti-HIV activity of R11-29

The role of the N-terminal hydrophobic patch of R11-29 was investigated by single alanine substitutions and by testing mutated peptides in HIV-1 envelope-mediated cell fusion assays against the CCR5-dependent isolate HIV-1 BaL. In line with previous results [9], dimeric, N-terminally acetylated and C-terminally amidated peptides were produced and studied. As illustrated in Fig. 3A, a significant loss of function was observed following alanine substitution of F12, Y14 and I15. Similar effects were previously reported with analogous substitutions in full-length RANTES, with the F12 substitution leading to the most dramatic drop of activity [9]. Targeted deletions of F12 and I15 were also performed. Both deletions led to a dramatic decrease in anti-HIV activity, similar to that observed by the respective alanine substitutions (Fig. 3B).

Altogether, these results confirmed the critical role played by residues F12, Y14 and I15 in this region of RANTES and provided evidence of the functional preservation of this region when shifting from native full-length RANTES to a short derivative synthetic peptide. In agreement with this hypothesis, preliminary structural data for this family of RANTES peptides show a structurally defined N-terminal portion (unpublished data).

Role of the C-terminal hydrophobic patch in the anti-HIV activity of R11-29

As for the N-terminal patch, the role of the C-terminal hydrophobic patch of R11-29 was investigated by alanine scanning analysis. Single or multiple alanine substitutions within this region resulted in a dramatic impairment of R11-29 anti-HIV activity (Fig. 3C). Of note, the Y27A substitution caused the least dramatic (~50%) loss of function. Conversely, the F28A mutation led to 80% reduction in R11-29 activity. Moreover, simultaneous mutation of F28-Y29 and Y27-F28-Y29 to alanine resulted in 80–90% activity reduction, confirming F28 as the major functional residue within the R11-29 C-terminus.

Effect of an increased hydrophobicity within the N-loop and β 1-strand clusters

Given their critical role in anti-HIV activity, we evaluated the functional consequences of substituting F12 and I15 in the N-terminal patch and F28 and Y29 in the C-terminal patch with non-natural amino acids of higher hydrophobicity. These substitutions were introduced into R11-29 homologues bearing single or double proline substitutions at positions 13 and 22, which will be described in detail elsewhere (unpublished data). Substitution of F12 with 3-(1-naphthyl)-alanine (1Nal), 3-(2-naphthyl)-alanine (2Nal) or β -cyclohexylalanine (Cha)

determined a slight decrease in peptide activity, indicating that the original phenylalanine is preferable for this position (Fig. 4A). Strikingly, substitution of I15 with α -cyclohexylglycine (Chg) resulted in a dramatic drop of activity (Fig. 4A), confirming the importance of an isoleucine for this position and suggesting that hydrophobicity is not the only feature required for an optimal CCR5 interaction.

Divergent results were obtained with non-natural amino acids at the C-terminal hydrophobic cluster. Replacement of F28 with 1Nal, 2Nal or Cha led to peptides with similar or even slightly higher activity, suggesting that phenylalanine in this position might be replaced by different moieties (Fig. 4B). Conversely, substitution of Y29 with 1Nal, 2Nal or Cha resulted in a marked decrease in anti-HIV activity, pointing to tyrosine as a key residue for this position (Fig. 4B).

Discussion

Growing interest is currently focused on the development of safe and effective HIV-entry inhibitors targeting CCR5. This strategy has a rationale for both systemic administration and local microbicide delivery at mucosal sites. However, the lack of information on the three-dimensional structure of CCR5 and, as a consequence, on the binding interface between RANTES and CCR5, limits the rational design of effective inhibitors of this interaction. Small chemical CCR5-targeted HIV inhibitors have been developed but several were withdrawn from clinical trials because of cardiac and hepatic toxicity, most likely due to crossreactivity with structurally related receptors in these organs. Yet, the field is highly active in the search for potent CCR5-targeted HIV inhibitors. Receptor antagonists would be preferable as they should not induce inflammatory side-effects. We have previously reported the generation of short synthetic RANTES-derived peptides that act as antagonists of CCR5, providing evidence for the N-loop and β 1-strand regions as the RANTES core involved in competition with HIV gp120 binding to CCR5 [9]. At the same time, several studies highlighted the prominent role of the RANTES N-terminus in inducing CCR5 activation and internalization [14]. Thus, a 2-site interaction can be postulated between RANTES and CCR5, in agreement with previous models of ligand interaction with 7-transmembrane-domain, G-protein-coupled receptors [15]. The first site, corresponding to the N-loop- β 1-strand regions, would be responsible for CCR5 binding, while the second site, mapped to the N-terminus, would induce receptor triggering. RANTES derivatives bearing hydrophobic chemical groups at the N-terminus, such as PSC-RANTES, show higher anti-HIV activity than the wild type molecule but this effect is mostly resulting from downmodulation of cell surface CCR5 rather than from binding competition. Conversely, another N-terminally modified mutant, C1C5-RANTES, as well as short synthetic peptides derived from the RANTES N-loop- β 1-strand regions, exhibit an impaired capability to induce CCR5 internalization [9,16]. In these cases, the RANTES N-terminus was either forced in a non-optimal conformation for CCR5 activation (C1C5-RANTES) or not present at all (R11-29), implying that HIV inhibition resulted from a direct competition with gp120. The remarkable increase in anti-HIV activity observed in homodimeric R11-29 may reflect a cooperative effect provided by tertiary interactions stabilizing its fold or, alternatively, suggest an important role played by avidity. Three-dimensional structure determination of RANTES-derived peptides in monomeric and dimeric conformations should help in shedding light towards the understanding of this issue.

