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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.

Keywords: Advillin; L cells; Process formation; Protein phosphatase; Scavenger receptor

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].

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

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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

grow in media lacking tryptophan leucine, histidine, and uracil and to express β-galactosidase, 50 cDNA clones were isolated and subjected to sequence analysis. Since sequence analysis of the positive clones revealed that they were usually incomplete, full-length sequences were determined from databases. Among the sequences determined, 8 out of 29 clones were determined to be protein phosphatase 1α (PP1α), which was the most frequently identified protein in this assay, suggesting that PP1α indeed bind to and plays a role in the process formation of L cells induced by SREC-I.

Identification of PP1α as an SREC-I-binding protein

To examine whether PP1α indeed binds to the cytoplasmic domain of SREC-I, binding of PP1α to GST-fused C-terminal fragments of the SREC-I protein was assayed. We prepared fragments termed GST-C1 (residues between 451 and 643) and GST-C2 (residues between 561 and 756) as reported previously [11] and employed for the binding experiments. The cytoplasmic fraction prepared from L cells was loaded onto either GST-C1 or GST-C2 affinity column, and the proteins bound to the respective column were co-eluted with GST-fused peptide by the addition of glutathione. As shown in Fig. 1, PP1α bound to GST-C2 but not to GST-C1. These results indicated that as in the case of advillin, PP1α bound to the amino acid sequence between residues 643 and 752 of the SREC-I cytoplasmic domain.

We then examined whether PP1α binds to SREC-I in intact cells. L cells transfected with cDNAs for both proteins were solubilized and soluble cell lysate was immunoprecipitated with anti-PP1α antibody. As shown in Fig. 2, SREC-I and PP1α were co-immunoprecipitated with anti-PP1α antibody in a dose dependent manner. On the other hand, the receptor was barely detectable in a control precipitate when co-immunoprecipitation was performed employing unrelated IgG prepared from pre-immune serum. In the absence of cell lysate, no protein corresponding to the molecular weight of SREC-I was detectable, indicating that the interaction indeed occurs in intact cells. When cells transfected only with PP1α cDNA were employed, no detectable band corresponding to the molecular weight of SREC-I was co-immunoprecipitated with anti-PP1α antibody (data not shown), confirming the specificity of the antibody employed.

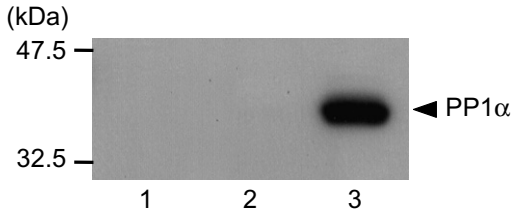

Fig. 1. Identification of PP1α as an SREC-I-binding protein. The L cell cytoplasmic fraction was loaded onto glutathione–Sepharose columns coated with GST (lane 1), GST-C1 (lane 2) or GST-C2 (lane 3). The bound proteins were eluted by the addition of glutathione. The eluates were subjected to SDS–PAGE followed by Western blot analysis with anti-PP1α antibody.

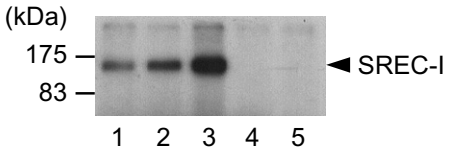

Fig. 2. Interaction of SREC-I and PP1α *in vivo*. After L cells transfected with cDNAs for SREC-I and PP1α were solubilized, SREC-I was immunoprecipitated with anti-PP1α antibody (lane 1, 1 μg; lane 2, 2 μg; lane 3, 4 μg antibody). An experiment shown in lane 4 was performed in the absence of cell lysate using 4 μg of the antibody. Lane 5 shows the results using 4 μg of unrelated IgG1. The resulting immunoprecipitates were then analyzed by Western blotting with anti-SREC-I antibody.

When cell lysate was immunoprecipitated with anti-SREC-I antibody first, co-precipitation of PP1α was also detected (data not shown).

