Molecular cloning of a novel C or γ type chemokine, SCM-1

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Abstract From human PBMC stimulated with PHA, we have isolated cDNA clones encoding a novel cytokine named SCM-1, which is significantly related to the CC and the CXC chemokines but has only the 2nd and the 4th of the four cysteines conserved in these proteins. Its gene is also distinctly mapped to human chromosome 1. SCM-1 is strongly induced in human PBMC and Jurkat T cells by PHA stimulation. Among various human tissues, SCM-1 is expressed most strongly in spleen. SCM-1 is found to be 60.5% identical to lymphotactin, a recently described murine lymphocyte-specific chemokine, which also retains only two cysteines. SCM-1 and lymphotactin may thus represent the human and murine prototypes of a novel C or γ type chemokine family.

Key words: Chemokine; Molecular cloning; Lymphotactin

1. Introduction

Emigration of leukocytes from blood into sites of inflammation and immune responses is essential for the host defense mechanisms. It is now known that a number of inducible cytokines collectively called as chemokines regulate migratory responses of leukocytes. From the arrangement of the first two of the four conserved cysteines, chemokines are grouped into two major families, the CXC or α family and the CC or β family. In CXC chemokines, the first two cysteines are separated by a single amino acid. The genes for these proteins are clustered in q12-21 region of human chromosome 4. They are potent chemoattractants for neutrophils. In CC chemokines, the first two cysteines are adjacent. The genes of this family are clustered on human chromosome 17. They are potent chemoattractants for monocytes (for review, see [1,2]). Recently, a novel chemokine named lymphotactin was identified from activated mouse pro-T cells [3]. Lymphotactin is unique because it has only two, the 2nd and the 4th, of the four cysteines conserved in other chemokines and also a C-terminal sequence much longer than those of other chemokines. In the present study, we have identified a novel human cytokine, SCM-1. The cDNA clones were isolated from PHA-stimulated PBMC. SCM-1 is significantly related to the CC chemokines but, like mouse lymphotactin, has only two instead of four cysteines and a C-terminal region much longer than those of other chemokines. In fact, SCM-1 is 60.5% identical to murine lymphotactin and may well be its human homologue. SCM-1 and lymphotactin may thus represent the human and murine prototypes of a new C or γ type chemokine family.

2. Materials and methods

2.1. Cells and cytokines

PBMC were isolated from heparinized whole blood from healthy donors by using Ficoll-Paque (Pharmacia) and cultured in the presence of PHA-M (Gibco) at a 1:100 dilution. Raji (a human B cell line), Jurkat (a human T cell line) and U937 (a human monocytoid cell line) were used. Recombinant TNF-α, IFN-γ, IL-1α, and IL-4 were purchased from Genzyme (Cambridge, MA). The cDNA for human MIP-1α [4] was cloned from a library generated from human PBMC stimulated with PHA (see below) by polymerase chain reaction (PCR) [5] using specific primers (5′-ATCATGCAGGTCTCCA-CTGCT-3′ and 5′-CCCTCAGGCACTCAGCTCTAG-3′). The identity of the cDNA was confirmed by DNA sequencing using T7 Sequencing kit (Pharmacia).

2.2. Signal sequence trap

Novel cDNA fragments were initially isolated by a modified method of signal sequence trap [6] (Imai et al., to be described elsewhere). In brief, poly(A)$^+$ RNA prepared from human PBMC stimulated with PHA for 72 h was converted to cDNA by using random primers (cDNA Synthesis Kit, Pharmacia). Oligo(dC) tails were added to the 3′ ends of cDNA by using terminal deoxynucleotidyl transferase (Gibco/BRL). The second strands were synthesized by using oligo(dG) primers carrying a unique sequence and a *Sal*I site. After sonication, cDNA fragments of about 300 base pairs (bp) were gel-purified, blunted and ligated to an adaptor carrying a unique sequence and an *Xba*I site. The 5′-portion-enriched cDNA fragments were amplified by PCR [5] using universal amplification primers, double-digested with *Sal*I and *Xba*I, and inserted between the promoter for EF-1α [7] and the coding sequence of CD4 (without the signal sequence) in an expression vector pDREF (Imai et al., to be described elsewhere). Raji cells were transfected with the cDNA library by electroporation using a GenePulser (Bio-Rad) and cultured under hygromycine selection. After three consecutive sortings, about 40% of cells were positive for surface CD4. Plasmid DNAs were recovered from these cells and transfected into *E. coli*. Plasmid clones prepared from 100 random transformants were then separately transfected into Raji and 42 clones were found to induce surface expression of CD4. Sequences of the inserted DNA fragments were determined by using AutoRead Sequencing kit and A.L.F. DNA Sequencer (Pharmacia), and compared in the GenBank database for homology. A novel cDNA fragment TY53 with significant homology to chemokines was selected for further study.

