Identification of Single C Motif-1/Lymphotactin Receptor XCR1*

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Single C motif-1 (SCM-1)/lymphotactin is a member of the chemokine superfamily, but retains only the 2nd and 4th of the four cysteine residues conserved in other chemokines. In humans, there are two highly homologous SCM-1 genes encoding SCM-1α and SCM-1β with two amino acid substitutions. To identify a specific receptor for SCM-1 proteins, we produced recombinant SCM-1α and SCM-1β by the baculovirus expression system and tested them on murine L1.2 cells stably expressing eight known chemokine receptors and three orphan receptors. Both proteins specifically induced migration in cells expressing an orphan receptor, GPR5. The migration was chemotactic and suppressed by pertussis toxin, indicating coupling to a Gα type of G protein. Both proteins also induced intracellular calcium mobilization in GPR5-expressing L1.2 cells with efficient mutual cross desensitization. SCM-1α bound specifically to GPR5-expressing L1.2 cells with a Kd of 10 nM. By Northern blot analysis, GPR5 mRNA of about 5 kilobases was detected strongly in placenta and weakly in spleen and thymus among various human tissues. Identification of a specific receptor for SCM-1 would facilitate our investigation on its biological function. Following the set rule for the chemokine receptor nomenclature, we propose to designate GPR5 as XCR1 from XC chemokine receptor-1.

The chemokines constitute a group of structurally related, mostly basic, heparin-binding chemotactic cytokines that recruit selective types of leukocytes and play important roles in inflammatory and immunological responses (for review, see Refs. 1–3). The chemokines are grouped into two major subfamilies, CXC and CC, according to the motif based on the first two of the four conserved cysteine residues; one amino acid separates the two cysteine residues in the CXC chemokines, whereas the two cysteines are adjacent in the CC chemokines. Furthermore, chemokine-related molecules with C and CX3C motifs have been identified, potentially representing C and CX3C subfamilies. Most CXC chemokines are directed to neutrophils, whereas most CC chemokines primarily act on monocytes. Notably, some recently described CC chemokines are highly specific for lymphocytes and may be involved in homeostatic recirculation and homing of lymphocytes (3).

Previously, we described a novel basic secretory protein that shows significant homology to chemokines, particularly to CC chemokines, but retains only the 2nd and 4th of the four cysteine residues conserved in other chemokines (4). We thus named this molecule as single C motif-1 (SCM-1)1 (4). We have further demonstrated that, in humans, there are two highly homologous SCM-1 genes, SCYC1 and SCYC2, that are closely localized on chromosome 1q23 and encode two SCM-1 proteins, SCM-1α and SCM-1β, with only two amino acid substitutions (5). These two genes were similarly induced in peripheral blood T cells and some T cell lines upon stimulation with phytohemagglutinin + phorbol myristate acetate (5). Independently, the same protein as SCM-1α was described with terms lymphotactin and ATAC (activation-induced T cell-derived and chemokine-related molecule) (6, 7). Zlotnik and colleagues (6, 8, 9) reported that murine and human lymphotactin were produced mainly by activated CD8+ T cells and natural killer (NK) cells and were specifically chemotactic for lymphocytes and NK cells. Chemotactic responses of fresh and activated NK cells to lymphotactin were also demonstrated by other groups (10, 11). As for ATAC, Kroczek and colleagues (7) also described its highly selective expression by activated CD8+ T cells. However, they were unable to demonstrate any chemotactic activity of their recombinant, natural, or synthetic ATAC (7, 12). They only observed enhanced locomotion (chemokinesis) in CD4+ and CD8+ T cells by natural and chemically synthesized ATAC (12). We have also experienced similar difficulties in demonstrating any significant chemotactic responses of lymphocytes or any other types of cells to our recombinant SCM-1 proteins.2

Thus, besides a growing list of producer cells that now includes γδ type T cells and mast cells (13, 14), we still know very little about the function of SCM-1. As a first step to elucidate its biological role, we decided to identify its specific receptor. Here we demonstrate that an orphan receptor GPR5 (15) functions as a specific high affinity receptor for SCM-1α and SCM-1β. When expressed in murine L1.2 cells, GPR5 mediates efficient chemotactic responses and calcium mobilization by SCM-1α and SCM-1β. SCM-1α binds to GPR5 with a high affinity. Among various human tissues, GPR5 is strongly expressed in placenta and weakly in spleen and thymus. Following the set rule on the nomenclature of chemokine receptors (CXC,CCR, and CXCR for CXC, CC and CX3C chemokines, respectively), we propose to designate GPR5 as XCR1 from XC chemokine receptor-1.

