Characterisation of the RANTES/MIP-1α receptor (CC CKR-1) stably transfected in HEK 293 cells and the recombinant ligands

Amanda E.I. Proudfoot*, Christine A. Power, Arlene Hoogewerf, Marc-Olivier Montjovent, Frederic Borlat, Timothy N.C. Wells

Glaxo Institute for Molecular Biology, 14, Ch. des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland

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Abstract The CC chemokines RANTES and MIP-1α are known to activate certain leucocytes and leucocytic cell lines. We have produced and fully characterised the recombinant proteins expressed in *E. coli*. They induce chemotaxis of the pro-monocytic cell line, THP-1 and T cells. THP-1 cells express three of the known CC chemokine receptors. In order to study the activation of a single receptor, we have expressed the shared receptor (CC CKR-1) for RANTES and MIP-1α stably in the HEK 293 cell line. We have examined the effects of RANTES and MIP-1α on the CC CKR-1 transfectants by equilibrium binding studies and in a chemotaxis assay. RANTES competes for [^{125}I]RANTES with an IC_{50} of 0.6 ± 0.23 nM, whereas MIP-1α competes for its radiolabelled counterpart with an IC_{50} of 10 ± 1.6 nM in the transfectants. These affinities are the same as those measured on the THP-1 cell line. The stably transfected HEK 293 cells respond to both these chemokines in the chemotaxis assay with the same EC_{50} values as those measured for THP-1 cells. This indicates that this cellular response can be mediated through the CC CKR-1 receptor.

Key words: Recombinant chemokine; Chemotaxis; RANTES/MIP-1α receptor; HEK 293 transfectant

1. Introduction

The chemokines are a family of pro-inflammatory polypeptides that act on a variety of leucocyte cell types [1,2]. The name is derived from their chemoattractant properties (*Chemo*tactic cy*tokine*), but they have also been shown to have more diverse effects on leukocytes including calcium mobilisation, histamine release, and degranulation. They are all small (8–10 kDa), basic proteins and despite a relatively low homology in primary sequence, have a highly conserved four cysteine motif which permits their classification into two sub-groups. The α chemokines have an amino acid between the first two Cys residues (CXC) whereas in the β subclass the two first Cys residues are adjacent (CC). In addition, their biological activities can be broadly separated between the two sub-classes, since the α chemokines act principally on neutrophils whereas the β chemokines activate the other leucocytes.

*Corresponding author. Fax: (41) (22) 794 69 65.

Abbreviations: RANTES, Regulated on Activation, Normal T expressed and Secreted; MIP-1α, macrophage inflammatory protein-1α; MCP-1, monocyte chemotactic protein-1; IL-8, interleukin-8; IPTG, isopropylthiogalactoside; EC_{50}, concentration of agonist giving half the maximal response; IC_{50}, concentration of inhibitor giving half the maximal inhibition.

The chemokines have been shown to mediate their responses through G protein coupled seven transmembrane spanning receptors. Two receptors have been identified for the α chemokines; IL-8-RA and IL-8-RB [3,4]. IL-8-RA has been shown to be specific in that it binds only IL-8 with high affinity, whereas IL-8-RB also binds NAP-2 and Gro-α with high affinity, and thus probably mediates the activities on neutrophils induced by these two peptides, and their homologue, ENA-78 [5]. The first CC chemokine receptor cloned, CC CKR-1 [6,7] was shown to be a shared receptor for two members of this sub-class, RANTES and MIP-1α. Subsequently, a second CC chemokine receptor, CC CKR-2, was identified as being a high affinity receptor for MCP-1 [8]. Recently two new RANTES/MIP-α receptors have been cloned: CC CKR-3 which is expressed in eosinophils [9] and CC CKR-4 from a basophilic cell line, KU812 [10].

