

Class A Scavenger Receptor-mediated Adhesion and Internalization Require Distinct Cytoplasmic Domains*

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Class A scavenger receptors (SR-A) are transmembrane glycoproteins that mediate both ligand internalization and cell adhesion. Previous studies have identified specific amino acids in the cytoplasmic tail of SR-A that regulate receptor internalization; however, the role of cytoplasmic domains in regulating cell adhesion has not been addressed. To investigate the role of cytoplasmic domains in SR-A-mediated adhesion and to address whether SR-A-mediated adhesion and internalization require distinct cytoplasmic domains, different SR-A constructs were stably expressed in human embryonic kidney (HEK 293) cells. Deleting the entire cytoplasmic tail (SR-A_{Δ1-55}) greatly reduced receptor protein abundance. Retaining the six amino acids proximal to the membrane (SR-A_{Δ1-49}) restored receptor protein abundance. Although SR-A_{Δ1-49} localized to the cell surface, cells expressing this receptor failed to internalize the ligand acetylated low density lipoprotein. Replacing the cytoplasmic tail of SR-A with that of the transferrin receptor (TfR/SR-A) resulted in retention of the chimeric receptor in the endoplasmic reticulum suggesting a specific role for the membrane-proximal amino acids in trafficking SR-A from the endoplasmic reticulum to the Golgi. Like SR-A expressing cells, cells expressing SR-A_{Δ1-49} displayed increased spreading and adhesion, demonstrating that the membrane-proximal amino acids were sufficient for SR-A-mediated cell adhesion. Together, our results indicate a critical role for the membrane-proximal amino acids in SR-A trafficking and demonstrate that SR-A-mediated adhesion and internalization require distinct cytoplasmic domains.

Class A scavenger receptors (SR-A)¹ are type II trimeric transmembrane glycoproteins with an amino-terminal cytoplasmic tail of 50 (human, bovine, and rabbit) or 55 (murine) amino acids (1, 2). The carboxyl-terminal extracellular region of SR-A contains a positively charged groove that allows SR-A

to bind a variety of polyanionic ligands including modified lipoproteins, bacterial products, and extracellular matrix proteins (3, 4). Because of its ability to bind these various ligands, SR-A is thought to be involved in many physiological and pathophysiological processes such as host defense and atherosclerosis (4, 5).

SR-A was originally identified on macrophages as the receptor that binds and internalizes modified lipoproteins via clathrin-coated pit-mediated endocytosis (6, 7). Consequently, most studies on SR-A have focused on its ability to internalize ligand. However, SR-A also mediates cell adhesion (8). Several components of the extracellular matrix, including modified types of collagen and certain proteoglycans present at sites of inflammation, have been identified as adhesion substrates for SR-A (9–12). Therefore, SR-A-mediated adhesion might play an important role in macrophage retention, specifically at sites of tissue injury.

It has not been defined whether SR-A-mediated adhesion results from an attempt to internalize an immobilized ligand or involves functional processes that are distinct from those involved in receptor internalization. Previous studies have indicated that SR-A-mediated adhesion and internalization have similar extracellular requirements (8–11). Consequently, it has been postulated that these functions involve a common mechanism (13). However, our recent results showed that clathrin does not co-localize with SR-A in the filopodia-like projections associated with the enhanced adhesion of SR-A expressing cells and this suggests that SR-A-mediated adhesion is a process that is distinct from that of receptor endocytosis (14).

In general, receptor-mediated endocytosis requires specific internalization motifs located within the cytoplasmic tail. For example, the transferrin receptor (TfR), which like SR-A, is a type II transmembrane receptor, has a well defined cytoplasmic motif (YTRF) that is required for receptor internalization (15). Previous studies with SR-A have identified specific amino acids in the cytoplasmic tail that are involved in regulating receptor internalization and cell-surface expression (13, 16, 17). However, the importance of cytoplasmic domains in SR-A-mediated adhesion has not been addressed. To investigate the role of the cytoplasmic tail in SR-A-mediated cell adhesion and to determine whether adhesion and internalization require distinct cytosolic domains, various SR-A constructs were stably expressed in human embryonic kidney (HEK 293) cells under the control of a tetracycline-inducible promoter.

EXPERIMENTAL PROCEDURES

Generation of the SR-A Construct Expression Vectors—The cDNAs encoding full-length SR-A, SR-A lacking all 55 amino acids of the amino-terminal cytoplasmic tail (SR-A_{Δ1-55}), and SR-A lacking the first 49 amino acids of the cytoplasmic tail (SR-A_{Δ1-49}) were amplified by PCR from SR-A type II murine cDNA (GenBankTM accession number U04275). Deleting the cDNA encoding the amino-terminal amino acids

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¹ The abbreviations used are: SR-A, Class A scavenger receptor; AcLDL, acetylated low density lipoprotein; EndoH, endoglycosidase H; ER, endoplasmic reticulum; HEK, human embryonic kidney; RT, reverse transcriptase; TfR, transferrin receptor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid.

eliminated the translation start codon. Therefore, the 5' PCR primers used to amplify SR-A_{Δ1-55} and SR-A_{Δ1-49} were designed to insert a methionine translation start codon and a spacer alanine codon. To replace the cytoplasmic tail of SR-A with that of the transferrin receptor (TR/SR-A), the cDNA encoding the amino-terminal 57 amino acids of the murine transferrin receptor (GenBankTM accession X57349) was amplified by RT-PCR (Access RT-PCR System, Promega, Madison, WI) from RNA isolated from mouse liver and inserted 5' to the cDNA encoding the SR-A transmembrane and extracellular domains. These cDNAs inserts were cloned into the pcDNA5/FRT/TO expression vector (Invitrogen, Carlsbad, CA). The sequence of the inserts was confirmed at the Macromolecular Structure Analysis Facility at the University of Kentucky. The amino acid sequences of the receptor constructs are shown in Table I.

