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Chondroitin sulfate B exerts its inhibitory effect on secondary lymphoid tissue chemokine (SLC) by binding to the C-terminus of SLC

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Abstract

We previously reported that certain glycosaminoglycans (GAGs) bind secondary lymphoid tissue chemokine (SLC, CCL21) and that the SLC-binding GAGs, including chondroitin sulfate B (CS B), negatively modulate the function of SLC, although the mechanism remains unknown [J. Biol. Chem. 276 (2001) 5228]. To gain insight into the mechanism of inhibition, we used a C-terminally truncated SLC (SLC-T) that lacked clusters of basic amino acid residues that have been implicated in GAG binding. While SLC-T failed to bind any GAGs, it induced prominent intracellular Ca^{2+} mobilization in CC chemokine receptor (CCR) 7-expressing cells, as did wild-type SLC. However, the SLC-T-induced Ca^{2+} influx was not inhibited by CS B, unlike the SLC-induced Ca^{2+} influx. These results demonstrate the requirement of the C-terminus of SLC for the inhibition of chemokine responses by CS B; that is, CS B exerts its inhibitory effect by binding to the C-terminus of SLC, thus defining the mode of action of CS B on certain chemokines.

Keywords: Chemokine; Secondary lymphoid tissue chemokine (SLC); CC chemokine receptor (CCR) 7; Glycosaminoglycan; Chondroitin sulfate B; Chondroitin sulfate E

1. Introduction

Chemokines bind not only to small-G-protein-coupled seven-transmembrane receptors but also to glycosaminoglycans (GAGs) such as chondroitin sulfate (CS) and heparan sulfate (HS) [1–3]. The binding of chemokines to GAGs on cell surfaces or in extracellular matrices has been thought to be important for chemokine function. The immobilization of chemokines by GAGs increases the effective local concentration of chemokines on the surface and in the vicinity of endothelial cells, resulting in the effective presentation of chemokines to extravasating leukocytes [4,5].

Secondary lymphoid tissue chemokine (SLC, CCL21) is a member of the CC chemokine subfamily and is a potent chemoattractant for naive lymphocytes [6]. SLC is mainly expressed in the high endothelial venules (HEVs) of the lymph node [6], which are pivotal sites for lymphocyte homing from the peripheral blood to lymph nodes and Peyer's patches. SLC can induce the activation of $\alpha L\beta 2$ (LFA-1) and $\alpha 4\beta 7$ integrin on lymphocytes, which results in the arrest and firm adhesion of lymphocytes to the integrin ligands expressed on HEV cells [7–9]. The critical role of SLC in lymphocyte homing has been shown in *plt*/*plt* (paucity in lymph node T cells) mice [10,11], whose HEV cells are deficient in SLC expression,

Abbreviations: GAG, glycosaminoglycan; CS, chondroitin sulfate; HS, heparan sulfate; SLC, secondary lymphoid tissue chemokine; HEV, high endothelial venule; CCR, CC chemokine receptor; CHO, Chinese hamster ovary; FCS, fetal calf serum; MACS, magnetic cell sorting

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and in mice that are deficient in CC chemokine receptor (CCR) 7, the receptor for SLC, which show severely reduced numbers of naive T lymphocytes in their peripheral lymph node [12]. These findings suggest that SLC is one of the essential chemokines for T lymphocyte recirculation.

We previously reported that SLC can bind a variety of GAGs and that the function of SLC is negatively regulated by CS B, but not by HS, while HS is known to augment chemokine functions [13]. This result suggests that SLC might exhibit different activities according to the feature of the GAGs that it binds to. However, the precise mechanism of how certain GAGs, such as CS B, can inhibit chemokine signaling remains unknown.