CC CKR-1 has been demonstrated to transmit activation of transfected *Xenopus* oocytes through calcium mobilisation induced by both RANTES and MIP-1α [7]. However, only high affinity binding has been described for MIP-1α whereas the affinity reported for RANTES in competition binding studies with RANTES has been reported to be very low, in the micromolar range [6]. Yet in THP-1 cells, high affinity binding (500 picomolar) for RANTES has been demonstrated [11].

In order to further characterise the interaction of the two ligands RANTES and MIP-1α, we report the characterisation of the recombinant proteins and the CC CKR-1 receptor stably expressed in the HEK 293 cell line.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were purchased from Sigma and were of the highest grade obtainable; enzymes were from Boeringer Mannheim and all chromatographic media from Pharmacia. [^{125}I]RANTES and [^{125}I]MIP-1α (2200 Ci/mmol) were either from NEN Research Products (Du Pont de Nemours, Brussels, Belgium) or were obtained by radiolabelling the recombinant proteins by Amersham Laboratories (Amersham, Bucks, UK). The specific activities of the [^{125}I]chemokines labelled by Amersham Laboratories was 2000 Ci/mmol

2.2. Chemokine cloning and expression

Human RANTES and MIP-1α were cloned from a human bone marrow or human peripheral blood monocyte λGT11 cDNA libraries (Clontech), respectively, by PCR. Briefly, total cDNA inserts in the cDNA libraries were first amplified using λGT11 primers which flanked the *Eco*RI cloning site in a 100 μl reaction containing 2 μl of phage stock (10^6 plaque-forming units), 10 mM Tris-HCl buffer, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 2.5 units Amplitaq (Perkin Elmer-Cetus) and 1 μM of each primer (λGT11PCR-1 (forward primer) 5′ GATTGGTGGCGACGACTCCT and λGT11PCR-2

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(reverse primer) 5′ CAACTGGTAATGGTAGCGAC) for 30 cycles of 95°C for 2 min, 55°C for 2 min and 72°C for 5 min in a Techne PHC-2 thermal cycler. One tenth of the reaction mixture was then subjected to a 2nd round of PCR in a 100 μl reaction now containing 1 μM each of either RANTES specific primers (forward primer 5′ CCATGAAGGTCTCCGCGGCAC reverse primer 5′ CCTAGCTCATCTCCAAAGAG antisense) based on the published RANTES cDNA coding sequence [12] or MIP-1α specific primers [13] (forward primer 5′ ATGCAGGTCTCCACTGCTGC and reverse primer 5′ TCAGGCACTCAGCTCCAGGTG) for 30 cycles of 95°C for 2 min, 55°C for 2 min and 72°C for 5 min. PCR products were visualized on 3% Nu-Sieve (FMC) agarose gels stained with 0.5 mg/ml ethidium bromide. Bands migrating at the predicted size of RANTES cDNA (278 bp) or MIP-1α cDNA (279 bp) were gel-purified by standard methods [14]. Gel-purified DNA was rendered blunt-ended by sequential treatment with T4 polynucleotide kinase and *E. coli* DNA polymerase I Klenow fragment (New England Biolabs) and subcloned into the *Eco*RV site of pBluescript II SK-plasmid (Stratagene). Ligation products were electroporated into electrocompetent *E. coli* strain XL-1 blue using a Bio Rad Gene pulser (2.5 kV, 200 W, 25 mF). Following electroporation, cells were grown up in LB medium at 37°C for 1 h and then plated on LB plates containing 100 mg/ml of ampicillin. Mini-prep DNA prepared from 3 ml overnight cultures of individual ampicillin resistant colonies was digested with restriction enzymes *Hin*dIII and *Eco*RI. Mini-prep DNAs which yielded an insert size of approximately 280 bp were then subjected to DNA sequence analysis using T3 and T7 primers and Sequenase (USB). Sequencing revealed that all clones obtained were identical to the published sequence encoding the mature RANTES protein or MIP-1α protein. Plasmid DNA (1 μg) from clone pMIP-1α-2 was used to generate the mature peptide coding sequence of MIP-1α by PCR as described above. Clone pRANTES-6 was similarly used to generate the mature coding sequence of RANTES, except that the forward PCR primer encoded a hexapeptide sequence ending in Arg at the 5′ end. The resultant PCR products were subcloned into the expression vector pT7–7 [15] and transformed into *E. coli* strain BL21 (DE3). T7 RNA polymerase and subsequent MIP-1α or RANTES expression was induced by addition of IPTG (isopropylthiogalactoside) to the medium.

