Extension of recombinant human RANTES by the Retention of the Initiating Methionine Produces a Potent Antagonist*

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RANTES is a member of a large family of cytokines, known as chemokines, which have the ability to recruit and activate a wide variety of proinflammatory cell types (1). They are small polypeptides of 8–10 kDa and have been further classified into two major families: C, for cytokines, which have a single cluster of cysteines (Cys4) and the CC chemokines, which have two similar clusters (Cys2Cys2). The CC chemokines are rich in infiltrating eosinophils (3). In addition, injection of RANTES into dog skin has been shown to induce a large eosinophilic infiltrate (5).

Methodology—The cDNA coding for RANTES was cloned from a human peripheral blood monocyte cDNA library by PCR using primers based on the published sequence (2). The resultant PCR product was subcloned into the E. coli expression vector pT7-7 (11) and transformed into the strain BL21(DE3). High level expression of T7 polymerase and RANTES was induced by addition of isopropyl-β-D-thiogalactopyranoside to the medium.

The protein was purified from inclusion bodies by gel filtration on a Sephacyr S-300 HR column equilibrated in 0.1 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine HCl and 1 mM dithiothreitol. Renaturation was carried out on a 20-fold dilution into 0.1 M Tris-HCl buffer, pH 8.0, containing 1 M oxidized glutathione and 0.1 M reduced glutathione and stirring overnight at 4°C. The renatured protein was concentrated by adjusting the pH to 4.5 with acetic acid, and loading onto a HiLoad SP26/10 column equilibrated in 50 mM sodium acetate buffer, pH 4.5. The protein was eluted with a gradient of 0.6–2.0 M NaCl in the same buffer. The Met-RANTES-containing fractions were dialyzed extensively against 1% acetic acid, and then against 0.1% trifluoroacetic acid and lyophilized. Recombinant full-length human RANTES and Met-RANTES were expressed, purified, and renatured from E. coli.

Analytical Methods—SDS-PAGE was carried out on 4–20% acrylamide mini-gels (Novex) according to the manufacturer’s instructions, and the proteins were visualized by staining with Coomassie Brilliant Blue R-250. Purified RANTES was quantified by the extinction coefficient of ε280 = 1.6 at 280 nm calculated from the amino acid sequence. Amino acid analysis was carried out by gas phase hydrolysis under vacuum in a nitrogen atmosphere for 24 h in 6 M HCl containing 1 mg/ml phenol at 112°C. The resultant amino acids were quantified using a Beckman 6300 system using norleucine as an internal standard. Protein sequence was obtained with a model 477A protein sequenator (Applied Biosystems) using on line quantification of amino acid phenylthiohydantoin derivatives with a model 120A phenylthiohydantoin-derivative analyzer. Electrospray ionization mass spectroscopy (ESI-MS) was carried out on a Trio 2000 instrument (VG Biotech, Altrincham, UK).

The abbreviations used are: Met-RANTES, methionylated RANTES; HPLC, high performance liquid chromatography; pI, isoelectric point; MAP, methionine amino peptide.
Samples from reverse phase HPLC (trifluoroacetic acid/acetonitrile system) were dried and then dissolved in methanol/water/acetic acid (49.5/49.5/1, v/v/v) and infused at a rate of 2 ml/min. Spectra were co-added by repetitively scanning until an acceptable signal to noise ratio was obtained (3-4 min). Circular dichroism spectra were obtained using a Jasco J600 spectropolarimeter. 1H NMR spectroscopy was according to Chung et al.

Bioassays—THP-1 cell chemotaxis was carried out using 96-well micro-Bouyden chambers (Neuro-Probe, Cabin John, MD) fitted with 5-μm filters. 5.6 × 10⁴ cells in 200 μl of medium (RPMI 1640 containing 0.01% HEPES, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 0.005% gentamicin) were placed in the upper chamber. 370 μl of the medium described above, but without fetal calf serum, containing the ligand and appropriate dilutions of Met-RANTES, were placed in the lower chamber. After 60 min of incubation at 37°C under 5% CO₂, the cells were removed from the upper wells, and 200 μl of phosphate-buffered saline containing 20 μM EDTA added to detach the cells bound to the filter. After 30 min of incubation at 4°C, the plate was centrifuged at 1800 g and the medium removed. The cells bound to the filter were fixed in 100 μl of methanol/acetone (1:1, v/v) for 20 min, washed with PBS, and stained with 0.01 M HEPES, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 0.005% gentamicin. The plates were read in a CytoFluor plate reader using the Cell Titer 96® apyrase assay (Promega), which monitors the conversion of tetrazolium blue into its formazan product. Cell counts were performed according to the Cell Titer 96® nonradioactive cell proliferation assay (Promega), which measures the conversion of tetrazolium blue into its formazan product. Cell counts were performed according to the Cell Titer 96® nonradioactive cell proliferation assay (Promega), which monitors the conversion of tetrazolium blue into its formazan product. The appearance of activity ( ) correlates with the removal of the amino-terminal methionine residue ( ). 0.8 mg of Met-RANTES was incubated with 0.08 mg of leucine aminopeptidase as described in the text. Comparison of the NMR spectra of the methionylated and nonmethionylated forms of RANTES showed that no major conformational change had been induced by addition of the amino-terminal amino acid. In both cases, the three-dimensional location of the amino terminus of RANTES is partially disordered in solution, similar to the results described for another CC chemokine MIP-1β (20). Since the only difference between active RANTES and our Met-RANTES was the initiating methionine, we postulated that this residue was responsible for the observed difference in activity between the two proteins. This was tested by treating Met-RANTES with CNBr to remove the methionine. Activity was observed even without a further renaturation following the harsh treatment in 70% formic acid. Enzymic removal of the amino-terminal methionine using leucine aminopeptidase (Fig. 1) also activated the protein. These results further confirm that the initial lack of activity in Met-RANTES is not due to misfolding of the recombinant methionylated protein.