Generation of HEK Cells Expressing SR-A Constructs and Cell Culture—To generate cells that express the different SR-A constructs, we used the Flp-InTM T-RExTM system (Invitrogen). This system allows tetracycline-inducible expression after integration of the cDNA into a specific genomic site. An important advantage of this system is that similar levels of expression can be obtained upon induction of cells transfected with the different SR-A constructs. Flp-InTM T-RExTM HEK 293 host cells were used, as HEK 293 cells do not endogenously express SR-A and have been used previously to study SR-A function (14). HEK 293 host cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing penicillin (10 units/ml), streptomycin (10 μg/ml), heat-inactivated fetal bovine serum (10% fetal bovine serum; Invitrogen) (DMEM/fetal bovine serum), and the selection antibiotics blasticidin (15 μg/ml) and zeocin (100 μg/ml). Cells were co-transfected with pcDNA5/FRT/TO containing the SR-A constructs and pOG44 encoding Flp recombinase, which mediates insertion of pcDNA5/FRT/TO into the genomic integration site, using the TransIT-293 transfection reagent according to the manufacturer's protocol (Mirus, Madison, WI). Integration of pcDNA5/FRT/TO eliminates zeocin resistance and confers hygromycin B resistance. Thus, transfected cells were selected for stable integration of the SR-A constructs with hygromycin B (200 μg/ml). Receptor expression was induced by adding tetracycline to the culture media for 16 h unless stated otherwise. Concentration response studies showed that a concentration of 0.5 μg/ml tetracycline induced maximal protein expression (data not shown).

RT-PCR—Total RNA was isolated using the SV total RNA isolation system (Promega, Madison, WI). The Access RT-PCR system (Promega) was used to assess mRNA expression of the SR-A constructs. The primers used (5'-cgggggaagcttgcaatggctgcctcattgctctc-3' and 5'-ggctc-gagttatactgatcttgatccgcc-3') amplify a 897-bp product corresponding to the SR-A transmembrane and extracellular domains (SR-A nucleotides 171–1067) common to all SR-A constructs. The parameters for the thermocycle reaction were: 48 °C for 45 min, 94 °C for 2 min, and 40 cycles of 94 °C for 30 s, 60 °C for 60 s, and 68 °C for 120 s. Reactions performed in the absence of RNA or of reverse transcriptase were used as negative controls. RT-PCR products were analyzed by agarose gel electrophoresis and visualized following ethidium bromide staining using a Kodak Image Station 440.

Preparation of Cell Lysates—Cell lysates were prepared by incubating cells in lysis buffer (25 mM MES, 150 mM NaCl, 60 mM octylglucopyranoside, 1% Triton X-100, pH 6.4) for 30 min on ice. Protein concentrations were determined using bovine serum albumin as standard (DC Protein Assay, Bio-Rad).

Western Blotting—Cell lysates were resolved by SDS-PAGE on a 7.5% gel under reducing or non-reducing conditions and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). In some experiments, SR-A receptors were immunoprecipitated from cell lysates or culture media using the rat SR-A monoclonal antibody, 2F8 (3 μg/ml; Serotec, Raleigh, NC) and anti-rat IgG-coated magnetic beads. Receptor proteins resolved under reducing conditions were detected using a guinea pig antiserum developed to a fusion protein of the extracellular SR-A domain as described previously (18). To detect oligomeric forms of the receptors, cell lysates were resolved under non-reducing conditions and receptor proteins were detected with 2F8 (3 μg/ml, Serotec), which also recognizes an extracellular epitope of SR-A (18). Primary antibody binding was detected with horseradish peroxidase-labeled anti-guinea pig (1:10,000 dilution; Jackson, West Grove, PA) or anti-rat (1:5,000 dilution; Santa Cruz) secondary antibodies. Horseradish peroxidase activity was visualized by chemiluminescence (Pierce) using a Kodak Image Station 440.

N-Glycanase (PNGase F) and Endoglycosidase H Digestion—To cleave all N-linked oligosaccharides, cell lysate protein (10 μg) was digested with N-glycanase (PNGase F; Sigma) in buffer (50 μl: 50 mM NaHPO₄, 0.1% SDS, 0.05 M β-mercaptoethanol, pH 7.5) according to the

manufacturer's protocol. Briefly, proteins were denatured by heating to 100 °C for 5 min. After cooling, Triton X-100 (0.75%, v/v) and N-glycanase were added and the reaction was incubated for 3 h at 37 °C. To cleave N-linked precursor oligosaccharides, cell lysate protein (10 μg) was digested with endoglycosidase H (EndoH, Sigma) in buffer (50 μl: 50 mM NaHPO₄, 0.1% SDS, 0.05 M β-mercaptoethanol, pH 5.5) according to the manufacturer's protocol, which was identical to that used for N-glycanase digestion with the exception of the addition of Triton X-100.

Tunicamycin and Brefeldin A Incubation—To inhibit N-linked glycosylation in the endoplasmic reticulum (ER), cells were cultured in the presence of tunicamycin (5 μg/ml, Sigma). To inhibit maturation of N-linked oligosaccharides in the Golgi apparatus, cells were cultured in the presence of brefeldin A (5 μg/ml, Sigma). These inhibitors were added 60 min before inducing cells with tetracycline and were present throughout induction. Cell lysates were prepared 6 h after addition of tetracycline.

Biotinylation of Cell-surface Proteins—Cells were cultured and induced in 6-well plates (0.5 × 10⁶ cells/well), or to study the effect of cell adhesion on cell-surface localization of receptor, in ultra-low attachment plates (Costar, Corning, NY). Induced cells were trypsinized, washed, and cell-surface proteins were biotinylated in suspension (1 mg/ml EZ-Link sulfo-NHS-LC-Biotin in PBS, Pierce) at 4 °C for 30 min according to the manufacturer's instructions. To remove non-bound biotin, cells were washed with ice-cold Tris-buffered saline (pH 7.4) followed by PBS. Subsequently, cell lysates were prepared and biotinylated surface proteins were precipitated from lysates using streptavidin-coated magnetic beads. Cell-surface proteins (bead pellet) and intracellular proteins (supernatant) of the precipitation were resolved under reducing conditions by SDS-PAGE and SR-A was detected by Western blotting.