2.3. Generation of stable cells lines expressing CC CKR-1

A full length cDNA encoding the MIP-1α/RANTES receptor (CC CKR-1) was obtained by reverse transcriptase-PCR from the human eosinophilic cell line EOL-3 [16] using specific primers based on the published sequence [6] and subcloned into the mammalian cell expression vector pcDNA1neo (Invitrogen) (C.A. Power and A.J. Hoogewerf, unpublished results). CsCl gradient-purified pcDNA1neo/CC CKR-1 plasmid DNA (30 μg) was transfected into HEK 293 cells by electroporation using a Bio-Rad Gene Pulser (260 V, 960 mF). Stable transfectants were selected in DMEM-F12 medium supplemented with 2 mM glutamine, 10% foetal calf serum and 600 μg/ml Geneticin (G418, Gibco-BRL). G418 resistant clones were individually ring cloned after 14 days. Clones expressing the receptor were then selected by their ability to bind [^{125}I]MIP-1α as described above.

2.4. Recombinant chemokine expression and purification

Recombinant MIP-1α and RANTES were purified from inclusion body pellets by solubilisation in 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM dithiothreitol and 6 M guanidine HCl followed by gel filtration on a Sephacryl HR S300 column equilibrated in the same buffer. The proteins were renatured by a 20-fold dilution into 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM oxidised and 0.1 mM reduced glutathione and the solution stirred overnight at 4°C. The renatured RANTES protein was concentrated by applying the solution to a HiLoad S (26/10) column equilibrated in 50 mM sodium acetate, pH 4.5, and eluting the adsorbed protein with a linear 0.6–2 M NaCl gradient in the same buffer. The hexapeptide leader sequence was removed from the fusion protein by incubation in 50 mM Tris-HCl buffer, pH 8.0, with Endoproteinase Arg-C (Boeringer Mannheim) (1:600, enzyme:substrate, w/w) overnight at 37°C. The cleaved product was separated by cation exchange chromatography as described above, except that 6 M urea was included in the buffers. The renatured MIP-1α was concentrated by anion exchange chromatography on a HiLoad Q16/10 equilibrated in 20 mM Tris-HCl buffer, pH 8.0, and eluted with a 0–0.5 M NaCl gradient in the same buffer. The purified proteins were dialysed extensively against firstly 1% acetic acid and finally 0.1% trifluoracetic acid and stored as lyophilised powders at −80°C.

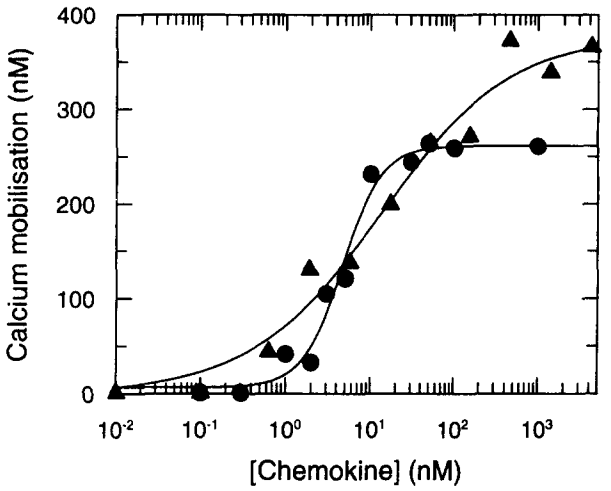

Fig. 1. Mobilisation of intracellular calcium in THP-1 cells by RANTES and MIP-1α. The assay was carried out using 1×10^6 cells loaded with Fura-2 dye as described in the text for RANTES (▲) and MIP-1α (●).