1 The abbreviations used are: SCM-1, single C motif-1; ATAC, activation-induced, T cell-derived and chemokine-related molecule; NK, natural killer; GPR5, G protein-coupled receptor 5; XCR, XC chemokine receptor; CXC, CC chemokine receptor; CCR, CC chemokine receptor-1; CX3CR, CX3C chemokine receptor; TARC, thymus and activation-regulated chemokine; LARC, liver and activation-regulated chemokine; SLC, secondary lymphoid tissue chemokine; ELC, EB11-ligand chemokine; PARC, pulmonary and activation-regulated chemokine; MCP, monocyte chemoattractant protein; RANTES, regulated on activation, normal T cell expressed and secreted; IP-10, interferon-γ-inducible protein 10.

2 T. Yoshida, T. Imai, M. Kakizaki, M. Nishimura, S. Takagi, and O. Yoshie, unpublished results.
EXPERIMENTAL PROCEDURES

Chemokines—Recombinant SCM-1α and SCM-1β were produced by using a baculovirus expression system and purified to essential homogeneity as described previously (16). In brief, High Five insect cells (Invitrogen) were infected with recombinant baculoviruses encoding SCM-1α and SCM-1β under the control of the polyhedrin promoter. After 2 days, recombinant SCM-1 proteins were purified from pooled culture supernatants by using cation-exchange chromatography on FPLC system (Amersham Pharmacia Biotech) and reverse-phase high performance liquid chromatography. SCM-1 proteins were eluted from the reverse-phase column as a single major peak. Fractions containing SCM-1 proteins were pooled and lyophilized. Protein concentrations were determined by BCA kit (Pierce). Endotoxin levels determined by the Limulus amoebocyte lysate assay (QCL-1000, BioWhittaker, Walkersville, MD) were always <4 pg/μg of recombinant protein. Production and purification of recombinant TARC, LARC, SLC, ELC, and PARC were described previously (see Ref. 3). Eotaxin and soluble fractalkine were described previously (17, 18). MIP-1α, MIP-1β, MCP-1, MCP-2, MCP-3, RANTES (regulated on activation normal T cell expressed and secreted), I-309, inter leukin-8, and IP-10 were purchased from Pepro tech (Rocky Hill, NJ).

Stable Expression of Cloned Receptor—A murine pre-B cell line L1.2 (19) was maintained in RPMI 1640 supplemented with 10% fetal calf serum. L1.2 cells stably expressing CCR1, CCR2B, CCR3, CCR4, CCR5, CX3CR1, Burkitt’s lymphoma receptor-1, and GPR-9–6 were described previously (20, 21). To express an orphan receptor, GPR5, the coding region of GPR5 was cloned from human genomic DNA by polymerase chain reaction using primers (‘+5’-GTCGAACGCCGCCC-ATGGGATCCCTCAACCCAGAGACGACG-3’ and ‘−5’-GGGCGGCCG-CACAGGGCTCTAGATAGA-3’) based on the reported sequence (15). After confirming the entire nucleotide sequence, the fragment was cloned into an expression vector pCAGGS-Neo (20). For stable expression of cloned receptors in L1.2 cells, 1 × 10⁶ cells in 0.5 ml of phosphate-buffered saline were transfected with 10 μg of linearized plasmids by electroporation at 260 V and 960 μF using Gene Pulsar (Bio-Rad). After selection with G418 (Sigma) at 600 μg/ml for 1–2 weeks, cells expressing transfected receptors at high levels were identified by limiting dilution culture and Northern blot analysis.