2.3. Isolation of full-length cDNA

Double-stranded cDNAs were synthesized from poly(A)$^+$ RNA prepared from PBMC stimulated with PHA for 72 h by using Superscript cDNA Synthesis kit (Gibco/BRL). cDNAs longer than 1 kb were inserted into pSPORT (Gibco/BRL) and transfected into *E. coli* DH10B by using a GenePulser (Bio-Rad). The library consisting of about 5×10^5 independent clones was screened with the TY53 cDNA and three independent clones were isolated. Two clones (0.5 kb and 1.2 kb) were completely sequenced from both sides by using AutoRead Sequencing kit and A.L.F. DNA Sequencer (Pharmacia). The other 0.5 kb clone was partially sequenced.

2.4. Southern and Northern blot analysis

Filters for Zoo blot and Multiple Tissue Northern blots were purchased from Clontech. Poly(A)$^+$ RNAs prepared from various cell

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The nucleotide sequence data have been deposited in DDBJ/EMBL/GenBank under the accession numbers of D43768 and D43769.

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sources were fractionated by electrophoresis on 1% agarose gel containing formaldehyde and blotted to a filter membrane (Hybond N^+) (Amersham). Filters were hybridized with probes labeled with ^{32}P using Multiprime DNA labeling system (Amersham) and subjected to autoradiography.

2.5. Chromosomal assignment

Genomic DNAs derived from human-rodent somatic cell hybrids were purchased from BIOS Lab. The presence of human SCM-1 gene in 100 ng of DNA from each hybrid was examined by PCR using a set of primers for human SCM-1 (5′-GTCTCAGATAAGAGGACCTG-3′ and 5′-GTGGCTTGTGGATCAGCACA-3′) that specifically amplifies 900 bp bands from human DNA but not from hamster or mouse DNA. Discordance is expressed as a percentage of cases in which the gene is detectable and a chromosome is absent and vice versa, and the values equal or less than 10% are considered significant for gene assignment.

3. Results

3.1. Molecular cloning of human SCM-1 cDNA

Signal sequence trap is a newly introduced technique aiming at selective isolation of 5′ portion fragments of cDNA species encoding secretory and type I integral membrane proteins [6]. A modified signal sequence trapping method (Imai et al., to be described elsewhere) was applied for isolation of cDNA fragments from PHA-stimulated human PBMC. Among a number of novel cDNA fragments, we noted that TY53 was translatable to a polypeptide showing homologies to chemokines. Interestingly, however, the peptide had only the 2nd of the two cysteines that are regularly found in the N-terminal region of chemokines in a motif of CXC or CC [1,2]. TY53 might, therefore, represent a new type of chemokine having a single C motif. We thus decided to isolate the full-length cDNA of TY53 from a library generated from PHA-stimulated PBMC.

By using TY53 as a probe, we isolated three independent cDNA clones, two 0.5 kb clones and one 1.2 kb clone. Their nucleotide sequences overlap and encode the same polypeptide (Fig. 1A). The 3′ non-coding regions differ in length (188 bp and 860 bp) probably because of alternative uses of poly(A) signals. The longer 3′ non-coding sequence contains a single mRNA destabilization signal (UUAUUUAUU)[8], which is frequently observed in cytokine genes. From the size of hybridizing bands detected in Northern blot analysis (about 0.8 kb) (see below), the major transcripts may carry the shorter 3′-non-coding sequence.