The GAG binding site on chemokines has been studied by various methods including in vitro mutagenesis, and clusters of basic amino acids, such as lysine and arginine, in the C-terminal portion of chemokines, have been implicated in the binding of heparin/HS [14–16]. However, it remains unknown whether CSs bind to the same site, or how certain CSs such as CS B can interact with SLC. Unlike other chemokine family members, SLC has a unique extended C-terminus of 32 amino acids that contains numerous basic amino acids [17,18]. These clusters of basic amino acids are positively charged and may interact directly with GAGs bearing strong negative charges, possibly making the extended C-terminus of SLC responsible for binding to GAGs. To gain insight into the mechanism of inhibition of SLC signaling by certain GAGs, we used a truncated mutant of the extended C-terminus of SLC to examine whether the C-terminus of SLC is involved in the functional modulation by GAGs. Here we demonstrate that the C-terminus of SLC is not required for SLC signaling, but essential for the inhibition of SLC signaling by CS B. This study may help to understand the inhibition mechanism of chemokine signaling by certain GAGs.

2. Materials and methods

2.1. Reagents

Truncated SLC (SLC-T) was generated by one of us (M.S.W.) as described below. The anti-human CCR7 monoclonal antibody (mAb) CCR7.6B3 was generated as described previously [19]. The anti-chondroitin sulfate mAb CS56 [20] and anti-heparan sulfate mAb 10E4 [21], chondroitin (whale cartilage), chondroitin sulfate (CS) A (whale cartilage), CS B (pig skin), CS C (shark cartilage), CS D (shark cartilage) and CS E (squid cartilage) were all obtained from Seikagaku Kogyo (Tokyo, Japan). Heparan sulfate (porcine intestinal mucosa) and heparin (porcine intestinal mucosa) were purchased from Sigma (St. Louis, MO). The other reagents used in this study have been described previously [13].

2.2. Cells

The proteoglycan-deficient variant pgsA-745 Chinese hamster ovary (CHOA745) cells [22], which are deficient in xylosyltransferase, were cultured in Ham's F-12 medium (Sigma) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (Ham's F-12–FCS).

2.3. Western blot analysis

Western blotting analysis was performed according to the standard procedures using biotinylated goat anti-human SLC polyclonal antibody (1 μg/ml, DAKO).

2.4. Production of truncated SLC

Recombinant truncated SLC (SLC-T, amino acids 1–79) was produced using the *Pichia pastoris* yeast expression system (Invitrogen). The coding sequence for SLC-T was generated by PCR using the sense primer 5′-GAA**CTC-GAG***AAAAGA*AGTGATGGAGGGGCT-3′ containing an *Xho*I restriction site (in bold), yeast α-secretion factor sequence before the Kex2 cleavage site (in italics) and the codons of the first five amino acids of human SLC (underlined), and the antisense primer 5′-ATT**TCTAGA***CTA*-GCCCTGGGCTGGTTTCTG-3′ corresponding to the codons for amino acids 74–79 (underlined), a stop codon (italics), and an *Xba*I restriction site (bold). The template for the PCR reaction was PCRII/huSLC (kindly provided by Dr. Kunio Hieshima, Kinki University). The PCR product was cloned into pPicZαA, transfected into *Escherichia coli* DH5α, and recombinant colonies were selected on plates containing 25 μg/ml zeocin. Plasmid from each colony was purified and the sequence of the insert confirmed. Plasmid (10 μg) containing the correct sequence was linearized using *Pme*I and transfected into GS115 *P. pastoris* using electroporation. Protein production was induced following the manufacturer's protocol. SLC-T was purified using cation exchange chromatography from the culture supernatant to a purity of ~98% as determined by SDS-PAGE and Coomassie staining.

2.5. ELISA

ELISA was performed as described previously [13].

2.6. Generation of CCR7 transfectants

Human CCR7 cDNA [17] was subcloned into the expression plasmid pCI-neo (Promega, Madison, WI). The plasmid pCI-neo/CCR7 was then transfected into CHOA745 cells using LipofectAMINE (GIBCO BRL, Life Technologies, NY). Following selection for resistance to geneticin (1 mg/ml, Sigma), positive colonies were selected by examining the binding of SLC on an EPICS-XL flow cytometer