Antagonistic Properties—Since it was correctly folded, we next attempted to antagonize the function of RANTES in chemotaxis assays using the methionylated protein (Fig. 2a). A concentration of 3.5 nM RANTES was used as the chemotactic stimulus for THP-1 cells, 5 times the EC₅₀ value for RANTES in this assay. The Met-RANTES clearly inhibits the chemotactic response under these conditions, with an IC₅₀ value of 6 nM, which is a 1.7-fold molar excess. Since RANTES and MIP-1α share a common receptor, (9, 10), we also studied the effect of Met-RANTES against MIP-1α induced chemotaxis of THP-1 cells. Again, nanomolar inhibition was seen, with an IC₅₀ value of 0.49 nM, using 1 nM MIP-1α (5 × EC₅₀) as a stimulus (Fig. 2b). RANTES also induces migration of T cells (2), and Met-RANTES was able to antagonize the response to 3 nM RANTES (5 × EC₅₀) with an IC₅₀ of 6 nM (Fig. 2b), which is the same concentration as the EC₅₀ for RANTES-induced response. As with the THP-1 cells, Met-RANTES antagonized the response induced by 0.5 nM MIP-1α (5 × EC₅₀), with an IC₅₀ of 6 nM, which is 60-fold higher than the EC₅₀ for MIP-1α-induced lymphocyte chemotaxis (Fig. 2b). No antagonism of MCP-1-induced chemotaxis of THP-1 cells or IL-8 induced chemotaxis of T cells was observed, in keeping with the known receptor selectivity of the MIP-1α/RANTES receptor, CC-CKR1 (9, 10).

As a second bioassay, we studied the ability of chemokines to mobilize calcium in THP-1 cells (Fig. 3). Here again, Met-RANTES was capable of antagonizing the effects of RANTES. The concentration required for half-maximal inhibition of the calcium mobilized by 66 nM RANTES (5 × EC₅₀) was 88 nM.

FIG. 1. Incubation of Met-RANTES with leucine aminopeptidase produces a protein that can mobilize a calcium signal in THP-1 cells. The appearance of activity ( ) correlates with the removal of the amino-terminal methionine residue ( ) 0.8 mg of Met-RANTES was incubated with 0.08 mg of leucine aminopeptidase as described in the text. Comparison of the NMR spectra of the methionylated and nonmethionylated forms of RANTES showed that no major conformational change had been induced by addition of the amino-terminal amino acid. In both cases, the three-dimensional location of the amino terminus of RANTES is partially disordered in solution, similar to the results described for another CC chemokine MIP-1β (20). Since the only difference between active RANTES and our Met-RANTES was the initiating methionine, we postulated that this residue was responsible for the observed difference in activity between the two proteins. This was tested by treating Met-RANTES with CNBr to remove the methionine. Activity was observed even without a further renaturation following the harsh treatment in 70% formic acid. Enzymic removal of the amino-terminal methionine using leucine aminopeptidase (Fig. 1) also activated the protein. These results further confirm that the initial lack of activity in Met-RANTES is not due to misfolding of the recombinant methionylated protein.

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Although Met-RANTES was capable of inhibiting the mobilization of calcium by 23 nM (EC50) MIP-1α, an inhibitory effect was only seen at concentrations above 100 nM, and the IC50 of 1.2 μM is 260-fold higher than the EC50 value (4.6 nM) of the agonist-induced response. It was again unable to antagonize the calcium mobilization produced as a result of stimulation with either MCP-1 or IL-8, which are known to act at distinct receptors (21–23).

Receptor Binding—Equilibrium competition binding studies using the promonocytic cell line, THP-1 (Fig. 4a) showed that Met-RANTES could effectively compete for both 125I-RANTES and 125I-MIP-1α, Met-RANTES competes for the binding of 125I-RANTES with an IC50 of 25 nM, and for 125I-MIP-1α binding with an IC50 of 28 nM.