AcLDL Internalization—Cells were cultured and induced in 6-well plates (0.5 × 10⁶ cells/well), or to study the effect of cell adhesion on receptor-mediated ligand uptake, cells were cultured in ultra-low attachment plates (Costar). Following incubation of induced cells in serum-free DMEM for 2 h, fluorescently labeled AcLDL (2.5 μg/ml, Alexa Fluor 488-AcLDL, Molecular Probes) was added to the media and incubation was continued for another 2 h. Adherent cells were trypsinized, washed, and resuspended in PBS (4 °C). AcLDL internalization was assessed by quantifying cell-associated fluorescence (Flow Cytometry Core Facility, University of Kentucky). Fluorescence was gated for individual live cells and the fluorescence associated with ~20,000 cells was determined for each sample. To assess nonspecific AcLDL cell association, polyinosine (10 μg/ml) was added 5 min before addition of fluorescently labeled AcLDL.

Microscopy—Cells were plated on 2-chamber (5 × 10⁴ cells/chamber) LAB-TEK glass slides (Nalge Nunc International, Naperville, IL) and receptor proteins were detected as previously described (14). Briefly, cells were induced where indicated, washed twice with phenol-red free DMEM (37 °C), fixed in paraformaldehyde (4% w/v), and permeabilized with Triton-X (0.1% in PBS). Nonspecific binding sites were blocked with bovine serum albumin (1% in PBS). To detect expressed receptors, cells were incubated with 2F8 (3 μg/ml, Serotec), followed by incubation with an Alexa Fluor 488-labeled goat anti-rat antibody (2 μg/ml; Molecular Probes). Cell nuclei were stained using 4,5-diamidino-2-phenylindole (Molecular Probes). Cells were mounted in Mowiol embedding medium containing n-propyl gallate (1%, w/v) and dried overnight at 4 °C. Images were captured digitally by fluorescence microscopy with exposure times kept constant.

Cell Adhesion Assay—Cells were plated in 96-well plates (2 × 10⁴ cells/well) in DMEM/fetal bovine serum and induced where indicated. Adherent cells were incubated in EDTA solution (0.2 g/liter, 37 °C) for 10 min to eliminate divalent cation-dependent adhesion. After washing with PBS (37 °C), cells remaining adhered were quantified using the CyQUANT assay (Molecular Probes) according to the manufacturer's instructions. Cell adhesion is expressed as a percentage of total cells plated.

RESULTS

The Membrane-proximal Amino Acids of the Cytoplasmic Tail Are Required for Post-transcriptional Processing of SR-A—To investigate the role of the cytoplasmic tail in SR-A-mediated adhesion and to determine whether SR-A-mediated adhesion and internalization require distinct cytoplasmic domains, we expressed full-length SR-A or different SR-A constructs (Table I) under the control of a tetracycline-inducible

TABLE I
Amino acid sequences of the amino-terminal cytoplasmic tail of SR-A receptor constructs

Full-length murine SR-A has a 55-amino acid amino-terminal cytoplasmic tail. SR-A $_{\Delta 1-49}$ lacks the first 49 amino acids, and SR-A $_{\Delta 1-55}$ lacks all 55 amino acids of the cytoplasmic tail. In Tfr/SR-A, all 55 amino acids of the cytoplasmic tail are replaced by the first 57 amino acids of the murine Tfr. The transmembrane and extracellular domains are identical for all receptor constructs and correspond to amino acids 56 to 354 of SR-A type II.

Receptor construct	Amino acid sequence of amino-terminal cytoplasmic tail						
	1	10	20	30	40	50	55
SR-A (full-length)	MTKMETENQRLCPHEREDADCSSSESVKFDARSMTASLPHSTKNGPSVQEKLSFK						
SR-A $_{\Delta 1-49}$							MAKLSFK
SR-A $_{\Delta 1-55}$	MA						
Tfr/SR-A	1	10	20	30	40	50	57
	MMDQARSFAFSNLFGEPLSYTRFSLARQVDGDNHSHVEMKLADEEENADNNMKASVR						

promoter in HEK 293 cells. Our initial approach involved deletion of the cytoplasmic tail, which we hypothesized would result in a loss of function, and the subsequent addition of amino acids to recover function. We found that deleting the entire 55-amino acid cytoplasmic tail (SR-A $_{\Delta 1-55}$) greatly diminished SR-A protein abundance compared with that of SR-A full-length expressing cells (Fig. 1A). SR-A protein was not detectable in the culture media indicating that the decreased cellular SR-A $_{\Delta 1-55}$ protein abundance was not because of secretion into the media (data not shown). In contrast to complete deletion of the cytoplasmic tail, retaining the membrane-proximal six amino acids by deleting amino acids 1 to 49 (SR-A $_{\Delta 1-49}$) restored protein abundance of SR-A (Fig. 1A). SR-A mRNA abundance was similar for all SR-A constructs (Fig. 1B). Thus, our results demonstrate that the six membrane-proximal amino acids are required for post-transcriptional processing of SR-A.

