Mimicking phosphorylation at Ser-48 strongly reduces surface expression of human macrophage scavenger receptor class A: implications on cell motility

Harald Heider*, Eva S. Wintergerst

Institute of Biochemistry, University of Basel, Vesalgasse 1, 4051 Basel, Switzerland

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Abstract The role of human macrophage scavenger receptor A1 (SRA1) in the development of atherosclerotic lesions is still scarcely defined. Substituting serine 48 in human SRA1 by an aspartate demonstrated that (1) surface expression of the mutated receptor was 13-fold decreased; (2) the amount of cell-associated Texas red-labeled acetylated low density lipoprotein (LDL) in mutant receptor-expressing cells was almost threefold reduced; (3) the migration of mutant receptor-transfected cells towards surfaces coated with oxidized LDL decreased by almost 60% compared to cells that were transfected with the wild type receptor. Phosphorylation of the cytoplasmic part of SRA1 may help to modulate the residence time of macrophages in atherosclerotic lesions.

Key words: Scavenger receptor; Macrophage; Acetylated low density lipoprotein; Cell motility; Surface expression

1. Introduction

Macrophage scavenger receptor A (SRA) is one of the best characterized receptors of the continuously growing family of scavenger receptors. Its primary sequence was already determined in 1990 [1]. But the role of SRA1 in foam cell formation is still not well understood. Apolipoprotein E and SRA1 double knockout mice develop larger lesions upon a high cholesterol diet, according to a recent study [2], whereas in another publication the opposite effect was described [3]. Overexpression of SRA1 in a mouse model yielded enhanced foam cell formation in vitro [4] but reduced atherosclerotic lesions in vivo [5], indicating that SRA1, despite its ability to mediate uptake of modified lipoproteins, is involved in antiatherogenic processes. The contribution of SRA to uptake of oxidized low density lipoprotein (OxLDL), a modified lipoprotein with a variety of proatherogenic properties [6], accounts for approximately 30% of total uptake, indicating that other scavenger receptors are involved in intracellular lipid accumulation [7].

SRA also plays an important role in cell adhesion and in the establishment and maintenance of cell–cell contact [8]. However, it is not clear how the adhesion properties of SRA contribute to atherosclerotic lesion development. It has been proposed that modulation of adhesion may influence the residence time of cells at sites of cellular activation, e.g. in atherosclerotic plaques [9]. Unfortunately, there exist no clear data showing how adhesion properties could be changed. Recently, Fong and Le [10] found evidence that preventing phosphorylation of Ser-49 (corresponds to Ser-48 in human SRA1) in mouse SRA1 reduces acetylated LDL (AcLDL) internalization rates, suggesting that posttranslational modification might be a mechanism to modulate scavenger receptor functionality.

Here we found evidence that mimicking phosphorylation at serine 48 by an aspartate in human macrophage SRA1 decreases surface expression, lipoprotein binding and uptake, and the mobility of cells expressing the mutated receptor. Implications for the anti-atherosclerotic properties of SRA1 are discussed.

2. Materials and methods

2.1. Cells and transfections

COS-7 cells (ATCC CRL-1651) were cultured as described [11]. One day prior to transfection, cells were seeded at 40% confluence into either 12-well plates on coverslips for fluorescence microscopy or directly into six-well plates for lipid analysis, or biotinylation assays, respectively. On the day of transfection, cell density was routinely 5×10^4 cells/cm^2. Lipid-mediated transfections with FuGENE™ (Roche Molecular Biochemicals, Rotkreuz, Switzerland) were carried out as described [11].

2.2. RT-PCR and construction of the plasmids

Total cellular RNA from human monocyte-derived macrophages cultured for 4 days in RPMI and 5% heat-inactivated human serum was extracted with the RNeasy kit from Qiagen (Qiagen, Basel, Switzerland) and reverse transcribed using SuperScript II RNase$^-$ Reverse Transcriptase™ and oligo(dT) primers (Life Technologies AG, Basel, Switzerland) according to the instructions of the manufacturer. The resulting cDNA was amplified in a PCRsprint thermocycler (Catalys AG, Wallisellen, Switzerland) using the sense primer 5′-CCC **AAG CTT** GGA TGG AGC AGT GGG ATC ACT TTC AC-3′ and the antisense primer 5′-GG**G GTA CC**G CAT TAT AAA GTG CAA GTG ACT CCA GCA-3′. The restriction sites used for cloning are shown in bold. PCR bands corresponding to the expected size for SRA1 were excised and purified using the NucleoSpin Extract kit (Macherey-Nagel, Düren, Germany). The purified PCR product was

*Corresponding author. Fax: (41)-61-2673566.
E-mail addresses: harald.heider@unibas.ch (H. Heider), eva-sabine.wintergerst@unibas.ch (E.S. Wintergerst).