Morphological changes of L cells by the cytoplasmic domain of SREC-I

To elucidate the role of C-terminal cytoplasmic domain of SREC-I in the induction of neurite-like outgrowth of L cells, cDNAs for truncated forms of SREC-I were prepared (Fig. 3A) and transfected into L cells. As shown in Fig. 3B, while full-length SREC-I induced the morphological change, SREC-I lacking C-terminal 370 amino acids (between 461 and 830) (SREC-I-ΔC370) did not at all. Intriguingly, when SREC-I lacking C-terminal 270 amino acids (between 561 and 830) (SREC-I-ΔC270) was expressed in L cells, there occurred the change of cell shape and cell elongation (sprouting) was observed. These results suggested that there are two distinguishable steps in the SREC-I-mediated morphological change of L cells. The first step is elongation/sprouting in which the C-terminal amino acid sequence between residues 461 and 560 is involved, since this process occurred by SREC-I-ΔC270, but not by SREC-I-ΔC370. The second is process formation mediated by that between 561 and 830, which includes PP1α-binding sequence (between 643 and 752), since this process occurred only by full-length SREC-I. Thus, the process formation but not elongation/sprouting step might require the binding of PP1α to the C-terminal region of SREC-I. It should be noted here that both SREC-I-ΔC370 and SREC-I-ΔC270 showed comparable AcLDL up-take activities to full-length SREC-I, indicating that in contrast to the morphological change, the scavenger receptor activity of SREC-I is independent of its cytoplasmic domain [10,11].

Effects of protein phosphatase inhibitors on the SREC-I-mediated morphological change of L cells

We then examined the effects of protein phosphatase inhibitors (i.e., tautomycin and okadaic acid) on the SREC-I-mediated morphological change of L cells (Fig. 4).

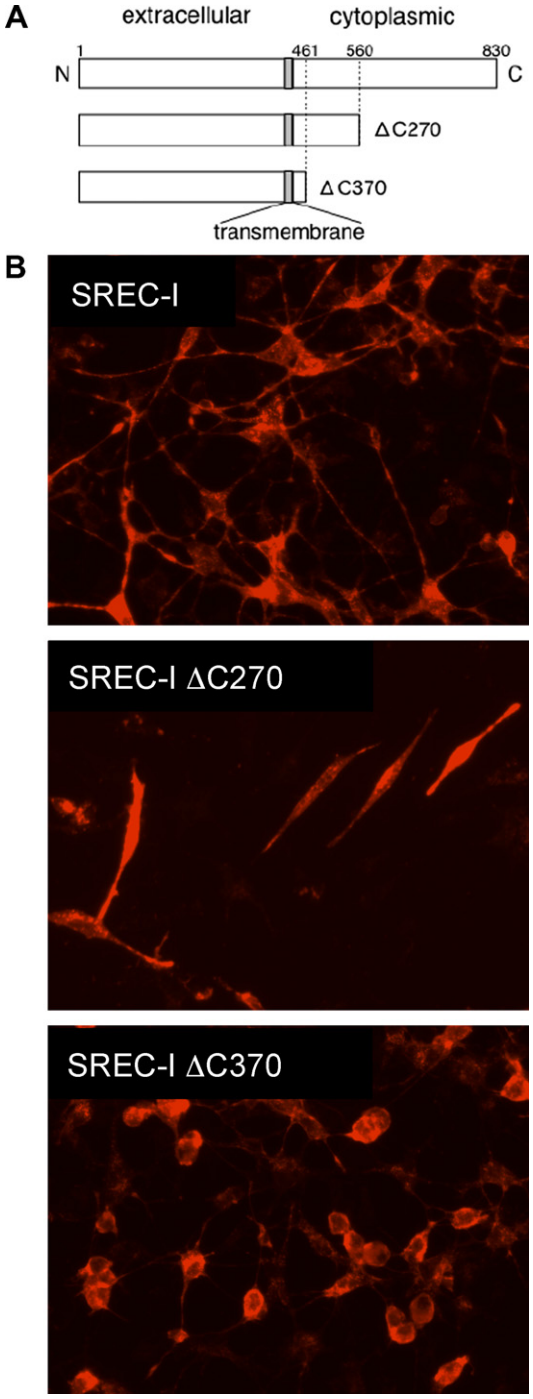

Fig. 3. Role of the cytoplasmic domain of SREC-I in the morphological change of L cells. (A) Schematic representation of full-length and truncated forms of SREC-I employed in this study. (B) L cells transfected with pcDNA3-SREC-I, SREC-I-ΔC270 or SREC-I-ΔC370 were incubated with DiI-AcLDL for 2 h to visualize the cells-expressing SREC-I or its truncated forms.