The Membrane-proximal Amino Acids of the Cytoplasmic Tail of SR-A Are Sufficient for Cell-surface Localization but Not for Receptor Internalization—To study the role of the membrane-proximal amino acids in regulating SR-A function, we analyzed cell-surface localization of SR-A $_{\Delta 1-49}$. Cell-surface localization of SR-A $_{\Delta 1-49}$ was greater than that of full-length SR-A (Fig. 2). Surface localization of SR-A $_{\Delta 1-49}$ was similar in cells grown adhered to tissue culture plates or in suspension demonstrating that the increased surface localization of SR-A $_{\Delta 1-49}$ was independent of SR-A-mediated adhesion. To address the role of the membrane-proximal amino acids for SR-A internalization, we examined internalization of the SR-A ligand AcLDL by quantifying cell-associated fluorescence after incubation with fluorescently labeled AcLDL for 2 h (Fig. 3). As shown previously, HEK 293 cells expressing full-length SR-A cells efficiently internalized AcLDL (14). However, cell-associated fluorescence detected in cells expressing SR-A $_{\Delta 1-49}$ was 14% of that detected in cells expressing full-length SR-A (Fig. 3B). The small amount of fluorescence associated with SR-A $_{\Delta 1-49}$ expressing cells most likely reflects AcLDL binding to cell-surface receptors. The increased cell-associated fluorescence observed in SR-A and SR-A $_{\Delta 1-49}$ expressing cells was completely blocked by the SR-A antagonist polyinosine demonstrating specificity for SR-A. As shown for cell-surface localization, AcLDL internalization by cells expressing SR-A $_{\Delta 1-49}$ was similar for both adherent and suspended cells (Fig. 3C), demonstrating that the failure of this receptor to internalize ligand was not the result of increased receptor-mediated adhesion. It is likely that the lack of SR-A $_{\Delta 1-49}$ internalization results in the increased cell-surface localization of SR-A $_{\Delta 1-49}$ (Fig. 2).

The Membrane-proximal Amino Acids of SR-A Are Required for Trafficking from the Endoplasmic Reticulum to the Golgi Apparatus—The ability of the membrane-proximal amino acids to restore protein expression and cell-surface localization suggest an important role for this motif in SR-A trafficking. Previous studies have associated SR-A cell-surface localization and

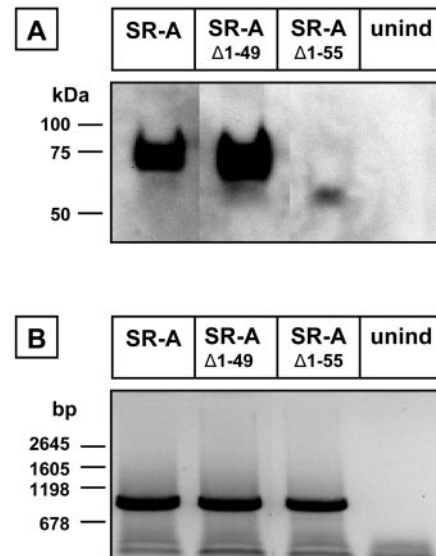


FIG. 1. Expression of SR-A constructs in HEK cells. A, SR-A protein was immunoprecipitated from cell lysates (100 μ g) prepared from induced cells. Precipitated proteins were resolved by SDS-PAGE under reducing conditions and SR-A was detected by Western blotting. SR-A protein expression was not detectable in lysates prepared from uninduced cells. Blot shown is representative of results obtained in two separate experiments. B, RT-PCR was performed using primers that amplify a 897-bp fragment spanning the transmembrane and extracellular part of SR-A from total RNA (500 ng) isolated from induced cells. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. SR-A mRNA expression was not detectable in uninduced cells. No products were detected in control reactions performed without RT or RNA. Data shown are representative of two separate experiments.

receptor internalization with a common cytoplasmic motif (17). However, the finding that SR-A $_{\Delta 1-49}$ localized to the cell surface, but did not internalize ligand, suggests that SR-A trafficking to the cell surface and internalization have distinct cytoplasmic requirements. To address the specific role of the membrane-proximal amino acids in SR-A trafficking and whether an internalization motif is sufficient to mediate cell-surface localization, we generated a chimeric receptor in which the entire cytoplasmic tail of SR-A was replaced by 57 amino acids of the Tfr cytoplasmic tail (Tfr/SR-A; Table I) omitting a similar membrane-proximal domain. Like SR-A, the Tfr is a type II transmembrane receptor that mediates internalization via clathrin-coated pits. However, whereas the cytoplasmic tail of SR-A is still poorly characterized, the cytoplasmic tail of Tfr has been studied extensively and contains a well characterized internalization motif (YTRF; Ref. 15).

To study the trafficking and intracellular processing of the chimeric Tfr/SR-A, we assessed the ability of Tfr/SR-A to form oligomers by resolving cell lysates from induced cells under non-reducing conditions. Tfr/SR-A was expressed and assembled into

FIG. 2. Cell-surface localization of full-length and SR-A $_{\Delta 1-49}$ in HEK cells. Cells were cultured and receptor expression induced in cells that were either adhered to tissue culture plates or growing in suspension. Cell-surface proteins were biotinylated and lysates were prepared. Biotinylated proteins were precipitated from lysates (25 μ g) using streptavidin-coated beads. Total cell-surface protein (pellet, *top panel*), 1/10 of the intracellular protein (supernatant, *middle panel*), and 10 μ g of lysate protein (*bottom panel*) were resolved by SDS-PAGE under reducing conditions and SR-A was detected by Western blotting. The blot shown is representative of three separate experiments.

