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Scavenger receptor expressed by endothelial cells (SREC)-I interacts with protein phosphatase 1α in L cells to induce neurite-like outgrowth

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Abstract

The scavenger receptor expressed by endothelial cells (SREC)-I was originally identified in a human endothelial cell line by expression cloning. Subsequently it was shown that the cytoplasmic domain of SREC-I mediates the neurite-like outgrowth of murine fibroblastic L cells through interaction with advillin, a member of gelsolin/villin family of actin regulatory proteins. In this work, we further searched for a binding protein to the cytoplasmic domain of the receptor, which might be required for the morphological change of L cells and identified protein phosphatase 1α (PP1α) as a binding protein to this domain. It was revealed that PP1α binds to the central region (i.e., residues between 461 and 560) of the cytoplasmic domain of the receptor. By the expression of truncated forms of SREC-I lacking C-terminal amino acids, it was suggested that the morphological change is a two step process (i.e., elongation/sprouting and process formation) mediated by two distinctive cytoplasmic regions of SREC-I and PP1α is required for the process formation. Our system may be useful for the elucidation of the mechanism of morphological maturation of neuronal cells such as dorsal root ganglion neurons that express SREC-I during early development.
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Scavenger receptors are defined by their ability to bind and metabolize modified low density lipoproteins (LDLs), such as acetylated LDL (AcLDL) and oxidized LDL (OxLDL), and have been regarded as relevant in the pathogenesis of atherosclerosis [1,2]. Mammalian cells have several different classes of scavenger receptors and endothelial cells express several distinct receptors such as SR-BI, LOX-1, and FEEL-1/stabilin-1 [2–8]. In addition to these receptors, we cloned a novel scavenger receptor from a cDNA library prepared from human umbilical vein endothelial cells (HUVEC) and termed it scavenger receptor expressed by endothelial cells (SREC)-I [9]. Subsequently, we cloned a homologous protein, SREC-II by a database search [10]. These two receptors are now classified into type F scavenger receptors [2].

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Both SREC-I and -II contain 10 repeats of epidermal growth factor-like cystein-rich motifs in their extracellular domains and unusually long C-terminal cytoplasmic domains with serine- and proline-rich regions. While extracellular domains are highly homologous to each other, cytoplasmic domains show less homology [9,10]. In contrast to SREC-I, SREC-II has little activity to uptake modified LDL (scavenger receptor activity) [10].

Recent functional analyses of SRECs suggest that they are multi-functional. In addition to scavenger receptor activity originally identified on SREC-I, SREC-I and -II display respective homophilic interaction through their extracellular domains between separate cells and strong SREC-I/SREC-II heterophilic interaction, suggesting that type F scavenger receptors can act as cell–cell adhesion molecules [10]. On the other hand, as in the case of scavenger receptor activity, SREC-I but not SREC-II induces the neurite-like outgrowth of L cells [11]. It was found that the

SREC-I-mediated morphological change requires the binding of advillin, a member of gelsolin/villin family of actin regulatory proteins, to the cytoplasmic domain of the receptor. Since both SERC-I and advillin are expressed in dorsal root ganglion neurons during embryonic development, SREC-I might play a role in the morphogenesis of some neuronal cells.

In this paper, we further examined the possible role of the cytoplasmic domain of SREC-I and found that protein phosphatase 1α (PP1α) bound to the domain and might play a role in the receptor-mediated morphological change of L cells. It was found that two distinctive regions of the cytoplasmic domain of SREC-I mediate the different steps of the morphological change of L cells. Our results suggest that L cells can be a model system for the SREC-I-mediated morphogenesis of neuronal cells.

Materials and methods

Cell culture. Murine L cells (CCL-1, American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Yeast two-hybrid screening. Proteins that bind to the cytoplasmic domain of human SREC-I were screened by the DupLEX-A Yeast Two Hybrid System (Origene Technologies). The cDNA fragment encoding the cytoplasmic domain of human SREC-I was subcloned into the BamHI site of pEG202 plasmid-fused in-frame with the DNA-binding domain of LexA (pEG202-SREC-IC). The EGY48 yeast strain was first transformed with pEG202-SREC-IC and pSH18-34 reporter plasmid and then transformed with HUVEC cDNA library in pJG4-5 plasmid. Co-transformants (2×10^7 transformants) were plated onto YNB(gal)-ura-his-trp-leu plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Positive clones were picked up and then grown in 2 ml of YNB(glu)-trp medium. Plasmids were recovered from yeast cells by glass beads preparation and cDNAs encoding potential clones that may interact with the cytoplasmic domain of SREC-I were amplified by PCR and sequenced.

Plasmid construction. The EcoRI–NotI fragment of the human cDNA for SREC-I was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and termed pcDNA3-SREC-I. The EcoRI–XhoI fragment of the human cDNA for SREC-I cytoplasmic domain deletion mutants that lacks amino acid residues 461–830 (SREC-I-ΔC370), 561–830 (SREC-I-ΔC270) and protein phosphatase α (PP1α) were subcloned into the mammalian expression vector pcDNA3 and expression plasmids were termed pcDNA3-SREC-I-ΔC370, pcDNA3-SREC-I-ΔC270, and pcDNA3-PP1α, respectively.