2.5. Analytical methods

Protein purification and purity were followed by SDS-PAGE using 4–20% acrylamide mini-gels (Novex) stained with Coomassie Brilliant Blue, R250 and reverse phase HPLC carrried out using a Beckman System Gold with a VarioPrep NUCLEOSIL 300–7 C_8 column (259 × 10 mm) (Macherey-Nagel). The purified proteins were quantified by the extinction coefficients of $A^{0.1\%}_{1\,cm} = 1.6$ for RANTES and 1.28 for MIP-1α at 280 nm calculated from the predicted amino acid sequence. Protein sequence was obtained with a model 477A protein sequencer (Applied Biosystems) using on line quantification of amino acid phenylthiohydantoin derivatives with a Model 120A PTH-amino acid analyser.

2.6. Bioassays

THP-1 and HEK 293 chemotaxis assays were carried out using 96-well micro-Boyden chambers (Neuro-Probe, Cabin John, MD) fitted with 5 μm filters for THP-1 cells and 8 μm filters for the HEK 293 cells. The HEK 293 cells were detached from the culture vessels by incubation with 5 ml Trypsin-EDTA (1×) (Gibco) for 2 min at 37°C, and then resuspended at a concentration of 2.8×10^6/ml in D-MEM F12 medium containing 10% FCS, 1 mM (L)-glutamine and 0.06% geneticin sulfate. THP-1 cells were resuspended in RPMI 1640 medium containing 0.01 M HEPES, 10% heat inactivated fetal calf serum (FCS), 2 mM (L)-glutamine and 0.005% gentamicin at a concentration of 2.8×10^6/ml. Cell suspensions (200 μl volume; 5.6×10^5 cells) were placed in the upper chamber. Appropriate dilutions of the chemokine in 370 μl of the media described above, but without FCS, were placed in the lower chamber. After 60 min incubation at 37°C under 5% CO_2, the cells were removed from the upper wells, and 200 μl PBS containing 20 μM EDTA added to detach the cells bound to the filter. After 30 min incubation at 4°C, the plate was centrifuged at 1800 × *g* for 10 min and the supernatants removed from the lower wells. The number of cells which had migrated were measured by the Cell Titer 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, USA) which monitors the conversion of tetrazolium blue into its formazan product. The number of viable cells is obtained from a standard curve relating cell number to the absorbance at 590 nm using a Thermomax microtitre plate reader (Molecular Devices, Palo Alto, CA). T cell chemotaxis was carried out according to [17]. Calcium mobilisation in THP-1 cells was carried out according to [18] with the modifications previously described [19]. The data obtained were fitted using Grafit 3.01 software [20] to a four-parameter logistic equation. The EC_{50} is defined as the concentration of agonist giving half the maximal response.