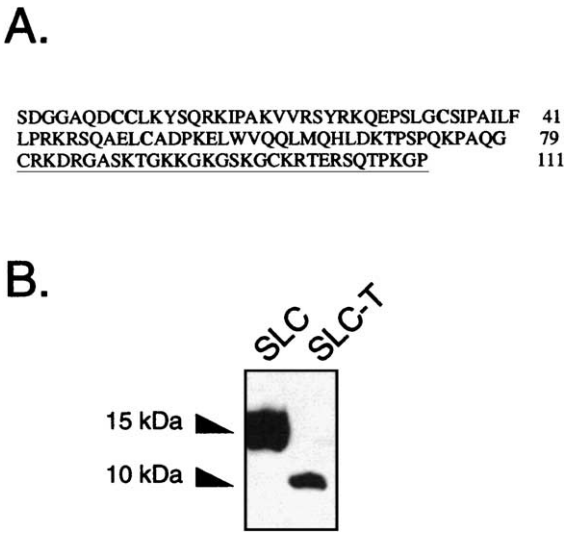

Fig. 1. Comparison between SLC and SLC-T. Amino acid sequence of SLC. The portion of the C-terminal sequence that was truncated to make SLC-T is indicated by an underline. (B) SLC and SLC-T (100 ng) were subjected to electrophoresis under reducing conditions on a 15–20% acrylamide gradient SDS-PAGE gel. Subsequently, the proteins were electroblotted onto an IPVH membrane and detected with anti-human SLC polyclonal antibody (1 μg/ml). Blots were developed with ECL. Black arrows indicate the molecular size of each protein.

(Coulter Electronics, Hialeah, FL). Finally, high-CCR7 expressors were isolated by magnetic cell sorting (MACS) separation using mouse anti-human CCR7 mAb, CCR7.6B3 [19] and goat anti-mouse IgG microbeads (Miltenyi Biotec, Germany).

2.7. Flow cytometric analysis

CCR7 transfectants (1×10^5 cells) detached from culture flasks with PBS containing 0.02% EDTA were incubated with various mAb indicated in Fig. 3 for 30 min on ice. After washing, the cells were incubated with FITC-goat

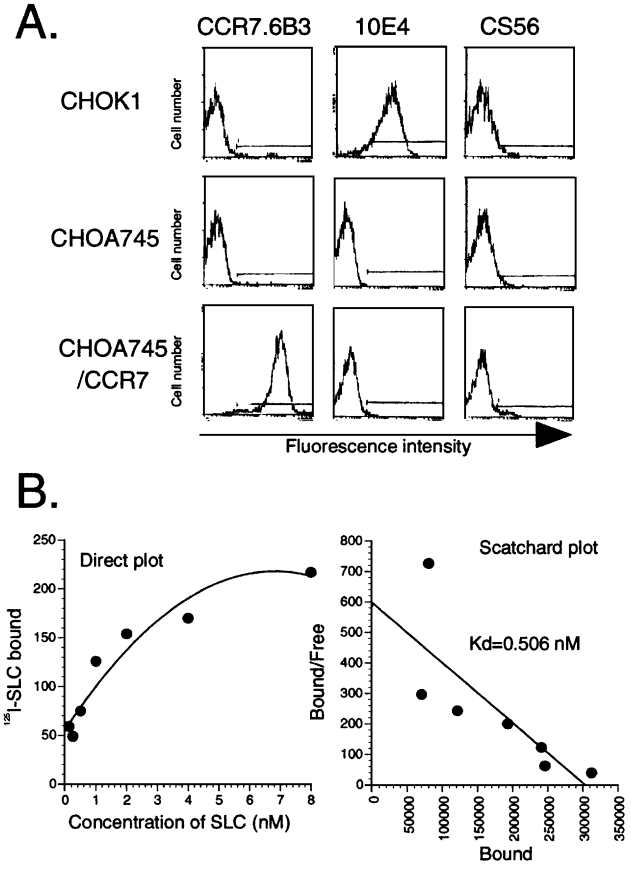

Fig. 3. Expression of CCR7 on transfectants. (A) Expression of CCR7 on CHOA745 cells. Cells (3×10^5) were first incubated with the anti-human CCR7 mAb CCR7.6B3 (2 μg/ml), the anti-heparan sulfate mAb 10E4 (5 μg/ml), or the anti-chondroitin sulfate mAb CS56 (1:500). After washing, the cells were stained with FITC-goat anti-mouse IgG $F(ab')_2$ (2 μg/ml) (for mAbs CCR7.6B3 and 10E4) or FITC-donkey anti-mouse IgM (for mAb CS56), respectively. The flow cytometric analysis was performed with an EPICS-XL flow cytometer. (B) Binding characteristics of ^{125}I-SLC to CHOA745/CCR7 transfectants. Saturation binding was performed with ^{125}I-SLC concentrations of 0.125–8 nM and Scatchard analysis was followed by standard protocols. Representative results from three separate experiments are shown.