The open reading frame starts from the first ATG which conforms well to the Kozak rule [9] and encodes a polypeptide of 114 amino acids. The predicted protein has a highly hydrophobic N-terminus characteristic of a leader sequence. From von Heijne's rule [10], the mature protein probably starts from valine-22 after cleavage of the signal peptide. This putative mature protein is quite hydrophilic, of a molecular weight of 10,271, and rich in basic amino acids with an isoelectric point of 11.1. No potential N-linked glycosylation site is found. Computer analysis revealed that the protein sequence of TY53 is related to a class of cytokines collectively called as chemokines and especially to CC chemokines. Fig. 1B shows the alignment of SCM-1 with RANTES (a CC chemokine) and IL-8 (a CXC chemokine). All the known chemokines have four conserved cysteines with the N-terminal two cysteines in either the CC motif or the CXC motif. Disulphide bonds are formed between the 1st and 3rd cysteines and the 2nd and the 4th cysteines [1,2]. Importantly, the TY53 protein carries only the 2nd and the 4th cysteines, thus presenting only the C motif and being capable of forming a single disulphide bond instead of two. The C-terminal region of TY53 is also much longer than those of other chemokines. We designated this cytokine as SCM-1 from Single Cysteine Motif-1.

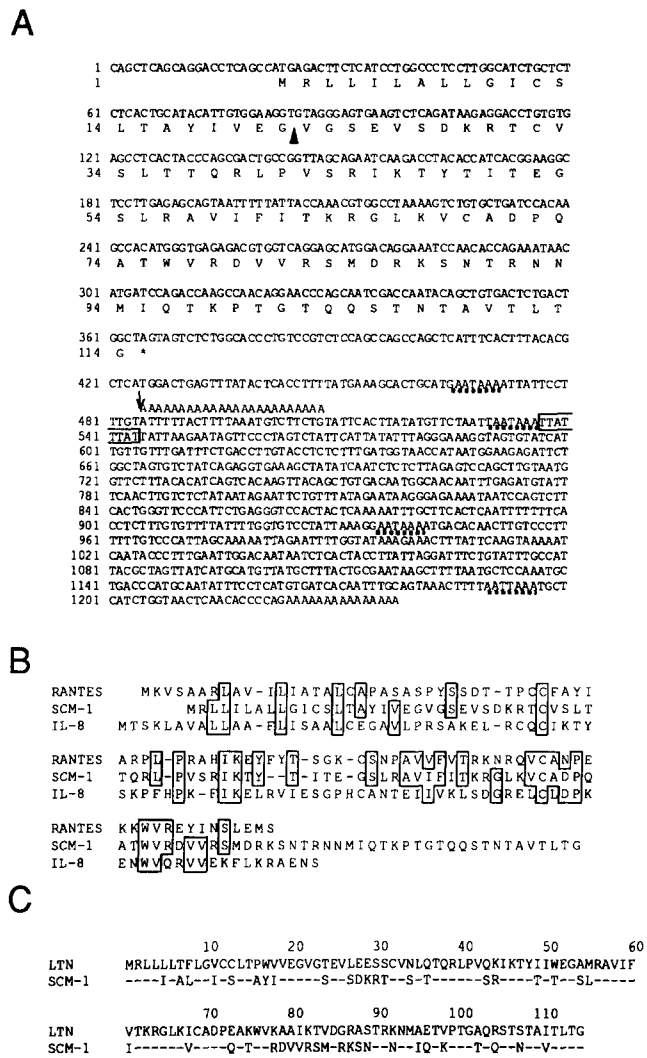

Fig. 1. (A) Nucleotide sequences and the predicted amino acid sequence of the SCM-1 cDNA clones. The probable cleavage site of the signal sequence is indicated by arrowhead. The polyadenylation signals are indicated by dotted lines. The alternative 3′ end of cDNA is indicated by arrow. The mRNA destabilization signal is enclosed. The nucleotide sequence of the human SCM-1 cDNA is available from DDBJ/GenBank/EMBL under accession number of D43768. (B) Alignment of SCM-1 with RANTES (a CC chemokine) and IL-8 (a CXC chemokine). Gaps are introduced for the maximal homology. Amino acids identical between SCM-1 and RANTES and/or IL-8 are enclosed. (C) Alignment of lymphotactin and SCM-1. The amino acid sequence of lymphotactin shown in this figure is in fact deduced from our murine SCM-1 cDNA (the DDBJ/GenBank/EMBL accession number: D43769) that is essentially identical to the lymphotactin cDNA [3] with two nucleotide differences in the coding region. This is because the deduced amino acid sequence of lymphotactin in the original report has a number of apparent translational mistakes [3]. The amino acid 78 should be lysine instead of leucine. An extra glycine is found between amino acids 99 and 100. The amino acid at 110 should be valine instead of isoleucine, which is thus possibly the only amino acid that is different between lymphotactin and our murine SCM-1 (isoleucine at 110).