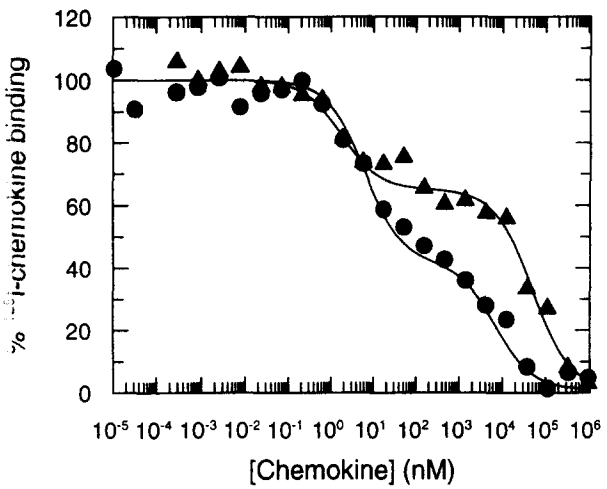

Fig. 2. Equilibrium competition binding of RANTES and MIP-1α to THP-1 cells. 10 μl of a THP-1 cell suspension in RPMI 1640 medium at a concentration of 2×10^7 cells/ml were incubated with 10 μl of 1 nM [^{125}I]RANTES (▲) or [^{125}I]MIP-1α (●) and 80 μl of chemokine diluted three fold from a 1.25×10^{-3} M solution in binding buffer for 2 h at 4°C. The cells were washed four times by filtration under vacuum with ice cold binding buffer containing 0.5 M NaCl. Each point represents two experiments.

2.3. Receptor binding

Equilibrium competition binding was carried out by incubating 10^5 THP-1 or HEK 293 cells in 100 ml 50 mM Hepes buffer, pH 7.2, containing 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA and 0.002% sodium azide, with 0.4 nM radiolabelled ligand except when indicated otherwise, and varying concentrations of competing ligand. After 2 h incubation at 4°C, the cells were washed four times with 1 ml ice cold binding buffer containing 0.5 M NaCl by filtration on 0.45 μm filters (Millipore) and aspiration by vacuum. The filters were suspended in 3.5 ml Ultima Gold scintillation fluid (Packard) and radioactivity measured on a Beckman LS5000 counter. The data were fitted using the Grafit 3.01 software [20] with simple weighting to the equation describing a competition for a single binding site $B = B_{max}/\{1 + [L]/IC_{50}\}$ where $IC_{50} = K_d$ + [radioligand] [21]. The results are expressed in terms of percentage binding in order to compare separate experiments.

2.4. Receptor distribution

Total RNA was prepared from FACS purified leukocytes and cell lines using the method of Chomczynski and Sacchi [22]. 10 μg of total RNA (1 mg/ml) and 0.5 ml oligodT_{15} (0.5 mg/ml) was heated at 70°C for 10 min and then cooled on ice for 2 min, followed by addition of 4 ml of 5× 1st strand buffer (250 mM Tris/HCl buffer, pH 8.3, containing 375 mM KCl and 15 mM $MgCl_2$), 2 ml of 0.1 M DTT, 1 ml of 10 mM dNTPs and 1 ml Superscript (Gibco-BRL) for 1 h at 37°C. 2 μl aliquots of each reverse transcriptase reaction was then subjected to 40 cycles of PCR (95°C for 2 min, 55°C for 2 min and 72°C for 2 min) in a 100 μl reaction mixture containing 100 pmoles each of specific primers for CC CKR-1. For THP-1 cells, the following primers were also used: CC CKR-2-B, IL-8R-B, IL-8R-A, CC CKR-4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously [10]. PCR reaction products were analysed on 1% agarose gels. The predicted size of PCR products for the chemokine receptors was approximately 1.1 kb, and 1.0 kb for GAPDH.

3. Results

The chemokines RANTES and MIP-1α were expressed at very high levels under control of the T7 RNA polymerase system. The final yield of purifed MIP-1α was 3.85 mg/g *E. coli* cells and that of RANTES, after the cleavage of the leader sequence, was 0.74 mg/g cells. Both proteins were estimated as >98% pure by SDS-PAGE and reverse phase HPLC analyses. In order to produce the RANTES protein with the correct amino terminus, it was necessary to express the protein with a cleavable leader sequence since retention of the initiating methionine when the cDNA encoding the mature form of the protein is expressed yields an inactive protein [23]). Amino terminal sequencing showed that the initiating methionine was removed from MIP-1α.