A shared receptor for RANTES and MIP-1α has been iden-
Met-RANTES therefore antagonizes cells. Met-RANTES competed for binding of \([125i]\)RANTES (25), interleukin-2 (26), and interleukin-5 (27). However, some such as granulocyte-macrophage colony-stimulating factor removes the Met residue. The retention of the initiating Met produced in inclusion bodies is not uncommon. This is not proteins expressed heterologously in \(E. coli\), particularly those produced in inclusion bodies, is not uncommon. This is not always the case, as the endogenous \(E. coli\) MAP (24) often removes the Met residue. The retention of the initiating Met does not impair bioactivity in the case of recombinant cytokines such as granulocyte-macrophage colony-stimulating factor (25), interleukin-2 (26), and interleukin-5 (27). However, some 5–10fold shifts in receptor binding affinity have been seen in certain cases such as hirudin (28) and interleukin-1\(\beta\) (29).

The retention of methionine in recombinant RANTES produces a protein that shows no agonist activity, despite the fact that it is correctly folded (13). Furthermore, it acts as a functional antagonist. The addition of a single amino acid to the amino terminus of RANTES creates an antagonist that is almost equipotent: a 1.7-fold molar excess is required for half-maximal inhibition of RANTES-induced chemotaxis of \(THP-1\) cells, whereas the RANTES-induced T cell chemotaxis is inhibited with an \(IC_{50}\) value equal to the \(EC_{50}\) for the response. Similarly, the calcium mobilization induced by RANTES is inhibited to 50% with an equimolar concentration to that used to induce the response. The same potency is observed for the inhibition of chemotaxis induced by \(MIP-1\alpha\) both in \(THP-1\) and T cells. Interestingly, however, the \(IC_{50}\) value for the inhibition of the MIP-1\(\alpha\) induced calcium mobilization in \(THP-1\) cells is 260-fold higher than the \(EC_{50}\). Met-RANTES therefore antagonizes RANTES and MIP-1\(\alpha\) with similar potency in the chemotaxis response but shows a clear difference in the other assay, calcium mobilization. This suggests that either there is an additional receptor for these chemokines or that there are two different signaling pathways for these two responses. A third possibility would be that different ligands evoke distinct signaling pathways at the same receptor.

The extension of RANTES by a single amino acid at the amino terminus thus produces a more potent antagonist than those described for other chemokines, which have been produced by amino-terminal deletions. Data from mutagenesis studies of the \(CXC\) chemokine interleukin-8 shows that the amino-terminal region is crucial for signaling, and deletion of five amino acids produces an antagonist (35, 36). However, a 30-fold molar excess is required for half-maximal inhibition of elastase release, and the reduction of neutrophil chemotaxis is not complete at 10 \(-6\) \(M\) (36). Residues 9–76 of the \(CC\) chemokine, monocyte chemotractant protein-1 (MCP-1) produce an antagonist that inhibits \(THP-1\) cell chemotaxis with an \(IC_{50}\) of 20 \(nm\) induced by 5 \(nm\) MCP-1 (37). Truncation of residues 2–8 from the amino terminus of MCP-1 produces an antagonist (38), which causes half-maximal inhibition of monocyte chemotaxis at a ratio of 75:1. This variant has been suggested to act as a dominant negative repressor of the active form of the ligand (39). However, the Met-RANTES antagonist appears to act as a competitive inhibitor on the shared MIP-1\(\alpha\)/RANTES receptor since, first, direct binding of \([125i]\)Met-RANTES has been demonstrated, and second, it can compete with both chemokines for binding.

Single amino acid changes have produced antagonists of other cytokines, such as interleukin-4 (32), interleukin-6 (33), and granulocyte-macrophage colony-stimulating factor (34). In the cases of these cytokines, they bind and signal through heterodimeric receptor complexes. Detailed studies have shown that the side chains involved in the initial binding between the ligand and its receptor, and the side chains involved in causing a signaling response are spatially separated. A mutant becomes an antagonist if its region interacting with the signaling part of the receptor can be eliminated without altering the binding region. To date, no mutagenesis data is available on RANTES, indicating whether the residues involved in binding and signaling differ. Chemokines, and other chemotactic peptides such as C5a, bind and signal through a seven-transmembrane \(G\)-protein coupled receptor. C5a interacts with its receptor through two distinct sites (30). Binding of C5a to the amino terminus of the receptor is proposed to produce a conformational change in the ligand, allowing it to properly interact with the second site at the carboxyl terminus to achieve functional activation (31). Such a dramatic change in bioactivity by the addition of a single amino acid emphasizes the importance of the amino terminus of RANTES. The extension of the sequence by one residue has removed the ability to form a functional interaction with the region of the receptor involved in \(G\)-protein-mediated signal transduction. However, since the overall structure of the protein is unaffected, receptor binding is unimpaired, producing a highly potent antagonist. In vivo studies with this readily available molecule will be extremely valuable in determining whether blocking the RANTES receptor(s) involved in the recruitment of leucocytes to inflammatory sites will relieve chronic inflammation.

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