Fig. 2. Zoo blot analysis of the SCM-1 gene. The zoo blot filter (Clontech) was hybridized with ^{32}P-labeled SCM-1 cDNA (the coding region) at low stringency conditions (5 × SSPE, 30% formamide, at 42°C) and washed (2 × SSC, at room temperature).

3.2. Southern blot analysis and chromosomal assignment

A Zoo blot filter was probed with the coding sequence of the SCM-1 cDNA (Fig. 2). Hybridizing bands were detected in DNAs from human and other mammalian species but not in those from chicken and yeast, suggesting that SCM-1 is well conserved among mammalian species. Chromosomal assignment of SCM-1 gene was carried out by using a panel of somatic hybrids containing known subsets of human chromosomes on hamster or mouse backgrounds. As shown in Fig. 3, the presence of the human SCM-1 gene that was determined by specific PCR correlated only with hybrids containing human chromosome 1 with 5% discordance. It is thus concluded that the SCM-1 gene is located on human chromosome 1.

3.3. Northern blot analysis

Expression of SCM-1 mRNA was next studied by Northern blot analysis using the coding region of SCM-1 cDNA as a probe. Poly(A)$^+$ RNA was prepared from PBMC at indicated time points after stimulation with PHA. As shown in Fig. 4A, no signals were detected in resting PBMC. Upon PHA-stimulation, transcripts of about 0.8 kb rapidly increased with a peak accumulation at 4 h and remained at a high level even after 72 h. The same filter was rehybridized with a probe for human MIP-1α, a CC chemokine. In contrast to SCM-1, MIP-1α transcripts were detected at a considerable level in resting PBMC. After PHA-stimulation, MIP-1α transcripts further increased with a peak accumulation at 4 h but returned to the basal level by 72 h. Induction of SCM-1 gene and that of MIP-1α were also studied in a human T cell line Jurkat and in a monocytoid cell line U937 (Fig. 4A). PHA-stimulation strongly induced SCM-1 mRNA in Jurkat cells with a peak accumulation at 48 h. In contrast, the induction of MIP-1α mRNA by the same treatment was quite rapid and transient with a peak level at 4 h. In the case of U937 cells, various cytokines, especially TNF-α and IL-1α, induced the expression of MIP-1α gene but not that of SCM-1 gene. The relatively long-lasting expression of SCM-1 mRNA after induction, rather unique among the known chemokines [1,2], may be in part related to the lack of the mRNA destabilization sequence in the shorter 3′ non-coding region (Fig. 1A).