The recombinant chemokines were tested in two measurements of cellular activation, chemotaxis and calcium mobilisation In the chemotaxis assay using the pro-monocytic cell line, THP-1, approximately 2% of the cells in the upper chamber migrated randomly in the absence of chemokine. RANTES consistently induced the migration of 10–12% of the cells from the upper chamber, whereas MIP-1α had less efficacy in this assay, inducing the migration of approximately 5% of the cells. RANTES had an EC_{50} value of 0.68 ± 0.27 nM, whereas MIP-1α was more potent with an EC_{50} value of 0.20 ± 0.1 nM. Similar values were obtained using commercially available chemokines (PeproTech). In a second chemotaxis assay on freshly isolated T cells, RANTES had an EC_{50} value of 3 nM and MIP-1α 0.11 nM. Both RANTES and MIP-1α are known to mobilise calcium in cells expressing endogenous chemokine receptors as well as recombinant receptors. We therefore established a dose response curve for the calcium mobilisation by the recombinant chemokines in THP-1 cells. In this assay RANTES has an 13 nM and MIP-1α an EC_{50} value of 4.7 nM. (Fig. 1).

Transfection of the HEK 293 cells with the cDNA encoding the CC CKR-1 receptor was successful as judged by reverse transcriptase PCR and Nothern blotting. Since we do not have an antibody against the receptor, we judged its integration into the membrane by functional assays. Firstly, RANTES was able to induce a mobilisation of calcium into the transfected cells, but not in un-transfected cells (results not shown). Both

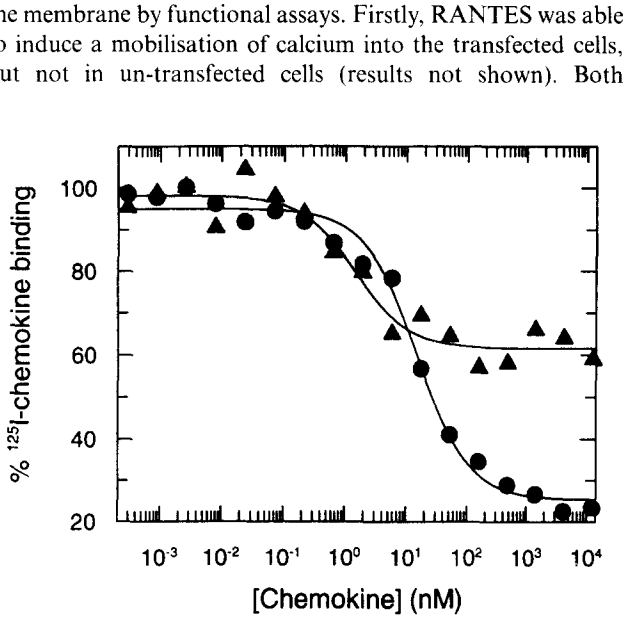

Fig. 3. Equilibrium competition binding of radiolabelled RANTES and MIP-1α to HEK 293 cells expressing the recombinant CC CKR-1 receptor. 10^5 cells were incubated with 0.4 nM [^{125}I]RANTES (▲) or 0.4 nM [^{125}I]MIP-1α for 2 h at 4°C in 50 mM Hepes buffer, pH 7.2, containing 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA and 0.002% sodium azide. The cells were washed four times by filtration under vacuum with ice cold binding buffer containing 0.5 M NaCl. Each point represents three experiments.

[^{125}I]RANTES and [^{125}I]MIP-1α could bind to the transfected cells. Scatchard analysis of saturation binding curves with [^{125}I]MIP-1α showed that the receptor was expressed at 18,000 sites per cell (results not shown). During initial binding experiments, we did not observe competition of [^{125}I]RANTES by unlabelled RANTES; rather the amount of radioactivity augmented with increasing concentrations of unlabelled ligand as has been previously reported [6]. However, inclusion of 0.5 M NaCl in the wash buffers as described [24] allowed us to observe high affinity binding of RANTES to both THP-1 cells and to the CC CKR-1 receptor expressed in HEK 293 cells. Competition of [^{125}I]RANTES by unlabelled RANTES resulted in an IC_{50} of 1 nM in THP-1 cells (Fig. 2) and 0.6 nM in HEK 293 cells expressing the recombinant CC CKR-1 receptor (Fig. 3). Competition of [^{125}I]MIP-1α on THP-1 and HEK 293 cells by MIP-1α was achieved with IC_{50} values of 7.4 and 10 nM, respectively. To our knowledge, this is the first published demonstration of high affinity binding of RANTES to CC CKR-1.