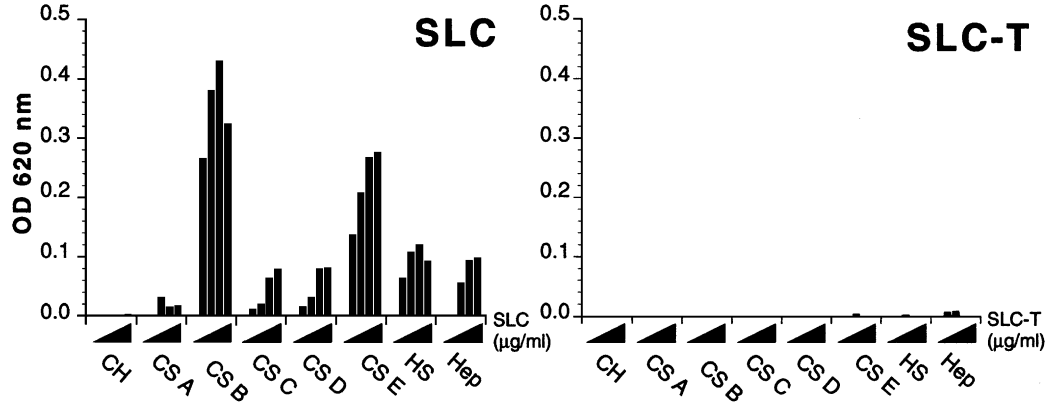

Fig. 2. SLC-T fails to bind GAGs. Binding of SLC or SLC-T to immobilized GAGs was determined by ELISA. GAGs (50 μg/ml) were immobilized on ELISA plates. After blocking with FCS, serially diluted SLC (0, 0.01, 0.03, 0.1, 0.3 μg/ml) or SLC-T (0, 0.1, 0.3, 1, 3 μg/ml) was added to the GAGs-immobilized wells. Binding of chemokines was detected as described in Materials and Methods and shown as bars.

anti-mouse IgG F(ab′)$_2$ or FITC-donkey anti-mouse IgM for 40 min on ice. The cells were then washed and analyzed on an EPICS-XL flow cytometer.

2.8. Radioligand binding analyses

The binding of ^{125}I-labeled SLC to CHOA745/CCR7 cells was detected as described previously [13].

2.9. Ca^{2+} mobilization assay

This was carried out as described previously [13].

3. Results

3.1. The extended carboxyl-terminal truncated SLC (SLC-T) fails to bind GAGs

Unlike other chemokines, SLC has a unique 32-amino-acid-long C-terminal tail that contains 2 additional cysteine residues and 12 basic amino acid residues (Fig. 1A). To examine the possibility that the extended C-terminus of SLC may interact with anionic sulfate groups present on GAGs, we generated a truncated mutant devoid of these C-terminal 32 amino acids (SLC-T). When resolved on SDS-PAGE and immunoblotted with an antibody against human SLC, SLC-T containing only amino acids 1–79 of human SLC was approximately 10 kDa, while the wild-type SLC is approximately 15 kDa (Fig. 1B).

To determine whether the extended C-terminus of SLC contributes to the binding ability of SLC to GAGs, we examined the ability of SLC-T to bind various GAGs using ELISA. SLC bound strongly to CS B and CS E, moderately to HS and heparin, and weakly to CS C and CS D, and the binding was dose dependent (Fig. 2). In contrast, SLC-T failed to bind any of the GAGs examined, even if a 10-fold higher dose of SLC-T than the wild-type SLC was used (Fig. 2). This result suggests that the extended C-terminus tail of SLC is critical for SLC's binding to GAGs.