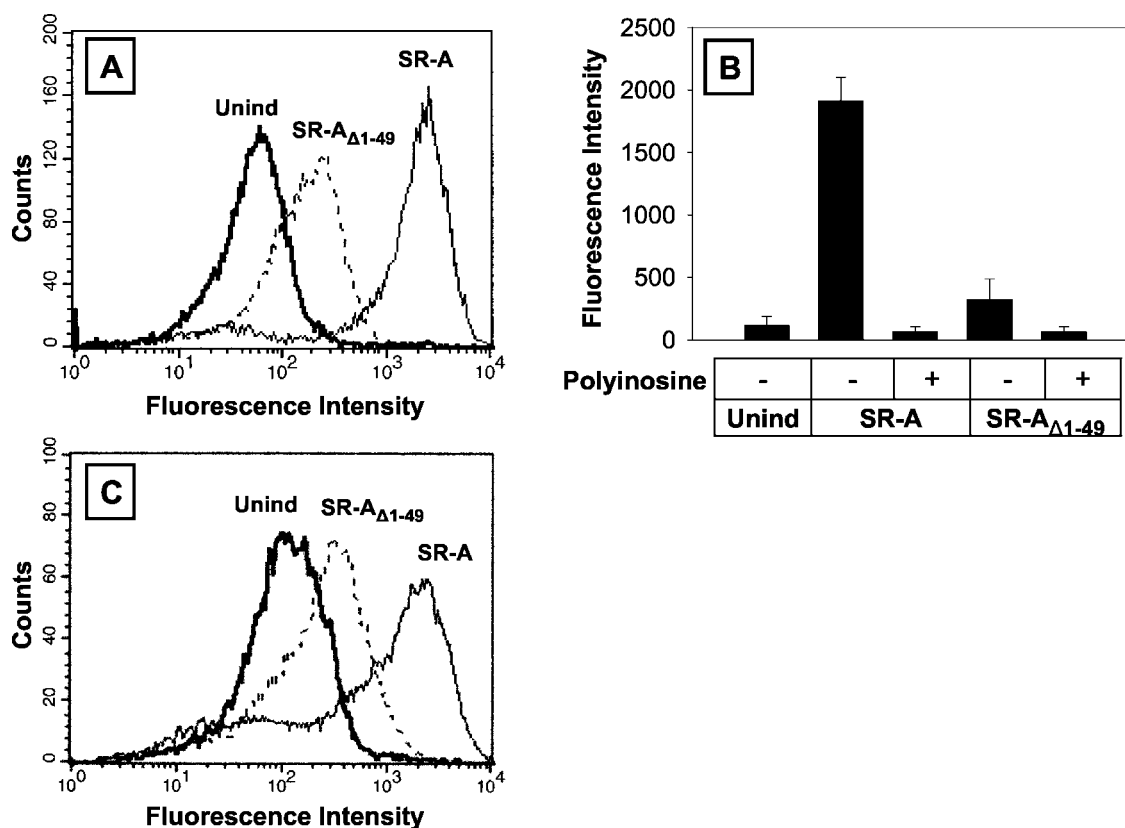
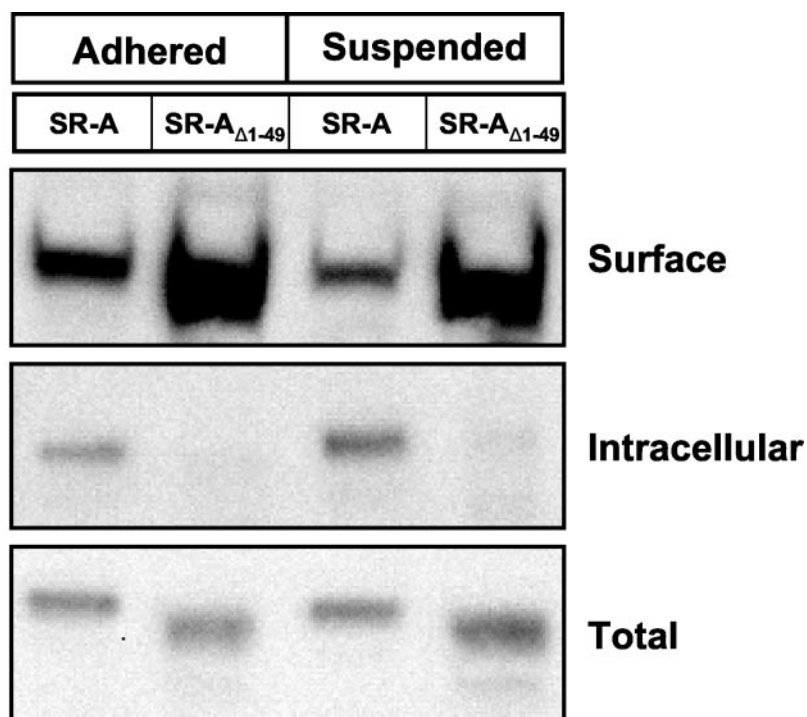


FIG. 3. AcLDL internalization by SR-A and SR-A $_{\Delta 1-49}$ expressing HEK cells. Cells were cultured and receptor expression induced as indicated in cells that were adhered to tissue culture plates (*A* and *B*) or growing in suspension (*C*). Following incubation of cells in serum-free DMEM for 2 h, fluorescently labeled AcLDL (2.5 μ g/ml, Alexa Fluor 488 AcLDL) was added and cells were incubated for another 2 h. To determine nonspecific AcLDL association, polyinosine (10 μ g/ml) was added before addition of AcLDL. Cell-associated fluorescence of uninduced, SR-A expressing, and SR-A $_{\Delta 1-49}$ expressing cells that were either adhered (*A*) or suspended (*C*) was assessed by flow cytometry. *B*, quantification of Alexa Fluor 488 AcLDL association with adhered HEK cells. Data are the mean fluorescence intensities \pm S.D. of two separate experiments.

oligomers similar to SR-A. Monomers of Tyr/SR-A and SR-A were detected at a molecular weight higher than predicted from the amino acid sequences (about 38 kDa) suggesting that both receptors were post-translationally modified (Fig. 4A). However, the

apparent molecular weight of Tyr/SR-A was lower than SR-A, indicating that the post-translational processing was different.