Abbreviations: SRA1, macrophage scavenger receptor A1; EGFP, enhanced green fluorescent protein; LDL, low density lipoprotein; AcLDL, acetylated LDL; TR-AcLDL, Texas red-labeled acetylated LDL; OxLDL, oxidized LDL; PBS, phosphate-buffered saline; PBST, PBS supplemented with 0.1% Tween 20

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cut and ligated into the identically cut vector pEGFP-C1 (Clontech Laboratories, Basel, Switzerland). Clones were analyzed by automated sequencing (Applied Biosystems, model 377 DNA sequencer).

2.3. Site-directed mutagenesis

The plasmid encoding the correct fusion protein (referred to as EGFP/SRA1) was mutagenized using the Stratagene Quickchange™ site-directed mutagenesis kit according to the instructions supplied by the manufacturer. The sense and antisense mutagenesis primers (sense: 5′-CCTTCAAGAGAAACTGAAG**GA**CTTCAAAGCTGC-AC-3′, antisense: 5′-GTGCAGCTTTGAAG**TC**CTTCAGTTTCTC-TTGAAGG-3′, altered nucleotides are shown in bold letters) were chosen to exchange the Ser-48 codon TCC to the aspartate codon GAC. Plasmids containing the correct mutation were identified by automated sequencing. We designated this plasmid EGFP/SRA1(S48D).

2.4. LDL isolation, modification and Texas red labeling

Human LDL was isolated under lipopolysaccharide-free conditions by discontinuous gradient density and flotation ultracentrifugation in a TL-100 ultracentrifuge (Beckman Instruments, Fullerton, CA, USA) and a TLA-100.4 fixed angle rotor as described previously [12]. AcLDL and OxLDL were prepared according to [13]. Protein concentrations of all LDL solutions were determined with bicinchoninic acid (Pierce, Boston, MA, USA) with bovine serum albumin (BSA) as a standard. AcLDL was labeled with Texas red (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's instructions.

2.5. Fluorescence microscopy

Transfected cells used for microscopy were cultured on 15-mm ethanol-washed glass coverslips (Merck Schweiz AG, Dietikon, Switzerland) in 12-well Falcon plates (Becton Dickinson, Basel, Switzerland), and harvested 15 min after addition of Texas red-labeled AcLDL (TR-AcLDL). They were washed twice with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 min at room temperature. Fixed cells were washed twice in PBS and mounted in Mowiol (Hoechst, Frankfurt, Germany), containing 1% *n*-propyl gallate (Sigma-Aldrich, Buchs, Switzerland). Cells were examined using a Zeiss Axiophot microscope (Carl Zeiss AG, Feldbach, Switzerland), equipped with a Hamamatsu video imaging system and a Bio-Point automated fluorescence filter wheel (all video imaging components including the software package were supplied by Paul Bucher AG, Basel, Switzerland). Images were assembled with the OpenLab software (Improvision, Coventry, UK).

2.6. Quantitation of the uptake of TR-AcLDL

The uptake of TR-AcLDL by transfected cells was calculated using the ROI (region of interest) measurement tool of the OpenLab software package. All images for quantitative measurements were recorded within one microscope session using identical exposure times for all slides throughout one series of experiments. The exposure times were chosen to be longer than 0.5 s in order to correct for fluctuations of UV intensity. The outlines of green fluorescent cells of comparable intensity were marked manually. Such a selected green fluorescence-positive cell was defined as one ROI. Then the appropriate excitation filter for red fluorescence was set and total gray level intensity of red fluorescence within single ROIs was recorded. Except for the vector (approximately 45 cells), at least 100 fluorescent cells were measured for each condition. Similar results were obtained in two independent experiments.