As shown in our previous work, neurite-like outgrowth was induced in SREC-I-expressing L cells. It was observed that treatment of tautomycin caused just elongation (sprouting) of the cells without process formation. Okadaic acid had little effect on the morphological change of the cells. Since tautomycin inhibits both PP1 and PP2A and okadaic acid

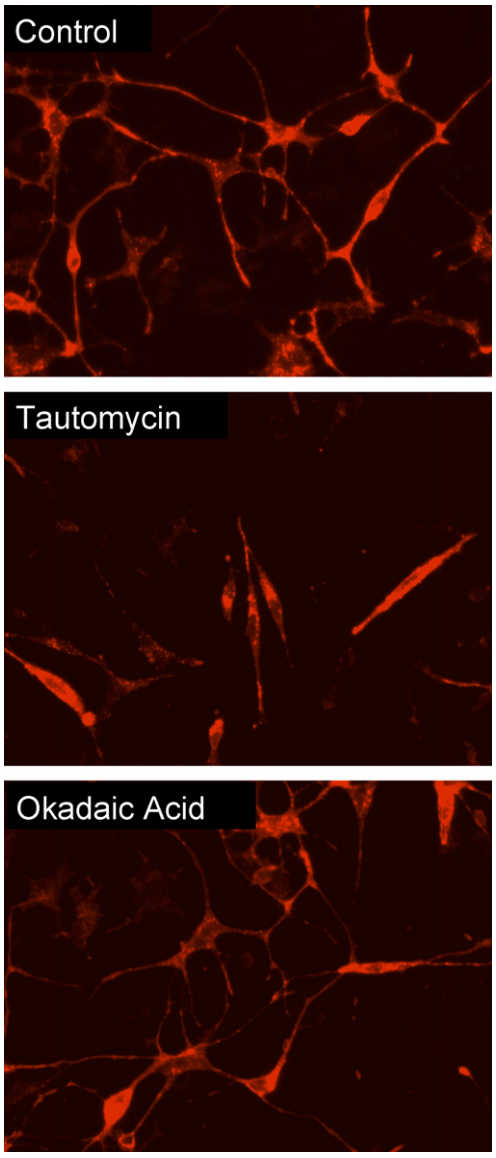

Fig. 4. Effects of protein phosphatase inhibitors on the SREC-I-mediated morphological change of L cells. L cells transfected with pcDNA3-SREC-I were treated with either tautomycin (100 nM) or okadaic acid (30 nM) 24 h after the transfection and further incubated for 24 h. The cells were then incubated with DiI-AcLDL for 2 h to visualize the SREC-I-expressing cells.

is rather a selective inhibitor of PP2A [12–14], these results suggested that PP1 is involved in the SREC-I-induced process formation in L cells. Taken together, our data strongly suggested that the process formation but not cell elongation/sprouting step required the binding of PP1α to SREC-I and the enzymatic activity of PP1α. Our data also implied that some protein(s) binding to the sequence between 461 and 560 might be required for the elongation/sprouting of the cells.

SREC-I expressed in L cells is a phosphorylated protein

The N-terminal half of the cytoplasmic domain of SREC-I is a serine- and proline-rich domain [9,10]. This

domain contains several serine and threonine residues that could potentially be phosphorylated by various protein kinases. Since PP1α binding to SREC-I is required for the process formation, we next examined whether SREC-I expressed in L cells was phosphorylated or not. Expression of SREC-I in L cells and metabolic labeling with ^{32}P revealed that the protein is constitutively phosphorylated (data not shown). These results might suggest that enzymatic activity of PP1α toward phosphorylated residues of SREC-I was involved in the morphological change of the cells.