To examine the post-translational processing of the SR-A receptor constructs, we digested cell lysates prepared from

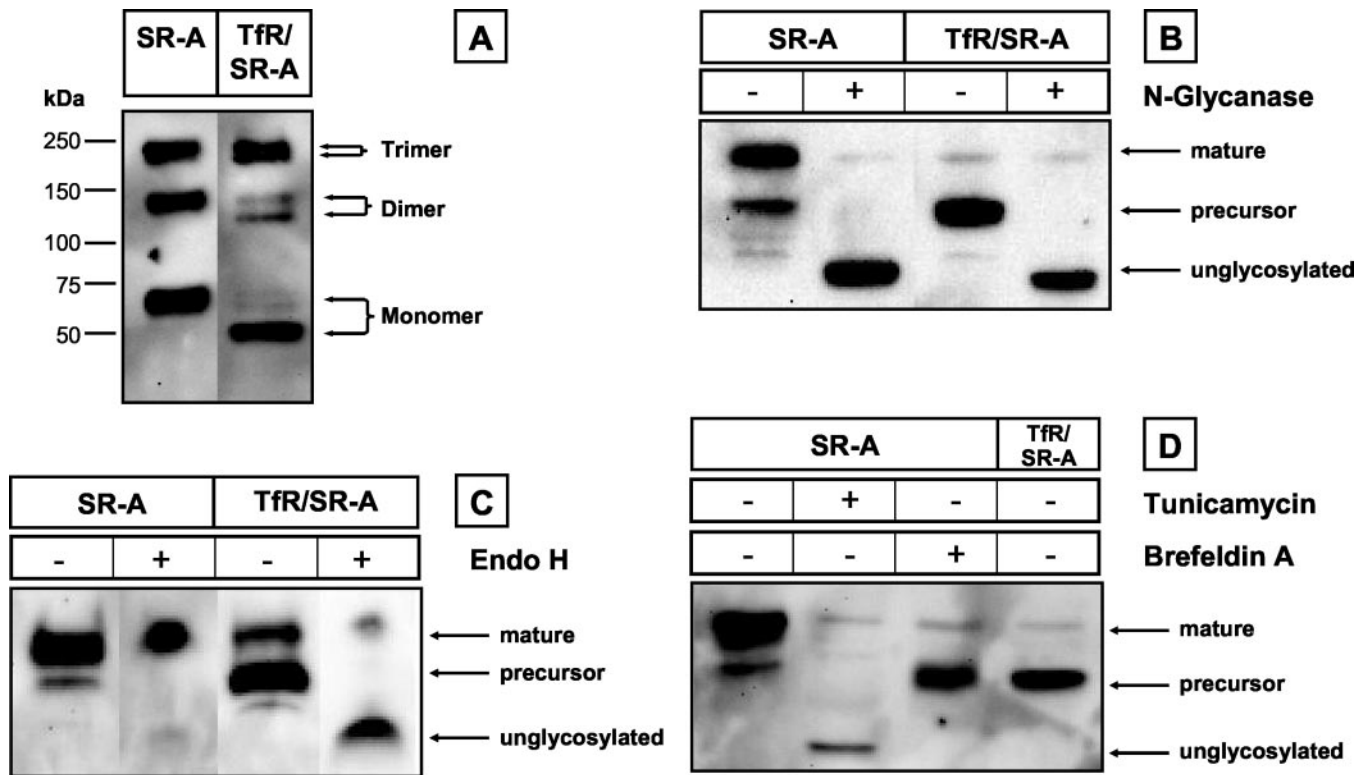


FIG. 4. Expression and processing of SR-A and Tfr/SR-A in HEK cells. *A*, cell lysates were prepared from induced cells and resolved by SDS-PAGE under non-reducing conditions. SR-A and Tfr/SR-A were detected by Western blotting. Arrows indicate the oligomeric forms of the receptors. *B*, cells were induced and cell lysates were prepared. As indicated, lysates were treated with *N*-glycanase to cleave all *N*-linked oligosaccharides and were then resolved under reducing conditions by SDS-PAGE. SR-A and Tfr/SR-A were detected by Western blotting. *C*, cells were induced and cell lysates were prepared. As indicated, lysates were treated with EndoH to cleave only precursor *N*-linked oligosaccharides and then analyzed as in panel *B*. *D*, cells were treated with tunicamycin (5 μ g/ml) to inhibit formation of *N*-linked precursor oligosaccharides or with brefeldin A (5 μ g/ml) to prevent maturation of the *N*-linked precursor oligosaccharides 60 min before and during induction (6 h). Then, cell lysates were prepared and analyzed as in panel *B*. Each blot is representative of results obtained in at least two separate experiments.

induced cells with *N*-glycanase (Fig. 4*B*), which cleaves all *N*-linked oligosaccharides. The deglycosylated monomeric proteins were detectable at 38 kDa for both SR-A and the chimeric Tfr/SR-A indicating that the differences in apparent molecular weight resulted from differences in *N*-linked oligosaccharides. To test whether the *N*-linked oligosaccharides of the receptors differ in Golgi processing, lysates prepared from induced cells were digested with EndoH (Fig. 4*C*). EndoH cleaves the high-mannose precursor *N*-linked oligosaccharides attached in the ER, but not the low-mannose mature oligosaccharides that are formed after processing in the Golgi. In contrast to SR-A (Fig. 4*C*) and SR-A $_{\Delta 1-49}$ (data not shown), Tfr/SR-A (Fig. 4*C*) was EndoH-sensitive indicating that Tfr/SR-A was not processed into the EndoH-resistant mature form by the Golgi apparatus.

The EndoH sensitivity of Tfr/SR-A suggests that the oligosaccharides of Tfr/SR-A are analogous to that of the EndoH-sensitive SR-A precursor. To confirm this, cells were treated before induction either with brefeldin A, which disrupts the Golgi apparatus thereby preventing conversion of the precursor into the mature form, or with tunicamycin, an inhibitor of *N*-linked glycosylation (Fig. 4*D*). We found that the apparent molecular weight of the SR-A precursor detected in brefeldin A-treated cells was the same as that of Tfr/SR-A in untreated cells but was greater than that of the unglycosylated receptor in tunicamycin-treated cells. Together, the data demonstrate that Tfr/SR-A is translated and subsequently glycosylated in the ER, but the glycosylated precursor is not further processed in the Golgi apparatus. Because Tfr/SR-A assembles into trimers, it seems unlikely that Tfr/SR-A is retained in the ER because of misfolding of the protein. As expected, Tfr/SR-A was

not detected on the cell surface using the biotinylation protocol described above (data not shown).

Overall, our results from experiments studying the trafficking and intracellular processing of the different SR-A constructs demonstrate that an internalization motif is not sufficient to mediate trafficking to cell surface. Furthermore, the membrane-proximal amino acids of SR-A are sufficient for cell-surface localization and are specifically required for trafficking of SR-A from the ER to the Golgi apparatus.