2.7. SDS–PAGE and Western blots

SDS–PAGE was performed with the NuPAGE System from Novex™ (Frankfurt/Main, Germany). Proteins were transferred to Poly-Screen™ PVDF transfer membranes (NEN Life Science, Zaventem, Belgium) in blotting buffer (25 mM Tris, 192 mM glycine and 10% methanol). First, blots were incubated in blocking buffer composed of 3% BSA (Fluka, Buchs, Switzerland) in PBS for 1 h at room temperature before they were incubated for another 2 h in horseradish peroxidase–streptavidin conjugate (Zymed Laboratories, San Francisco, CA, USA) diluted 1:2500 in blocking buffer. After four washing steps in PBS plus 0.1% Tween 20 (PBST) detection was performed using the enhanced chemiluminescence method (ECL™ system from Amersham, UK). Bands were quantified densitometrically. Upon detection membranes were reprobed with green fluorescent protein (GFP) antibodies, followed by anti-mouse serum coupled to alkaline phosphatase. Color detection was performed according to standard procedures.

2.8. Biotinylation and immunoprecipitation

Transfected cells were grown for 24 h in six-well Falcon plates (Becton Dickinson, Basel, Switzerland), washed twice in 4 ml ice-cold borate buffer (pH 8.3) and incubated for 30 min at room temperature with 0.5 mg biotin-sulfo-*N*-hydroxysuccinimide with gentle agitation. Then cells were washed twice in 4 ml ice-cold PBS and solubilized in 300 μl lysis buffer (PBST supplemented with 10 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM Pefabloc®SC, 2 μg/ml aprotinin, 2 μg/ml pepstatin and 2 μg/ml leupeptin). Immunoprecipitation was performed with monoclonal GFP antibodies (Roche Molecular Biochemicals, Rotkreuz, Switzerland) according to the procedure described by Altin and Pagler [14].

2.9. Cell migration

Cell migration assays were performed in tissue culture-treated Transwell™ plates (6.5 mm diameter, 10 μm thickness, 8 μm pores; Costar, Cambridge, MA, USA). Half of the wells were coated on the upper side of the membrane, the other half of the wells were coated on the underside. For coating we used either 10 μg/ml rat tail collagen type I (Upstate Biotechnology, Lake Placid, NY, USA), or 20 μg/ml OxLDL for 2 h at room temperature followed by a washing step in PBS. 40 000 transfected cells were added into the upper chambers 4 h after transfection. Cells were allowed to migrate to the underside of the membrane during 20 h. Subsequently non-migrating cells in half of the wells were removed with a cotton swab. The number of all green fluorescent cells was counted with an inverted microscope using a 20× objective (Nikon, Küsnacht, Switzerland). We subsequently calculated the relative amount of migrating cells by dividing the number of migratory fluorescent cells by the number of all fluorescent cells, migrating and non-migrating. We counted at least 400 cells in either of two independent wells.

3. Results

3.1. Plasma membrane expression of SRA1 in EGFP/SRA1- and EGFP/SRA1(S48D)-transfected cells

First we investigated whether mutation of Ser-48 to aspartate in human SRA1 influences surface expression. In order to efficiently monitor expression we fused wild type and mutant receptor to the C-terminal end of EGFP. Then we transfected COS-7 cells with both constructs, and biotinylated all proteins at the surface of the cells as described in Section 2. Immunoprecipitation of fusion proteins with GFP-specific antiserum revealed that no biotinylated proteins in vector-transfected cells could be isolated (Fig. 1A, 'EGFP'). In contrast, massive amounts of biotinylated proteins were precipitated in cells transfected with EGFP/SRA1 (Fig. 1A, 'SRA1'). Two bands appeared, but only the faster migrating band corresponds to EGFP/SRA1 as shown by reprobing the blot with GFP antiserum (Fig. 1B, 'SRA1'). Biotinylation analysis of cells transfected with EGFP/SRA1(S48D) demonstrated that the surface expression of this receptor is strongly reduced (Fig. 1A, 'S48D'). The overall expression levels of the mutated receptor increased more than two-fold (Fig. 1B, 'S48D'), though the amount of vector DNA used for transfection was identical for both wild type and mutant receptor (1 μg/well). Quantification of the biotinylation of the EGFP–receptor fusion protein revealed that cell surface expression of the phosphorylation site mutant receptor was approximately five times lower compared to the wild type receptor. Normalizing the expression levels of the plasmids revealed that the surface expression of EGFP/SRA1(S48D) was even 13 times lower than the surface expression of EGFP/SRA1 (Fig. 1C).