3.2. CS B regulates SLC-induced Ca^{2+} mobilization via the C-terminus of SLC

We previously demonstrated using Ca^{2+} mobilization assays that CS B negatively regulates SLC signaling [13]. To understand the mechanism of the inhibition of SLC signaling by CS B, we examined whether CS B exerts its inhibitory effects on SLC by binding to its extended C-terminus. First, we generated a transfectant cell line that expressed CCR7, the SLC receptor, using a GAG-deficient parental cell line, CHOA745, so that the interaction between SLC and CCR7 could be examined in the absence of any cell-surface-associated GAGs that might otherwise bind SLC. Fig. 3A shows that cells transfected with the CCR7 cDNA expressed high levels of CCR7, whereas cells transfected with vector alone expressed no CCR7. Neither the anti-HS mAb, 10E4, nor the anti-CS mAb, CS56, reacted with CHOA745/CCR7 or CHOA745 (Fig. 3A). Scatchard analysis showed that SLC bound to a single class of high-

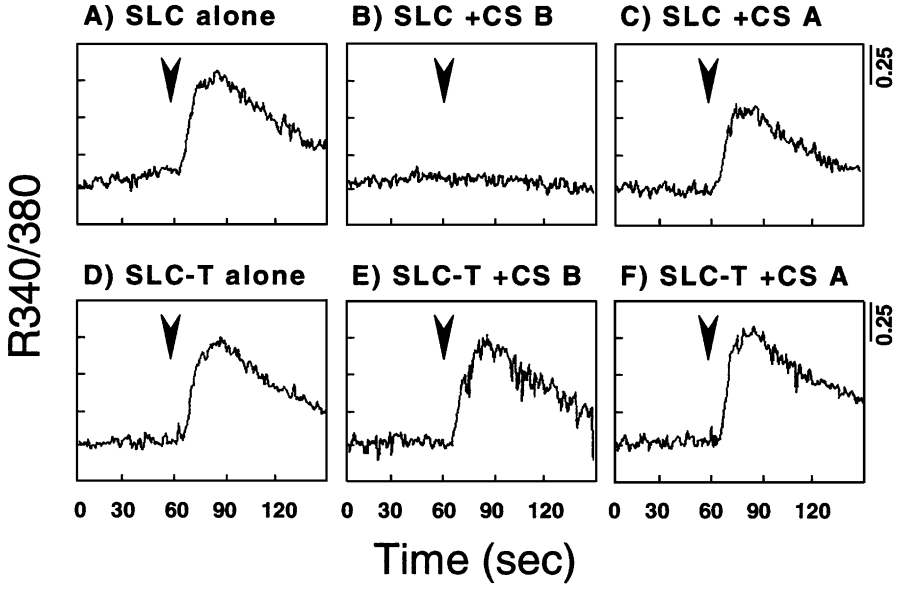

Fig. 4. CS B exerts its inhibitory effect on SLC by binding to the C-terminus of SLC. CHOA745/CCR7 (1×10^6 cells/ml) were loaded with Fura-2 and stimulated with SLC or SLC-T (100 ng/ml) in the presence or absence of GAGs (50 μg/ml). Arrowheads indicate the time of application of the stimulator. Intracellular calcium concentration was monitored by measuring the fluorescence ratio (F340/380). The scale is shown as a bar on the right. Representative results from two separate experiments are shown.

affinity receptors expressed on CHOA745/CCR7 cells with a K_d of 0.51 nM (Fig. 3B), indicating that SLC interacted directly with CCR7.

When cell line CHOA745/CCR7 was used, CS B completely inhibited the SLC-induced Ca^{2+} response but not the SLC-T-induced Ca^{2+} response (Fig. 4). CS A, which does not bind SLC, affected neither of the responses (Fig. 4). In addition, this inhibition was not observed when SLC-T was preincubated with a high concentration (100 μg/ml) of CS B (data not shown). These observations demonstrate that the inhibitory effect of CS B on SLC signaling is mediated through the C-terminus of SLC.

4. Discussion

We have previously shown that CS B binds certain chemokines such as SLC and inhibits the SLC-induced integrin activation and intracellular Ca^{2+} influx [13]. However, the precise mechanism underlying how CS B inhibits these signaling events has not been fully understood. In this study, we have demonstrated, using a C-terminally truncated mutant of SLC, that CS B exerts an inhibitory effect on SLC via its extended C-terminus.