Discussion

In our previous work, we have shown that SREC-I-expressing L cells could be a model system for the analysis of the receptor-mediated neurite outgrowth in neuronal cells [11]. It was found that SREC-I was expressed in dorsal root ganglion neurons during embryonic development, suggesting its role in the morphogenesis of the neurons during development through interaction with advillin. In fact, co-expression of SREC-I and advillin in cultured Neuro-2a cells induced long process formation [11].

We have shown in this paper that in addition to advillin, PP1α also play important roles in the morphological change of L cells via the interaction with large Ser/Pro-rich cytoplasmic domain of SREC-I. Comparing the ability to induce the morphological change of full-length and truncated forms of SREC-I, it was found that the process formation could be divided into two steps (i.e., elongation/sprouting and process formation steps). The elongation of the cells was observed when SREC-I-ΔC270 but not SREC-I-ΔC370 was expressed. It is also observed that treatment of the cells with tautomycin caused the elongation/sprouting without process formation. Thus, both by the treatment of SREC-expressing L cells with PP1α inhibitors and the deletion of PP1α-binding region of SREC-I, elongation/sprouting without process formation of the cells was observed. Taken together, these results strongly suggested that the interaction with and the enzymatic activity of PP1α were required for the process formation but not elongation/sprouting of L cells mediated by SREC-I. Since advillin binds to the same GST-C2 fragment with PP1α, it is also required for the process formation but not elongation/sprouting of the cells. It is elusive to identify the fine binding sites of these two proteins for further analysis of the mechanism of process formation. It is also plausible that the elongation/sprouting step is regulated by other unidentified binding proteins at the N-terminal side of the cytoplasmic domain. In fact, several putative binding proteins were identified by a yeast two-hybrid system including transglutaminase and vimentin. It is necessary to elucidate the role of these proteins in the morphological change of the cells. Since SREC-II has no activity to induce neurite-like outgrowth in L cells [10], it might be interesting to compare the binding proteins of the cytoplasmic domain of SREC-II with those of SREC-I. Although ~20% homology was observed in the entire cytoplasmic domains of SREC-I and -II, PP1α- and advillin-binding region of SREC-I is less homologous (less than 10%).

Consistent with the notion that PP1α plays a role in the morphological change, SREC-I expressed in L cells was constitutively phosphorylated. However, the receptor expressed in Chinese hamster ovary (CHO) cells was also phosphorylated even though SREC-I did not induce neurite-like outgrowth (data not shown), indicating that PP1α is required in the morphological change not merely for the de-phosphorylation of SREC-I molecules. PP1α may de-phosphorylate other SREC-I-binding signal molecule(s), and thus induce the neurite-like outgrowth of L cells, although we could not rule out the involvement of the de-phosphorylation of SREC-I in the morphological change of the cells at present. An interesting possibility is that as shown in the endothelial cell sprouting, some GTPase might play a role in the morphological change of L cells [15]. PP1α might regulate the activity of this putative GTPase. It will be important to identify the molecular target of PP1α in this system to elucidate the signal transduction mechanism of the morphogenesis of neuronal cell.

Protein phosphatase 1 (PP1) is a major eukaryotic protein serine/threonine phosphatase that regulates many biological processes including embryonic development, and cell proliferation and death [16]. There are four isoforms of the PP1 catalytic subunit (PP1c) (i.e., PP1α, -β, -γ1, and -γ2). PP1cs form complexes with over 50 different regulatory subunits. The formation of these complexes converts PP1c into many different forms, which have distinct substrate specificities and intracellular localizations [16,17]. To elucidate the SREC-I-mediated morphological change, it is necessary to examine whether SREC-I acts as a regulatory protein of PP1α activity affecting the substrate specificity of the enzyme or just a binding protein restricting PP1α molecules to the cytoplasmic domain of SREC-I.

In conclusion, it is most likely that SREC-I induced the neurite-like outgrowth of L cells through interaction with PP1α as well as advillin. This system may be useful for the elucidation of the mechanism of morphological maturation of some neuronal cells such as dorsal root ganglion neurons that express SREC-I during early development.

Acknowledgments

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