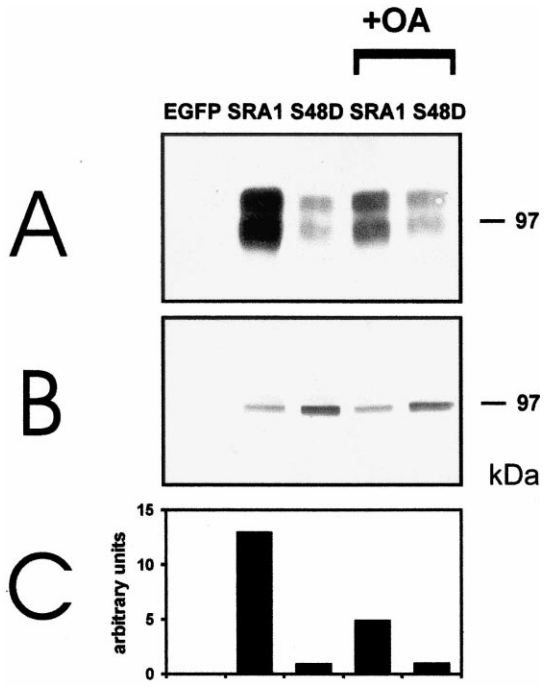

Fig. 1. Plasma membrane expression of SRA1 in transfected COS-7 cells. COS-7 cells were transfected with the EGFP vector ('EGFP'), with EGFP/SRA1 ('SRA1') as well as EGFP/SRA1(S48D) ('S48D'). After 24 h membrane proteins of the living cells were biotinylated. Then the cells were lysed and fusion proteins isolated by immunoprecipitation with GFP antiserum. The precipitated proteins were separated on SDS–PAGE and blotted onto PVDF membranes. The resulting blots were incubated with horseradish peroxidase-coupled streptavidin and bands were visualized with chemiluminescence (A). Then blots were washed and reprobed with GFP antiserum, followed by mouse antiserum coupled to alkaline phosphatase. Bands were visualized with the appropriate color reaction (B). In C the amount of biotinylated protein was normalized to the amount of fusion protein expressed.

In order to exclude that an unspecific structural modification of the mutated receptor accounts for the reduced surface expression, we treated the cells prior to biotinylation with 1 μM okadaic acid for 1 h. Okadaic acid is a well described membrane-permeable inhibitor of serine/threonine phosphatases of type 1 and 2A [15]. This treatment resulted in a marked attenuation of biotinylation of EGFP/SRA1 molecules. Less than 40% of the molecules labeled in untreated cells were biotinylated under these conditions (Fig. 1A,C, 'SRA1+OA'), confirming that phosphorylation is involved in surface expression of SRA1. The efficiency of biotinylation was hardly affected by okadaic acid treatment of cells transfected with the phosphorylation site mutant (Fig. 1A, 'S48D+OA'). Fig. 1B shows that the expression pattern of okadaic acid-treated, EGFP/SRA1- and EGFP/SRA1-(S48D)-transfected cells is identical to the one seen in non-treated cells.

3.2. Binding and uptake of TR-AcLDL

COS-7 cells were transfected with the EGFP vector alone and incubated with TR-AcLDL. Double immunofluorescence showed that vector-transfected COS-7 cells could be easily detected due to the bright cytoplasmic and nuclear green fluorescence (Fig. 2A, GFP). These cells were neither able to bind nor to take up TR-AcLDL (Fig. 2A, Texas red). COS-7 cells that were transfected with the EGFP/SRA1 fusion construct showed a punctate to reticular green fluorescent staining pattern in the cytoplasm with a concentration of the green fluorescence in a polarized, perinuclear region (Fig. 2B, GFP). At 4°C TR-AcLDL staining was concentrated at the plasma membrane region and overlapped with green fluorescence in these areas (Fig. 2B, Texas red and overlay). At 37°C the cells took up the fluorescently labeled modified lipoprotein already within 15 min of incubation (Fig. 2B, Texas red). The staining patterns of red and green fluorescence partially colocalized (Fig. 2B, overlay). COS-7 cells that were transfected with the mutated receptor (Fig. 2C) showed a weaker binding of TR-AcLDL at 4°C. In addition, the amount of TR-AcLDL internalized at 37°C appears to be smaller than in cells transfected with the wild type receptor.