Previous investigations attempting to elucidate HS/heparin binding regions on chemokines have shown that some basic amino acid residues in their primary amino acid sequences are critical for GAG binding [14–16,23–26]. For instance, four conserved basic amino acids, R18, K45, R46, and R48, are critical for GAG binding of MIP-1α [23] and MIP-1β [24], and K58 and H66 are also essential for GAG binding of MCP-1 [16]. SLC possesses in its C-terminal region numerous basic amino acid residues, and in particular two pieces of the consensus sequence for GAG recognition, that is, (-X-B-B-X-B-X-) [27] where B represents any basic residue and X represents any non-basic residue. We thus hypothesized that the deletion of the extended C-terminus would result in the abolition of the GAG-binding ability of SLC. As expected, the truncated mutant, SLC-T, failed to bind any GAGs, including CS B (Fig. 2).

To evaluate the functional capacity of SLC and SLC-T, its truncated mutant, we expressed receptors for SLC in a variant of the CHO cell line that is defective in its ability to synthesize GAGs [22]. While the parental CHOK1 cell line expressed endogenously produced GAGs on the cell surface (Fig. 3A), allowing the binding of IP-10 [25], SDF-1 [26], and SLC, a type of binding that is sensitive to heparitinase treatment (J. Hirose, unpublished observation), the CHOA745 cell line, which is deficient in xylosyltransferase [22], expressed no detectable cell-surface GAGs (Fig. 3A) and showed no binding of SLC (data not shown). Transfection of CCR7 cDNA into the CHOA745 line yielded the CHOA745/CCR7 cell line, which expressed a single class of high-affinity receptors for SLC (Fig. 3B). In this cell line, SLC induced an intracellular Ca^{2+} influx that was inhibited by CS B, whereas SLC-T yielded a comparable level of intracellular Ca^{2+} influx, which was not inhibitable with CS B, demonstrating that the C-terminus of SLC was required for the CS-B-induced inhibition of the chemokine response. However, the conclusion whether or not the C-terminus of SLC makes any measurable contributions to receptor binding or biological activity awaits further characterization.

CS B is composed of repeating disaccharide units of L-iduronic acid and *N*-acetylgalactosamine and can bind SLC. Other GAGs bearing L-iduronic acid, such as heparin and HS, can also bind SLC, whereas CS A, which bears L-glucuronic acid instead of L-iduronic acid in the repeating disaccharide units, cannot bind SLC (Fig. 2 and Ref. [13]). Although these observations may indicate the importance of L-iduronic acid residues in chemokine binding, the observation that CS E, which is devoid of L-iduronic acid residues, can also bind SLC (Fig. 2) and inhibit SLC-induced Ca^{2+} response [28] indicates that SLC binding to GAGs is also controlled by some other factor(s). Understanding the precise features of the carbohydrate structure that are required for chemokine binding will require further investigation.

In the present study, we found that CS B can inhibit SLC-induced intracellular Ca^{2+} influx even in a GAG-deficient cell line expressing only high-affinity receptors for SLC, thus demonstrating that the ability of CS B to inhibit SLC signaling is not due to competition with low-affinity receptors. However, we are still unable to completely exclude the possibility that CS B interferes with the high-affinity binding of SLC to its cognate receptors because the experimental condition used in the present study was different from our previous study in which we had suggested this possibility unlikely [13]. Currently, there are at least two other possible mechanisms for the inhibition of SLC-mediated signaling by CS B that remain to be tested. These are: (a) that CS B somehow interferes with signal generation from the high-affinity receptors for SLC without inhibiting high-affinity binding, and (b) that the CS B-SLC complex generates an inhibitory signal. Future studies will be required to test these possibilities.

In conclusion, this study demonstrates that CS B negatively regulates SLC-induced Ca^{2+} mobilization via the extended C-terminus of SLC. Given the importance of chemokines in immune and inflammatory responses, it is essential to understand how chemokine activities are regulated in vivo. Thus, further studies examining the mechanism of inhibition of chemokine signaling by certain naturally occurring GAGs such as CS B are warranted.

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