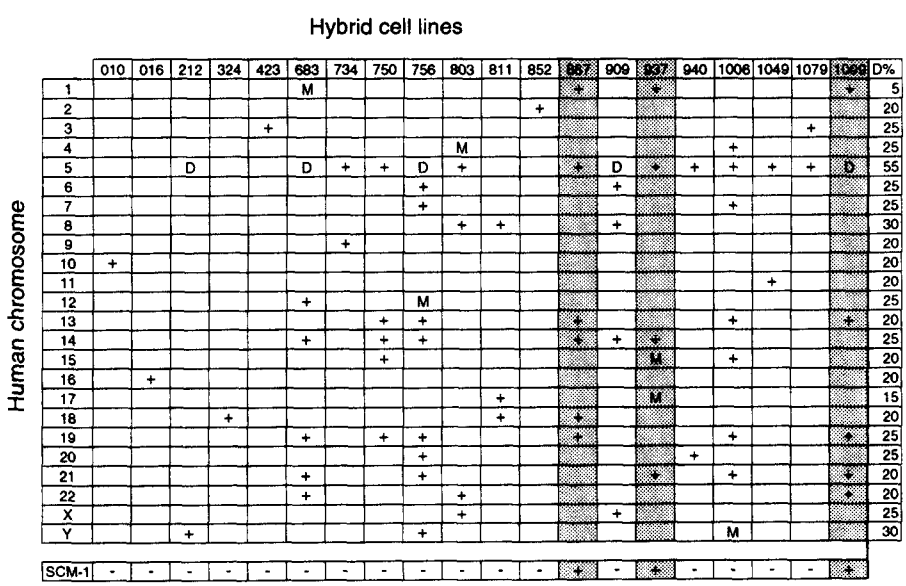

Fig. 3. Chromosomal mapping of the SCM-1 gene. A total of 20 rodent–human somatic cell hybrids were analyzed for the presence of the SCM-1 gene by using specific PCR. Karyotyping symbols are as follows: +, chromosome present in the cell line; M, chromosome present but at low frequency; D, chromosome present but with multiple deletions; blank, chromosome absent. D% is the percent of discordance between the presence of a chromosome and the PCR detection of the SCM-1 gene.