The Membrane-proximal Amino Acids of SR-A Are Sufficient to Mediate Cell Spreading and Adhesion—SR-A is involved in both ligand internalization and cell adhesion. As we described previously, SR-A-mediated cell adhesion involves an increase attachment of cells and distinct changes in cell morphology (14). To determine whether SR-A $_{\Delta 1-49}$ mediates cell adhesion, we investigated the morphology of cells expressing the SR-A constructs (Fig. 5). Consistent with our previous findings (14), SR-A expressing HEK cells exhibited a cell morphology characterized by filopodia-like projections and increased cell spreading (Fig. 5, panels *A* and *B*). Consistent with the results indicating a lack of surface expression, cells expressing Tfr/SR-A (panels *C* and *D*) exhibited an intracellular immunostaining pattern and no increased cell spreading. Interestingly, SR-A $_{\Delta 1-49}$ expressing cells (panel *E* and *F*) exhibited filopodia-like projections and increased cell spreading that was similar to that observed for cells expressing full-length SR-A.

As the presence of the filopodia-like projections and increased cell spreading are associated with increased cell adhesion of SR-A expressing cells, we analyzed cell adhesion of cells expressing the different SR-A constructs. Consistent with in-

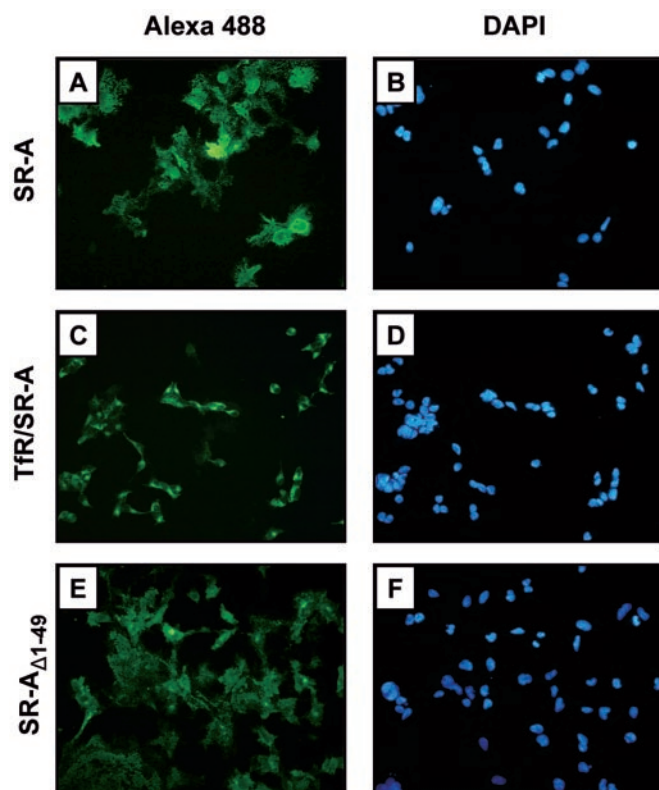


FIG. 5. Cellular morphology of SR-A, Tfr/SR-A, and SR-A $_{\Delta 1-49}$ expressing HEK cells. Cells were plated on glass slides, and SR-A (A and B), Tfr/SR-A (C and D), and SR-A $_{\Delta 1-49}$ (E and F) expression was induced. After the induced cells were fixed and permeabilized, SR-A construct expression was detected using 2F8 and a secondary Alexa Fluor 488-labeled anti-rat antibody (A, C, and E). No fluorescence was detectable in uninduced cells (not shown). The nuclei were stained with 4,5-diamidino-2-phenylindole (DAPI) (B, D, and F).

creased spreading of SR-A and SR-A $_{\Delta 1-49}$ expressing cells, both receptors were able to increase cell adhesion to the same extent (Fig. 6), demonstrating that the membrane-proximal amino acids are sufficient for SR-A-mediated adhesion. As expected from the lack of surface expression, neither Tfr/SR-A (Fig. 6) nor SR-A $_{\Delta 1-55}$ (not shown) increased cell adhesion.

DISCUSSION

To investigate the role of the cytoplasmic tail in SR-A-mediated adhesion, and to address whether SR-A-mediated adhesion and internalization require distinct cytoplasmic domains, we created different SR-A constructs and inducibly expressed them in stably transfected HEK 293 cells. Our results show that the cytoplasmic amino acids proximal to the membrane are required for SR-A post-transcriptional processing and play a critical role in SR-A trafficking to the cell surface. Furthermore, these amino acids were sufficient for SR-A-mediated adhesion, but not for ligand internalization. Thus, our results demonstrate that SR-A-mediated adhesion and internalization require distinct cytoplasmic domains.

The trafficking and post-translational processing of SR-A have been described previously (19). An initial step in the post-transcriptional processing of SR-A is insertion of the nascent protein into the membrane of the ER. The process whereby transmembrane proteins are inserted into the ER membrane and adopt a type I (carboxyl-terminal cytoplasmic tail) or type II (amino-terminal cytoplasmic tail) topology is not fully understood (20). It is generally thought that type I and II transmembrane proteins are targeted to the ER and then inserted and anchored in the membrane via a process involving signal-anchor sequences located in the hydrophobic amino acids of the

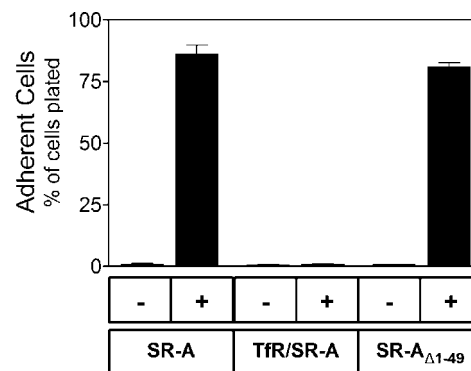


FIG. 6. Divalent cation-independent cell adhesion of HEK cells. Cells were plated in 96-well plates (20,000 cells/well) and induced as indicated. Then, cells were incubated in EDTA solution (0.2 g/liter, 37 °C) for 10 min to eliminate divalent cation-dependent adhesion. Cells that remained adhered were quantified by CyQUANT assay. Adhesion is expressed as percent of total cells plated. Data shown are representative of two separate experiments.