Complete displacement of the radiolabelled ligands was not achieved using concentrations of unlabelled ligand ranging between 10^{-12}–10^{-6} M routinely used for the experiments to determine high affinity binding. At micromolar concentrations of cold ligand, maximal competition of only 60–80% was achieved for MIP-1α and 30–40% for RANTES. The remaining radiolabelled ligand could only be displaced by increasing the concentration of unlabelled ligand up to millimolar concentrations as shown in Fig. 2 for THP-1 cells. Scatchard analysis using [^{125}I]MIP-1α showed that the THP-1 cells had 8200 high affinity sites per cell, which is comparable to the number of RANTES high affinity sites determined on THP-1 membranes [24].

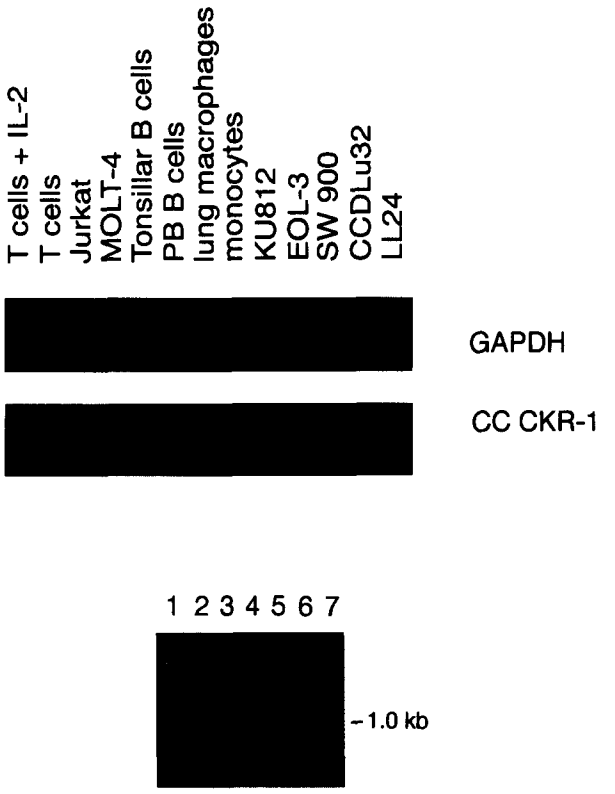

Fig. 4. Expression of chemokine receptor mRNA. PCR reactions were performed on total RNA derived from cells as described in text. One tenth of the reaction products were analysed on 1% agarose gels containing 0.5 μg/ml ethidium bromide. (A) THP-1 cells: Lane 1 = molecular mass markers (1 kb ladder, Gibco-BRL); lane 2 = IL-8 receptor A; lane 3 = IL-8 receptor B; lane 4 = CC CKR-1; lane 5 = K5–5; lane 6 = MCP-1 receptor B; lane 7 = GAPDH control. (B) Distribution of the CC CKR-1 receptor mRNA in leucocytes and cell lines.