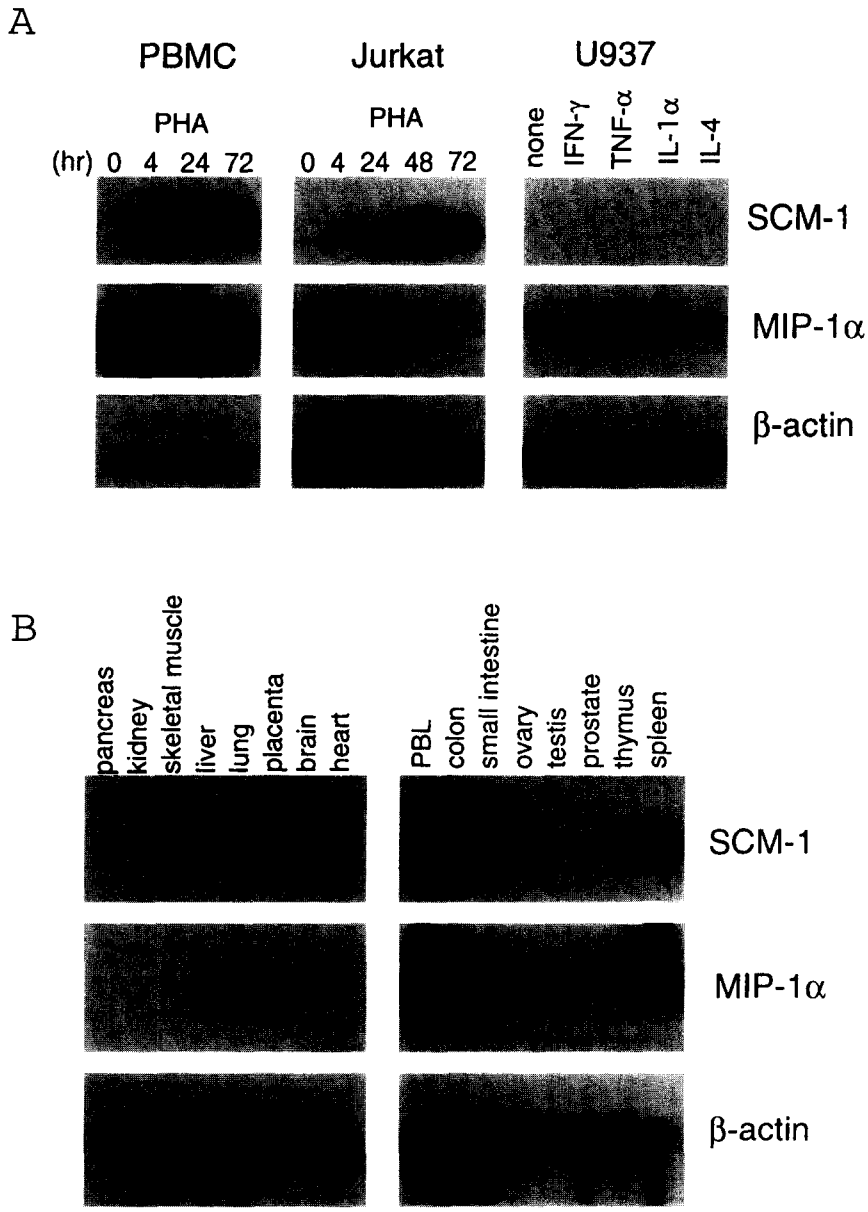

Fig. 4. Northern blot analysis for expression of SCM-1 mRNA in comparison with that of MIP-1α mRNA. (A) Induction of SCM-1 gene as well as that of MIP-1α gene in PBMC, Jurkat (a $CD4^+$ T cell line), and U937 (a monocytoid cell line). PBMC and Jurkat were cultured in the presence of PHA for indicated times. U937 cells were treated without or with IFN-γ (100 U/ml), TNF-α (70 ng/ml), IL-1α (5 ng/ml), or IL-4 (1 ng/ml) for 5 h. Poly(A)$^+$ RNA (2 μg/lane) was subjected to Northern blotting analysis with ^{32}P-labeled SCM-1 cDNA as a probe. The same filters were rehybridized with a probe for MIP-1α as well as with that for β-actin. (B) Multiple Tissue Northern blots filters (Clontech) were hybridized with ^{32}P-labeled SCM-1 cDNA. The same filters were rehybridized with a probe for MIP-1α as well as with that for β-actin.

The expression of SCM-1 in various human tissues was next studied by using Multiple Tissue Northern blots filters. As shown in Fig. 4B, SCM-1 mRNA is most abundantly expressed in spleen. Expression is also seen in peripheral leukocytes (PBL) and thymus. Very low levels of expression are detected in lung, colon and small intestine. The transcripts are below detection levels in other tissues. MIP-1α mRNA was similarly but more broadly detected in spleen, PBL, lung, thymus and some other tissues. Since we never detected SCM-1 mRNA in normal PBMC (Fig. 4A), the presence of SCM-1 mRNA in PBL of the Multiple Tissue Northern blots may be due to induction of SCM-1 gene in vivo for some reasons particular to the source of mRNA.

4. Discussion

No less than 16 different human chemokines are now known. All the chemokines have four cysteines, forming one disulphide bond between the 1st and the 3rd cysteines and another one between the 2nd and the 4th cysteines. From the arrangement of the first two cysteines, chemokines are grouped into two major types, the CXC or α type and the CC or β type. The CXC chemokines act mainly on neutrophils, while the CC chemokines act more broadly on monocytes, lymphocytes, basophiles and eosinophiles. The genes of the CXC chemokines are clustered on human chromosome 4, while those of the CC chemokines map to human chromosome 17 (for review, see [1,2]). In

the present study, we identified a new human cytokine SCM-1. Computer analysis shows that SCM-1 is related to the chemokines and particularly to the CC chemokines. SCM-1 is, however, quite unique because it has only two cysteines, the 2nd and the 4th, instead of four cysteines found in other chemokines. SCM-1 also has a C-terminal sequence much longer than those of other chemokines. Furthermore, the gene for SCM-1 distinctly maps to human chromosome 1. SCM-1 may thus be a new C type chemokine.

Recently, a new murine chemokine named lymphotactin was identified from a cDNA library derived from activated pro-T cells of Balb/c mouse [3]. Like SCM-1, lymphotactin has also only the 2nd and the 4th of the four cysteines conserved in other chemokines. High levels of lymphotactin mRNA were detected in activated $CD8^+$ T cells from thymus and spleen as well as in activated $CD4^-CD8^-TcR\alpha\beta^+$ thymocytes. Expression of lymphotactin gene was not detected in any other tissues. Recombinant lymphotactin was shown to act specifically on lymphocytes and not on monocytes or neutrophils. The gene encoding lymphotactin was mapped to the distal region of mouse chromosome 1 and not to mouse chromosome 11 where the genes for the murine CC chemokines are clustered or mouse chromosome 5 where those for the murine CXC chemokines are probably clustered [1,2]. These characteristics of lymphotactin are in fact quite similar to those of SCM-1. Furthermore, the homology between SCM-1 and lymphotactin is 60.5% at the amino acid level and 70.0% at the nucleotide level (Fig. 1C). It is thus possible that SCM-1 may be the human homologue of murine lymphotactin. In fact, by using SCM-1 cDNA as a probe and from a cDNA library derived from PHA-stimulated Balb/c mouse spleen cells, we had already isolated the murine cDNA encoding putative mouse homologue of SCM-1 which turned out to be essentially identical to the lymphotactin cDNA recently reported [3]. SCM-1 and lymphotactin may thus respectively represent the human and murine prototype of a new C or γ type chemokine family. The biochemical characteristics and the biological activities of SCM-1 are now under investigation.

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