Uptake of DiI-AcLDL (scavenger receptor activity). L cells (1×10^5 cells/well) in 24-well plates were mock transfected or transfected with either pcDNA3-SREC-I, pcDNA3-SREC-I-ΔC370, pcDNA3-SREC-I-ΔC370 or pcDNA3-PP1α using LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. The cells were incubated for 48 h, further incubated in the presence of 2 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchorate (DiI)-AcLDL (Biomedical Technologies Inc.) for 2 h, washed, and then fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. The presence of fluorescent DiI in the fixed cells was determined by visual inspection using fluorescence microscopy.

Preparation of GST-fusion proteins. The EcoRI–SalI fragment encoding the amino acid residues 451–643 (C1) or those 561–752 (C2) of the cytoplasmic domain of human SREC-I was subcloned into a multi-cloning site downstream of the sequence for glutathione *S*-transferase (GST) in pGEX-4T-1 (Pharmacia). This plasmid was transformed into the JM109 strain of *Escherichia coli* and induced with isopropyl-1-thio-β-D-galactopyranoside to produce GST-fusion proteins. The bacteria were suspended in PBS, and vigorous sonication was performed before centrifugation at 10,000*g* for 20 min. The resulting supernatants were applied to a glutathione–Sepharose column and then eluted with an elution buffer (50 mM Tris–HCl, pH 9.6, 120 mM NaCl, 10 mM glutathione). Purified GST-fusion proteins were dialyzed against PBS containing 2 mM EDTA and 1 mM dithiothreitol.

GST-affinity chromatography. L cells (6×10^7 cells) were harvested and homogenized in 1 ml of PBS and then centrifuged at 100,000*g* for 1 h at 4 °C. The resultant supernatant was used as cytosolic extract. Recombinant GST-C1 or -C2 fusion proteins, bound to the glutathione–Sepharose column, were used to affinity-purify C1- or C2-binding protein(s). L cell cytosolic extracts were loaded onto the GST-C1 or -C2 glutathione–Sepharose columns and then eluted with the elution buffer. The eluted fractions of affinity chromatography were collected, precipitated by 10% trichloroacetic acid, and then subjected to SDS–PAGE. Bound PP1α was detected by Western blot analysis employing anti-PP1α monoclonal antibody (Transduction Laboratories).

Preparation of anti-SREC-I antibody. The polyclonal antibody against human SREC-I was prepared as follows. Peptide corresponding to the C-terminal domain of human SREC-I (NH_2-ERQEEPEYENVVPISRP-PEP-COOH) was synthesized. The synthesized peptide was conjugated with keyhole limpet hemocyanin using an Imject sulfhydryl-reactive antibody production Kit (Pierce). The keyhole limpet hemocyanin peptides were gel-purified and emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories). Female Wistar rats were immunized with the emulsions. These rat sera were collected and purified using an affinity column (Sulfolink Coupling Gel, Pierce) to which the antigen peptide was coupled.

Immunoprecipitation. L cell extracts were prepared in lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% (w/v) Triton X-100, 0.5% (w/v) Nonidet P-40, 1 mM EDTA, protease inhibitor mixture (Sigma), 1 mM phenylmethylsulfonyl fluoride). The extracts were precleared for 2 h with protein G-agarose beads (Amersham Biosciences) and then incubated overnight with either anti-SREC-I or anti-PP1α antibody at 4 °C. Immunocomplexes were precipitated with protein G-agarose beads for 45 min, washed three times with lysis buffer, and boiled in SDS sample buffer containing 2-mercaptoethanol. The supernatants were subjected to SDS–PAGE and Western blotting.

Metabolic labeling. L cells-expressing SREC-I were incubated for 30 min in phosphate-depleted DMEM. Cultures were then labeled in labeling media containing 0.3 mCi (1 Ci = 37 GBq) of [^{32}P]orthophosphate for 4 h. Cells were then lysed in lysis buffer. Lysates were immunoprecipitated with anti-SREC-I antibody. The immunoprecipitates were then analyzed by Western blotting and autoradiography.

Materials. Tautomycin and okadaic acid were purchased from Wako Pure Chemicals.

Results

Screening of SREC-I-binding proteins by a yeast two-hybrid system

In our previous work, we have shown that SREC-I induced the morphological change of L cells and neurite-like outgrowth was observed through the interaction between SREC-I and advillin [11]. To further elucidate the mechanism of SREC-I-mediated morphological change of L cells, we searched for proteins that interact with the cytoplasmic domain of SREC-I by a yeast two-hybrid system. A yeast strain EGY48 expressing the sequence between 451 and 830 of SREC-I fused to the LexA DNA-binding domain as the bait was transformed with a cDNA library in which the LexA activation domain was fused to cDNAs prepared from HUVEC. Based on the ability of positive clones to

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