The qualitative data described above were confirmed by quantitative measurements of cell-associated TR-AcLDL. We calculated the amount of Texas red fluorescence associated with transfected cells as described in Section 2. At 4°C, cells transfected with EGFP/SRA1 bound almost three times more TR-AcLDL than EGFP/SRA1(S48D)-transfected cells (Fig. 3, white bars). Normalizing to the higher rate of surface expression of the wild type receptor-transfected cells (5.2-fold) and subtracting the value obtained for binding to vector-transfected cells (0.12), we calculated that binding of TR-AcLDL to the mutant receptor at 4°C reached 90% of the amount that bound to the wild type receptor. At 37°C, wild type transfected cells bound and/or took up almost three times more TR-AcLDL than cells that expressed the phosphorylation site mutant (Fig. 3, black bars). Normalizing these values as described above shows that the mutant receptor at the cell surface takes up TR-AcLDL even more efficiently than the wild type receptor. Obviously, the uptake underlies further regulatory mechanisms.

3.3. Cell motility of the transfected cells

In a next step, we analyzed the migration behavior of transfected cells. The cells were allowed to attach to and spread and migrate on porous polycarbonate membranes that were coated on the upper or lower side of the membrane with either collagen type I or OxLDL. On collagen-coated surfaces, EGFP-, EGFP/SRA1- and EGFP/SRA1(S48D)-expressing cells all showed similar cell migration behavior, irrespective of whether the membranes were coated on their undersides or on their upper sides. The overall difference in the number of migrating cells on collagen-coated surfaces was less than 8% (data not shown). On membranes coated either on the upper side (Fig. 4, white bars) or on the lower side (Fig. 4, black bars) with OxLDL, the majority of cells expressing only the EGFP vector remained on the upper side of the membrane. In contrast, more than 70% of the cells expressing the wild type receptor moved to the lower side of the membrane, provided this side was coated with OxLDL. If the upper side was coated with OxLDL, only around 33% of the cells transmigrated, whereas approximately 45% of the mutant receptor-transfected cells transmigrated, irrespective of which side was coated with OxLDL.

4. Discussion

First we showed that, at the cell surface, the steady-state expression level of the phosphorylation site mutant is mark-