Chemotaxis Assay—Cell migration was assessed by using Transwell chambers with 3-μm pores (Costar) as described previously (22). Cell suspension in 100 μl of assay medium (RPMI 1640 supplemented with 20 mM HEPES, pH 7.4, and 0.5% bovine serum albumin) was placed in upper compartments (10⁶ cells/well), and 0.6 ml of assay medium without or with a chemokine was placed in lower compartments. After incubation for 4 h in 5% CO₂ atmosphere, cells migrated into lower chambers were counted on FACStar Plus (Becton Dickinson, Mountain View, CA).

Calcium Mobilization Assay—This was carried out as described previously (22). In brief, cells at 1 × 10⁶/ml in Hank’s balanced salt solution supplemented with 1 mg/ml of bovine serum albumin and 10 mM HEPES, pH 7.4, and 0.5% bovine serum albumin) were incubated with 2 μM Fura-2/AM (Molecular Probes, Eugene, OR) at 37°C for 1 h in the dark. After washing, 2 ml of cell suspension at 2.5 × 10⁶/ml was placed on a luminescence spectrometer (LS50B, Perkin-Elmer) with constant stirring. Emission fluorescence at 510 nm was measured upon excitation at 340 and 380 nm with a time resolution of 5 point/s. Data were presented by the ratio of fluorescence intensity at 340 nm divided by that of 380 nm (R340/R380).

Binding Assay—This was carried out as described previously (16). In brief, purified recombinant SCM-1α was radiolabeled by using 125I-labeled Bolton and Hunter reagent (Amersham Pharmacia Biotech, Japan) to a specific activity of 1.3 × 10⁶ cpm/μg. For saturable binding experiments, cells were incubated with increasing concentrations of 125I-SCM-1α without or with 100-fold excess unlabeled SCM-1α in 200 μl of RPMI 1640 containing 20 mM HEPES, pH 7.4, and 0.5% bovine serum albumin. After incubation for 1 h at 15°C, cells were separated from the radiolabeled 125I-SCM-1α by centrifugation through a mixture of dibutyl phthalate/oil (4:1) and counted in a gamma counter. All samples were determined in duplicate. The binding data were analyzed by the LIGAND program (23).

Northern Blot Analysis—This was carried out as described previously (22). Northern blot filters were purchased from CLONTECH. The GPR5 probe of 1.2-kilobases in length was generated by polymerase chain reaction using GPR5-specific primers described above.

RESULTS

Production of Recombinant SCM-1α and SCM-1β—Recombinant SCM-1α and SCM-1β were produced by insect cells using the baculovirus expression system and purified from pooled culture supernatants by cation-exchange chromatography and reverse-phase high performance liquid chromatography. As shown in Fig. 1, SCM-1α was eluted from the reverse-phase column as a single major peak and migrated as a major 17-kDa band and a minor 15-kDa band on a gradient SDS-polyacrylamide gel electrophoresis. Previously, natural ATAC (identical to SCM-1α) was shown to be highly O-glycosylated,
giving rise to species ranging from 12 to 19 kDa (12). Thus, these two forms of recombinant SCM-1α were most probably because of difference in the level of glycosylation. Amino acid sequence analysis demonstrated that the mature recombinant SCM-1α started at Val-22. The signal peptide cleavage site was thus exactly as estimated (4) and was also identical to the one used by natural ATAC (12). SCM-1β was also purified through the same procedure (data not shown).

Induction of Chemotaxis via GPR5—We examined the ability of recombinant SCM-1α and SCM-1β to induce migration in murine L1.2 cells stably expressing the following chemokine receptors and orphan receptors: CC chemokine receptors CCR1 to CCR7 (see Refs. 1–3), CX3CR1 (18), GPR5 (15), GPR-9–6 (GenBank™ accession number U45982), and Burkitt’s lymphoma receptor-1 (24). Both SCM-1 proteins were found to induce efficient migration in L1.2 cells expressing GPR5 but not in those expressing any other of the 11 receptors listed above (data not shown). As shown in Fig. 2A, SCM-1α and SCM-1β attracted L1.2 cells expressing GPR5 with typical bimodal dose-response curves. SCM-1α showed a maximal level of migration at 100 ng/ml, whereas SCM-1β induced maximal migration broadly from 100 to 1000 ng/ml. Although the cause for such differences is not known at present, both proteins gave an EC_{50} of about 50 ng/ml. A checkerboard-type analysis revealed that the migration of GPR5-expressing L1.2 cells toward SCM-1α was mostly chemotactic (Fig. 2B). Furthermore, the migratory response of GPR5-expressing L1.2 cells toward SCM-1α was completely suppressed by pretreatment with pertussis toxin (Fig. 2C), indicating coupling of GPR5 with a Gαi class of G-proteins in murine L1.2 cells. Aside from SCM-1α and SCM-1β, 15 other recombinant chemokines tested so far (TARC, LARC, PARC, ELC, SLC, MIP-1α, MIP-1β, MCP-1, MCP-2, MCP-3, RANTES, eotaxin, I-309, interleukin-8 and IP-10) were unable to induce chemotaxis in GPR5-expressing L1.2 cells (data not shown). Thus, GPR5 is a highly specific functional receptor for SCM-1α and SCM-1β.