transmembrane domains. However, we found that the transmembrane domain of SR-A (SR-A $_{\Delta 1-55}$) was not sufficient for the post-transcriptional processing of SR-A. Retaining the membrane-proximal amino acids of the cytoplasmic tail (SR-A $_{\Delta 1-49}$) restored intracellular processing and trafficking to the cell surface. Three of the six retained SR-A membrane-proximal amino acids (KLKSFK) are positively charged. Charged membrane-proximal amino acids have been shown to affect the topology of transmembrane proteins (21). Thus, the membrane-proximal charged residues might be critical during ER membrane insertion of SR-A. However, replacing the cytoplasmic tail of SR-A with that of the transferrin receptor (Tfr/SR-A), omitting a similar charged membrane-proximal domain, was sufficient for protein expression, glycosylation, and oligomerization in the ER, but not for ER to Golgi trafficking. Therefore, the membrane-proximal amino acids of SR-A are specifically required for trafficking from ER to the Golgi.

After translocation of the carboxyl-terminal extracellular domains into the ER, the monomeric SR-A protein is N-linked glycosylated and the glycosylated monomers assemble into trimers. These “precursor” SR-A trimers are transported through the Golgi apparatus, where further processing of the N-linked oligosaccharides converts the EndoH-sensitive precursor into the EndoH-resistant mature form that is expressed on the cell surface. The mechanism by which proteins traffic from the ER to Golgi remains controversial (22). The *bulk-flow model* proposes that proteins are transported from the ER to the Golgi by default. In contrast, the *selective export model* proposes that proteins are selectively exported based on the presence of ER-export signals (23). Several cytosolic proteins are involved in trafficking of newly synthesized transmembrane proteins. Thus, the membrane-proximal amino acids might provide a critical recognition site for proteins involved in selective ER export.

Although SR-A $_{\Delta 1-49}$ was expressed primarily on the cell surface, SR-A $_{\Delta 1-49}$ failed to internalize ligand. A well defined internalization motif is not present in the cytoplasmic tail of SR-A. However, Morimoto *et al.* (17) have suggested that a single motif, VXFD, is required for both SR-A internalization and cell-surface localization. In contrast, our results show that the membrane-proximal amino acids are sufficient for cell-surface localization, but that receptor internalization depends on a distinct motif, possibly the VXFD motif.

It has been hypothesized that SR-A-mediated adhesion and internalization are regulated by the same mechanism and that SR-A-mediated adhesion results from the attempt to internalize an immobilized ligand (13). The inability to separate SR-A-

mediated adhesion from ligand internalization using various approaches supports this hypothesis (8–11). For example, structure-function studies have shown that SR-A-mediated adhesion depends on the same region in the extracellular collagen-like domain that mediates binding of soluble ligands during internalization (11). In contrast to data supporting a common mechanism for adhesion and internalization, we recently showed that clathrin does not co-localize with SR-A in the filopodia-like projections associated with the enhanced adhesion of SR-A expressing cells (14). This observation provided indirect evidence that SR-A-mediated adhesion is a process that is distinct from clathrin-coated pit internalization. The results of the current study demonstrating that SR-A-mediated adhesion and internalization require distinct domains of the receptor provide direct evidence to support this hypothesis.

The mechanism by which SR-A mediates cell adhesion remains unclear. It has been shown that SR-A-mediated adhesion involves changes in the actin cytoskeleton and the formation of focal adhesions. The ability of SRA $_{\Delta 1-49}$ to enhance cell adhesion suggests that the membrane-proximal amino acids of SR-A are sufficient to mediate these processes. Likewise, the cytoplasmic membrane-proximal amino acids of the adhesion molecule L1 have been shown to be sufficient for association of this receptor with the cytoskeleton (24). Thus, the membrane-proximal amino acids of SR-A might be a binding site for cytosolic proteins involved in formation of focal adhesions.

SR-A-mediated adhesion may have important physiological roles, particularly at sites of tissue inflammation. For example, we recently demonstrated a role of SR-A-mediated adhesion in an *in vivo* model of inflammation using SR-A overexpressing mice (25). Macrophage-specific SR-A overexpression enhanced granuloma formation after subcutaneous injection of carrageenan. Enhanced granuloma formation was associated with an increase in macrophage numbers suggesting that SR-A enhanced macrophage recruitment and/or retention. SR-A mediates adhesion specifically to modified extracellular matrix proteins such as collagen types I, III, and IV (9, 10). Such modifications of the extracellular matrix may occur during certain pathophysiological conditions such as hyperglycemia or inflammation. Recently, SR-A also has been shown to mediate adhesion to proteoglycans of the extracellular matrix that are present in atherosclerotic lesions (12). Furthermore, SR-A-mediated adhesion of microglial cells to β -amyloid fibrils might play a role in Alzheimer's disease (26). Thus, SR-A-mediated adhesion might be a mechanism of macrophage retention specifically at sites of tissue injury associated with inflammation.

In summary, we have shown that the cytoplasmic amino acids proximal to the membrane are required for SR-A post-translational processing and play a critical role in SR-A traf-

ficking to the cell surface. Furthermore, these amino acids were sufficient for SR-A-mediated adhesion, but not for ligand internalization. These are the first data to demonstrate that SR-A-mediated adhesion and internalization require distinct cytoplasmic domains and therefore must be distinct cellular processes. The development of a SR-A mutant such as SRA $_{\Delta 1-49}$ that specifically mediates cell adhesion will make it possible to specifically study the role of SR-A-mediated adhesion in various physiological and pathophysiological processes.

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