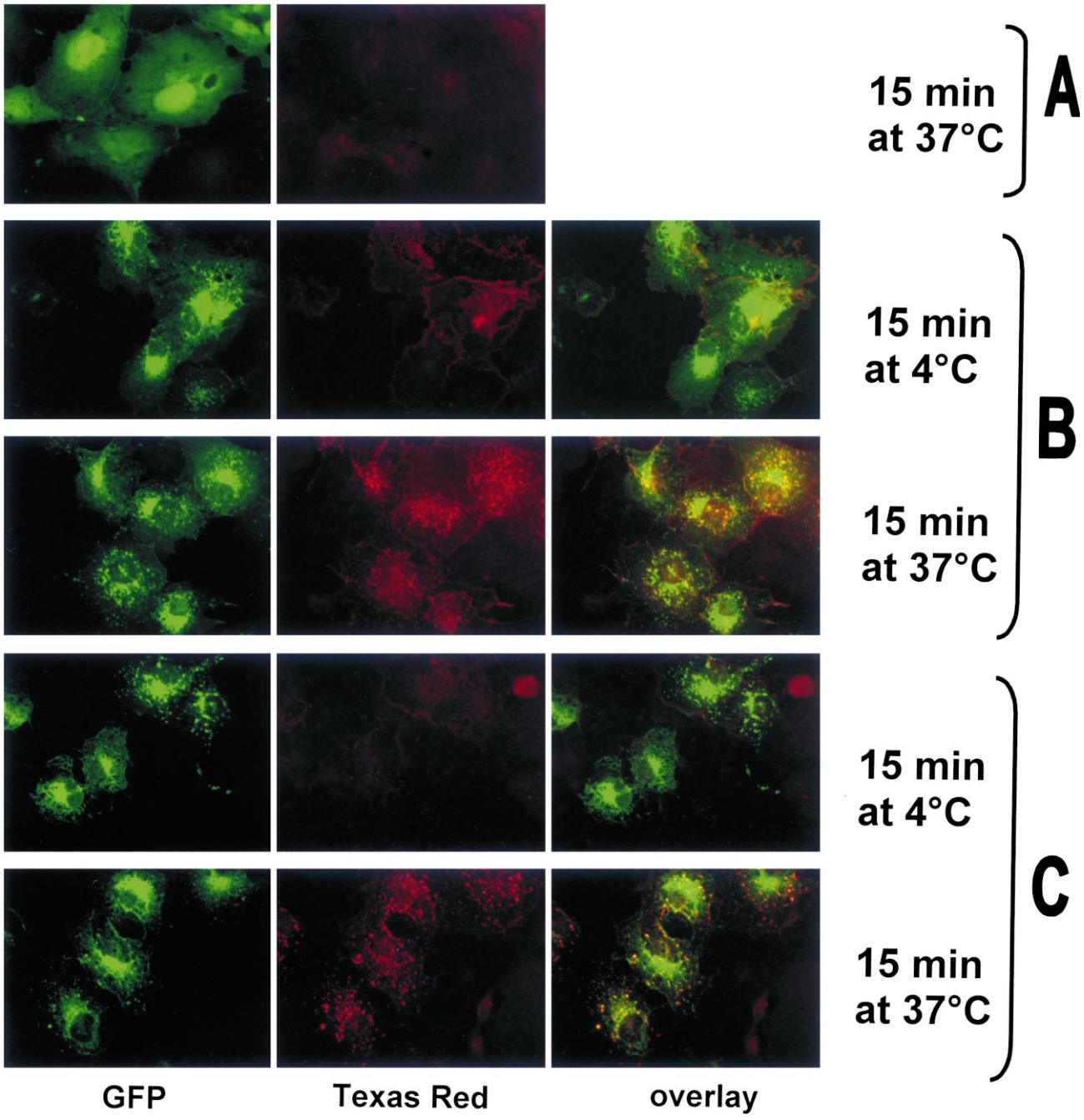

Fig. 2. Immunofluorescence analysis of TR-AcLDL uptake by transiently transfected COS-7 cells. COS-7 cells were transfected with the EGFP vector (A), EGFP/SRA1 (B) or EGFP/SRA1(S48D) (C). After 24 h of growth on glass coverslips in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, cells were incubated with TR-AcLDL (50 μg/ml) for the times indicated at either 4°C or 37°C. Then cells were fixed and analyzed by fluorescence microscopy using the appropriate filter sets for GFP and Texas red. The overlay of the corresponding images is shown in column 3 ('overlay').

edly decreased. A substantial decrease in surface expression was also observed in cells that were transfected with the wild type receptor and that were treated with a serine/threonine phosphatase inhibitor. It has been shown recently that recycling of the T cell receptor back to the membrane was critically dependent on dephosphorylation of one of its subunits [16]. The authors present evidence that a serine/threonine phosphatase of type II is involved in dephosphorylation of the receptor subunit and its return to the cell surface. Similarly, Innamorati et al. [17] present evidence that vasopressin V2 receptor recycling includes a step regulated by dephosphorylation of a specific C-terminal domain. Our data from okadaic acid treatment as well as the data obtained with the mutated receptor are in agreement with recycling mechanisms that are critically dependent on dephosphorylation of human SRA1.

Reduction of surface expression of SRA1 impaired ligand binding and/or uptake. We measured a substantial decrease in cell-associated TR-AcLDL in cells transfected with the phosphorylation site mutant. Interestingly, Fong and Le [10] pre-

viously demonstrated that COS cells transfected with mouse SRA1 in which serine 49 (which corresponds to serine 48 in human SRA1) was inactivated by replacing it with an alanine also display significantly decreased amounts of cell-associated AcLDL. Obviously, both conditions, phosphorylation and dephosphorylation, result in a net decrease in ligand binding and uptake. At first sight this seems contradictory, but Fong and Le also showed that decrease of AcLDL binding and/or uptake was counterbalanced by inactivating an additional putative phosphorylation site in the cytoplasmic part of SRA1, suggesting that receptor function is critically dependent on the pattern of phosphorylation of several sites. Complex phosphorylation patterns within the trimeric assembly of functional SRA1 may therefore be involved in fine-tuning surface expression and ligand uptake via this receptor.

Further support for the involvement of additional molecule moieties in uptake of ligand comes from our observation that mimicking phosphorylation indeed accelerates uptake of TR-AcLDL, at least for those receptor molecules that reach the cell surface.