Induction of Calcium Mobilization via GPR5—We next examined calcium mobilization in GPR5-expressing L1.2 cells by SCM-1α and SCM-1β. As shown in Fig. 3A, SCM-1α induced vigorous calcium mobilization in GPR5-expressing L1.2 cells with a dose-dependent manner. Furthermore, SCM-1α and SCM-1β efficiently desensitized each other (Fig. 3B).

Specific Binding of SCM-1 to GPR5—We next examined specific binding of SCM-1α to GPR5. As shown in Fig. 4A, 125I-labeled SCM-1α bound to GPR5-expressing L1.2 cells with a saturable manner. The Scatchard analysis revealed a K_{d} of 10 nm.

Expression of GPR5 mRNA in Human Tissues—We examined the expression of GPR5 mRNA in various human tissues. A filter blotted with 2 μg/lane of poly(A)^+ RNA from indicated human tissues (CLONTECH) was hybridized with the 32P-labeled cDNA probe for GPR5. PBL, peripheral blood leukocytes.

Expression of GPR5 mRNA in Human Tissues—We examined the expression of GPR5 mRNA in various human tissues. A filter blotted with 2 μg/lane of poly(A)^+ RNA from indicated human tissues (CLONTECH) was hybridized with the 32P-labeled cDNA probe for GPR5. PBL, peripheral blood leukocytes.
ined expression of GPR5 mRNA in various human tissues by Northern blot hybridization. As shown in Fig. 5, a message of about 5-kilobases was detected at low levels in spleen and thymus. Strikingly, however, placenta expressed GPR5 mRNA at high levels. A very low level of expression of GPR5 mRNA was also detected in peripheral blood leukocytes after a long exposure (not shown).

**DISCUSSION**

Even though SCM-1/Lymphotactin/ATAC is a member of the chemokine superfamily, it retains only the 2nd and 4th of the four cysteine residues conserved in other chemokines and has a C-terminal sequence much longer than those of other chemokines (4, 6–8). In humans, there are two highly homologous genes, SCYC1 and SCYC2, which encode SCM-1α and SCM-1β, respectively, with only two amino acid substitutions at positions 7 and 8 in mature proteins: Asp-Lys in SCM-1α and His-Arg in SCM-1β (5). These two genes are likely to have originated from a very recent gene duplication event because, besides their highly homologous sequences including the 5′ and 3′ noncoding regions, the mouse has apparently only one gene (5). In human tissues, SCM-1 mRNA was found to be expressed in spleen, thymus, and peripheral blood leukocytes (4). Similar tissue expression pattern was also described for human lymphotactin (8). At cell levels, it has been shown to be produced mainly by activated CD8+ T cells, NK cells, γδ-type T cells, and mast cells (6–10, 13, 14). Zlotnik and colleagues (6, 8–10) demonstrated that their murine and human lymphotactin induced chemotactic responses and calcium mobilization in lymphocytes and NK cells. Nevertheless, we and others (7, 12) have experienced difficulties in getting such results. These discrepancies may be because of differences in recombinant proteins and/or experimental conditions. Or else the main activity of SCM-1 proteins may be different from induction of chemotaxis.

To elucidate the biological function of SCM-1 rationally, identification of its receptor would be of great help. Here we propose to designate GPR5 as XCR1 from XC chemokine receptor-1.

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