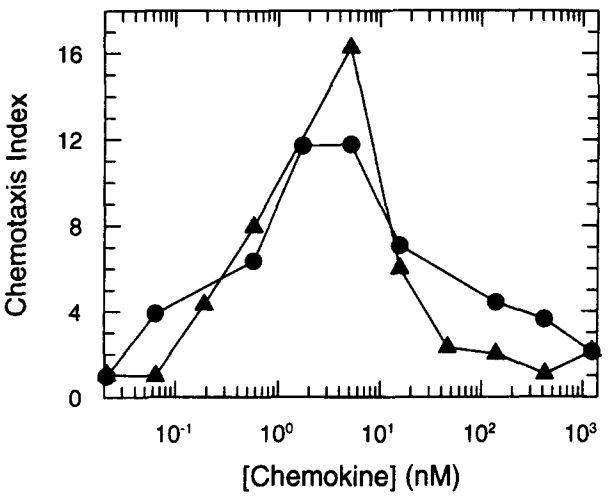

Fig. 5. HEK 293 chemotaxis induced by RANTES and MIP-1α. The HEK 293 cells were detached from the culture flasks with 5 ml Trypsin-EDTA (1×) (Gibco) for 2 min at 37°C, and then resupended at a concentration of 2.8×10^6/ml in D-MEM F12 medium containing 10% FCS, 1 mM L-glutamine and 0.06% geneticin sulfate. The chemotaxis assay was carried out as described for THP-1 cells, but using 8 μm filters.

Reverse transcriptase PCR has shown that the CC CKR-1 receptor is expressed in several leucocytes and cell lines (Fig. 4a). In addition, the pro-monocytic cell line THP-1 expresses several chemokine receptors (Fig 4b). Recently, it has been shown that HEK 293 cells transfected with the IL-8 receptor undergo chemotaxis in response to this chemokine [25]. We therefore examined the chemotaxis of HEK 293 cells stably transfected with the CC CKR-1 receptor in response to RANTES and MIP-1α. As shown if Fig. 5, the EC_{50} values measured for RANTES, 0.6 nM, and MIP-1α, 0.2 nM, are the same as those measured on the THP-1 cell line. Mock transfected HEK 293 cells do not respond to either chemokine in the same assay, so the effect measured can be unequivocably attributed to the CC CKR-1 receptor.

4. Discussion

In both the in vitro assays tested, chemotaxis of THP-1 cells and freshly isolated T cells, as well as calcium mobilisation in THP-1 cells, MIP-1α is more potent than RANTES. However, it has less efficacy in inducing the response. In THP-1 cells the chemotactic response induced by RANTES, 11, is more than twofold higher than that induced by MIP-1α, which was never greater than 5. Similarly, RANTES mobilised 375 nM calcium, whereas the maximum mobilised by MIP-1α was 275 nM.

Both of these chemokines have been shown to activate the CC CKR-1 receptor when expressed in HEK 293 cells and *Xenopus* oocytes and induce mobilisation of calcium [6,7]. However, only high affinity binding was demonstrated for MIP-1α. On the other hand, high affinity binding sites for RANTES have been demonstrated on THP-1 cells [24] and on butyrate-differentiated HL-60 cells [11]. The affinities reported

here as IC_{50} values can be translated to the affinity binding constant, by the equation $IC_{50} = [L^*] + [K_d]$ where $[L^*]$ is the concentration of radioligand [21]. Thus MIP-1α has a K_d of 7 nM for THP-1 cells and 9.6 nM for the HEK 293 cells expressing the CC CKR-1 receptor, whereas RANTES has a K_d of 600 pM for THP-1 cells and 200 pM for the CC CKR-1 receptor expressed in HEK 293 cells. The CC CKR-1 receptor is therefore probably the high affinity binding site observed for RANTES in the THP-1 and HL-60 cell lines, both of which express transcripts for the receptor [7].

The in vitro chemotaxis assay using the recombinantly expressing HEK 293 cells indicates that the CC CKR-1 receptor certainly functions as a mediator of chemotaxis. The EC_{50} values for the induction by both chemokines of chemotaxis are identical for THP-1 cells and that mediated by the recombinant CC CKR-1 receptor. Although it was first reported that T cells do not have the transcript for this receptor [7], we have been able to show the contrary. It is therefore possible that this receptor also mediates chemotaxis in lymphocytes.

We therefore conclude that RANTES binds 10–50 fold more tightly than MIP-1α to their shared receptor, CC CKR-1. However, MIP-1α is more potent in inducing biological responses, at least in vitro.

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