Far-UV CD analysis provided evidence that peptide R11-29 possesses a certain degree of secondary structure, although in aqueous solution the peptide appears to be random coil. It is possible that RANTES-derived peptides assume a defined secondary structure upon binding to CCR5 in an induced-fit mechanism; however, their structural features are likely to become more stable upon development of peptides with higher activity. Clearly, these results are only indicative, and more refined structural data are emerging that precisely define the secondary and tertiary structure of RANTES-derived peptides (unpublished data).

To investigate the contribution of residues within RANTES N-loop and β 1-strand, a series of analogues of R11-29 were produced. Residues in the N and C terminal hydrophobic patches were systematically mutated to alanine, deleted or substituted with non-natural amino acids. The N-terminal portion evidenced a key role of F12, Y14 and I15, in line with a similar study on full-length RANTES [9]. In full-length RANTES, F12 was identified as the predominant functional residue, with Y14 and I15 contributing significantly less to the blockade of HIV entry. These results may indicate a direct interaction of RANTES F12 with CCR5, in agreement with the observation that, in the three-dimensional structure of RANTES, F12 is solvent-exposed and thus available for interaction with a hydrophobic cavity on CCR5. Concerning Y14 and I15, the differences in anti-HIV activity within the context of full-length RANTES *versus* R11-29 may result from a better structural stability of R11-29 with the original residues.

Next, the R11-29 C-terminal patch was investigated, showing that residues in this region are of major relevance for the mechanism of action of the peptide. Within the motif Y27-F28-Y29, Y27 appears to contribute only marginally, since its alanine substitution decreased R11-29 activity to 50%. Conversely, substitution of F28, F28-Y29 and Y27-F28-Y29 induced a marked reduction of activity (80-90%), implying a major contribution of F28 to the interaction with CCR5. Again, a role of F28 in the stabilization of R11-29 could also be possible, as well as a dual effect resulting in a synergic R11-29 stabilization-CCR5 interaction. Interestingly, increase of activity by substitution of F28 with non-natural hydrophobic amino acids suggests that improvement at this site is possible.

Overall, the present data attest to the importance of the hydrophobic N and C termini in RANTES-derived peptides spanning the N-loop and β 1-strand, and provide a platform for further improvement of the R11-29 prototype. Currently, the central hydrophilic region of this peptide is being probed by substantial modifications in order to verify its role, suggested herein, as a linker of the two CCR5-interacting hydrophobic patches. This linker is most likely a rather flexible region in R11-29, while both termini might be capable of assuming discrete conformational states, providing the necessary scaffold to exhibit anti-HIV activity and the reported capability to inhibit RANTES chemotaxis [9]. Stabilization of this flexibility, coupled with structure-guided refinement of the CCR5-interactive sites of R11-29, may lead to the design of more effective RANTES-derived peptides for potential use in HIV therapy or prevention.

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Fig. 1.

N-loop and β 1-strand hydrophobic clusters in the RANTES-derived peptide R11-29 and RANTES 3D structure. The disulphide-bonded dimeric R11-29 sequence is represented on the top. N- and C-terminal hydrophobic stretches are underlined in yellow and red, respectively. Shown below is a surface representation of RANTES dimer 3D structure (PDB entry 1HRJ). The two hydrophobic patches (aa 11-16 and 27-29) are indicated on the surface of the RANTES dimer in yellow and red, respectively. RANTES monomers are represented in two different shades of blue. RANTES N-termini are in blue, while, in this orientation, the RANTES C-termini are hidden as they point to the back.

Fig. 2. Far-UV CD analysis of peptide R11-29. Dimeric R11-29 was analyzed in 100 mM NaPO₄ pH 7.4 (spectrum 1) and in presence of 2, 10 and 25 mM SDS (spectra 2, 3 and 4, respectively). Spectra were recorded at 22 °C and expressed as mean residue ellipticity ($[\Theta]$) at a given wavelength.

Fig. 3. Anti-HIV activity of R11-29 analogue peptides bearing alanine substitutions or deletions of hydrophobic residues. (A) Alanine substituted peptides at the N-terminal region. (B) F12 and I15 deleted peptides. (C) Alanine substituted peptides at the C-terminal region. Results represent the mean of two replicates, and are representative of two or three experiments performed.

Fig. 4.

Anti-HIV activity of R11-29 analogue peptides bearing substitutions with non-natural hydrophobic amino acids. (A) And (B) modifications at the N and C termini, respectively. Results represent the mean of two replicates, and are representative of two or three experiments performed.