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Recognition of RANTES by Extracellular Parts of the CCR5 Receptor

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The chemokine RANTES (regulated upon activation, normal T-cell expressed and secreted) is a natural ligand of CCR5, one of the major HIV-1 coreceptors. It is secreted as part of the immune response to human immunodeficiency virus 1 (HIV-1) and inhibits infection by CCR5-dependent (R5) HIV-1 isolates. We have investigated the interaction of RANTES with several peptides derived from the extracellular domains of CCR5 by heteronuclear NMR spectroscopy in aqueous solution. We show that a peptide comprising the first 25 amino acid residues of the CCR5 N-terminal domain and sulfated at the Y10 and Y14 side-chains binds with micromolar affinity exclusively to the monomeric form of RANTES. In contrast to the tight binding of the sulfated peptide, the affinity of the same peptide in non-sulfated form was reduced by more than two orders of magnitude. Peptides derived from the CCR5 extracellular loops ECL1, ECL2 and ECL3 showed only very moderate and mostly non-specific binding. Chemical shift mapping of the interaction of the sulfated N-terminal peptide reveals a contiguous binding surface on RANTES, which comprises amino acid residues of the first β-strand, the N-loop, the fourth β-strand and the turns around residues 30 and 40. This binding surface largely overlaps with the dimer interface and is strongly positively charged, providing a rationale for the exclusive binding of the monomer to the peptide and the requirement of the negative sulfate groups at the Y10 and Y14 side-chains. The binding surface also largely overlaps with the segments that were identified previously as crucial for HIV blockade by peptide scanning and mutagenesis studies. These data offer new insights into the structure–function relation of the RANTES–CCR5 interaction and may be helpful for the design of novel HIV-1 inhibitors.

Keywords: chemokine; HIV-1; sulfation; heteronuclear NMR; chemical shift mapping

Introduction

Chemokines (chemotactic cytokines) are small, secreted proteins, that regulate the immune response by signaling leukocytes. The chemokine superfamily has been divided into subclasses C, CC, CXC, and CX_3C, depending on the arrangement of a specific cysteine motif near the N terminus.[1] The CC-chemokines RANTES, MIP-1α and MIP-1β have been identified as natural suppressors of infection by the human immunodeficiency virus (HIV).[2] Subsequently, the chemokine receptors CCR5[3–5] and CXCR4[6] were discovered as crucial coreceptors (together with CD4) of HIV-1 on the cell surface. Both are seven transmembrane domain, G protein-coupled receptors, recognizing CC (CCR5) or CXC (CXCR4) chemokines, respectively. During the early stages of HIV infection, viral isolates tend to use exclusively CCR5 for viral entry, while later in the disease, isolates that use CXCR4 emerge in some patients.[7] HIV-1 infection *via* CCR5 is blocked specifically by RANTES, MIP-1α and MIP-1β.[2]

The inherent proinflammatory activity[8,9] of the most potent HIV-suppressive chemokine RANTES may limit its use *in vivo* as an HIV-blocking agent in

Abbreviations used: HSQC, heteronuclear single quantum coherence; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide.

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an unmodified full-length form. However, functional peptide mapping[10] has shown that isolated peptides (residues T7–A22) from the RANTES N-loop or from the turn around residue 30 (residues H23–V42) can be effective in CCR5 recognition and HIV-1 blockade. By contrast, the receptor-triggering function that mediates the proinflammatory activities was shown to map to the N-terminal domain.[11,12] These findings suggest that the antiviral activity can be uncoupled from the proinflammatory function, implying that it may be possible to develop smaller compounds with uniquely HIV blocking function. A further promising route is the use of the entire RANTES protein as a "microbicide" for the topical, external prevention of HIV infection. Indeed, recent studies have shown that topical applications of N-terminally modified forms of RANTES, i.e. PSC-RANTES (N^{α}-(n-nonanoyl)-des-Ser^1-[L-thioproline^2, L-α-cyclohexyl-glycine^3]-RANTES) and "−2 RANTES" (having a deletion of the first two residues) provide potent protection against vaginal challenge in macaques,[13] or mice and rabbits,[14] respectively.

Atomic details about the interaction of RANTES with CCR5 have not been determined. Besides the RANTES amino acids identified in the functional peptide mapping study,[10] it is clear that the RANTES N terminus plays a crucial role, since chemical modifications of the RANTES N terminus such as PSC-, AOP- (aminooxypentane[15] or C1-C5-RANTES[16] lead to significantly stronger inhibition of HIV-1 replication. NMR experiments have shown that RANTES undergoes dimerization in the micromolar range at pH 3.8.[17] Above pH 4, RANTES is strongly aggregating solution. Several structures of RANTES dimers have been solved by solution NMR spectroscopy[17,18] and X-ray crystallography.[19–21] The physiological role of the dimerization is under debate.[22]

On the side of CCR5, the extracellular N-terminal tail is essential for RANTES binding, since a deletion of residues 2 to 13 shows strongly reduced affinity,[23] and several site-directed mutagenesis studies indicate the involvement of specific amino acids in this region.[23,24] This tail is crucial also for virus entry and interactions with the HIV-1 gp120 protein.[25] The CCR5 N terminus is modified post-translationally and contains two to four sulfate moieties at tyrosine residues 3, 10, 14, and 15, as well as an O-linked oligosaccharide at serine 6.[26,27] Whereas the role of glycosylation is less clear, sulfation is critical for virus entry, interactions with gp120, MIP-1α and MIP-1β.[26,28] A similar role of N-terminal tyrosine sulfation has been reported for the interaction of CXCR4 with the CXC chemokine SDF-1α.[29] Recent NMR studies have mapped the binding site of the CXCR4 tail in non-sulfated and sulfated form on the surface of SDF-1α.[30,31] Besides the sulfated N-terminal tail of CCR5, its second extracellular loop (ECL2) has been ascribed to chemokine binding and selectivity by CCR5/CCR2b chimera experiments.[32]

In the present study, we have characterized quantitatively the interaction of RANTES with several CCR5-derived extracellular peptides by heteronuclear NMR spectroscopy in aqueous solution. The peptides comprise the first 25 amino acid residues of the N terminus in sulfated and non-sulfated form at the Y10 and Y14 side-chains, as well as the CCR5 extracellular loops ECL1, ECL2 and ECL3, derived from a model of the CCR5 receptor according to the rhodopsin X-ray structure.[33] Significant binding was found only for the sulfated form of the N terminus, which binds to only the monomeric form of RANTES. The RANTES binding surface is strongly positively charged, and largely overlaps with the dimer interface as well as with segments crucial for HIV blocking that were identified previously from peptide scanning and mutagenesis experiments.[10]

Results

Since the RANTES N terminus is crucially involved in chemokine function, special care was taken to obtain RANTES by heterologous expression from *Escherichia coli* with a mature N terminus that lacks the *E. coli* initiating methionine residue. For this purpose, the protein was expressed as a C-terminal fusion to the first IGG-binding domain of protein G and cleaved by enterokinase. A good yield of uniformly ^{15}N or ^{15}N,^{13}C-labeled RANTES, which starts at residue serine 1, was obtained from this system after an appropriate purification procedure. Two constructs of RANTES were investigated; one that contains the wild-type sequence (WT-RANTES) and a second where glutamic acid 66 is replaced by serine (E66S-RANTES). E66S-RANTES has a much lower tendency for aggregation at pH >4, while retaining its G protein-coupled receptor-mediated biological activities.[34,35] Apparently, this disaggregated E66S-RANTES variant has much lower inflammatory activity.[35] Very similar results for CCR5-peptide binding and RANTES dimerization were obtained for both RANTES constructs at pH values <4, whereas higher pH values could only be investigated with the E66S-RANTES construct. In addition, the stability of samples against long-term precipitation was better for E66S-RANTES.

Monomer–dimer equlibrium

The RANTES monomer–dimer equilibrium has been investigated by one-dimensional ^{1}H proton NMR spectroscopy.[17] Monomer and dimer species are observable as separate sets of resonances. This indicates that the exchange between the two forms is slow compared to the chemical shift differences, i.e. slower than about milliseconds. To understand the interactions with the CCR5-derived peptides, it was necessary to characterize the RANTES monomer–dimer equilibrium in detail. Backbone and partial side-chain ^{1}H, ^{15}N and ^{13}C resonance assignments for both monomeric and dimeric forms were obtained from standard sets of three-dimensional triple-resonance experiments and confirmed by exchange spectroscopy. ^{1}H and ^{15}N resonance