In the meantime, Fong and coworkers also constructed the Ser-49/Asp mutant of mouse SRA1. The amount of AcLDL that was associated with COS cells transfected with this construct dropped to less than 35% of the amount associated with wild type receptor-transfected cells (Fong, personal communication). These numbers are in good agreement with the results presented here.

In addition, it turned out that expression of SRA1 strongly increased the mobility of cells towards OxLDL. Expression of the phosphorylation site mutant increased cell motility, irrespective of whether the upper side or the lower side was coated with OxLDL. Therefore, we suggest that chemotaxis is operative in the case of the wild type receptor-transfected cells, whereas the migration behavior of mutant receptor-transfected cells indicates that their movement is best described as chemokinesis.

It has been shown previously that AcLDL binding induces tyrosine phosphorylation of a variety of intracellular proteins in a macrophage-like cell line [18]. These phosphorylations

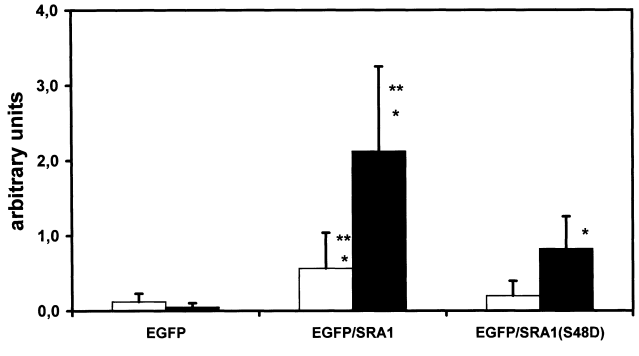

Fig. 3. Quantitation of cell-associated TR-AcLDL. Cells were treated and prepared for fluorescence microscopy as described in Fig. 2. Cells were kept, upon addition of TR-AcLDL, for 15 min at 4°C (white bars), or were transferred for 15 min to 37°C (black bars). The intensity of red fluorescence associated with transfected cells was quantified using the OpenLab Software from Improvision as described in Section 2. Data represent the mean fluorescence intensity of at least 40 (EGFP) or 100 cells (EGFP/SRA1, EGFPSRA1(S48D)). One asterisk denotes data that differ significantly from vector ($P < 0.001$). Two asterisks denote data that differ significantly from mutant receptor ($P < 0.001$).

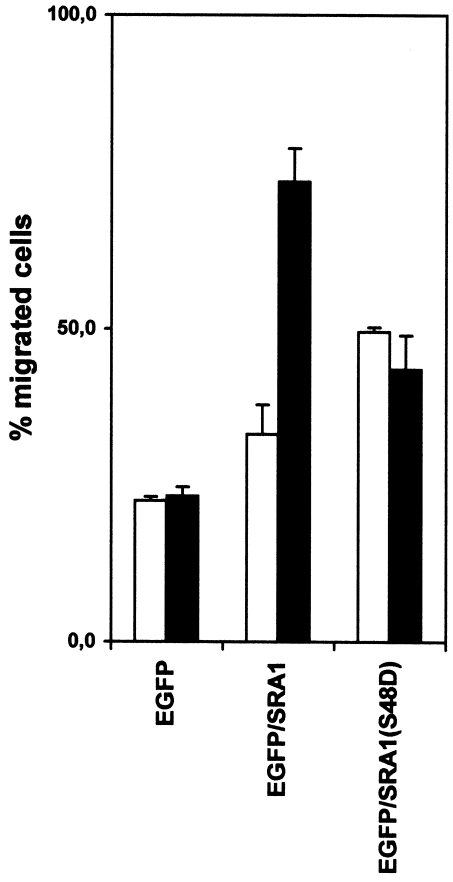

Fig. 4. Mobility of SRA1-expressing COS-7 cells on membranes coated with rat tail collagen or human OxLDL. Transfected cells were allowed to attach to and spread and migrate for 20 h on porous polycarbonate membranes that were coated on their upper sides (white bars) or on their lower sides (black bars) with 20 μg/ml OxLDL. Represented here is the percentage of cells that migrated to the underside of the membranes. For each condition, we counted at least 400 cells in either of two independent wells.

may trigger reorganization of the cytoskeleton, finally leading to mobility changes of the cells.

Increased expression of SRA1 at the cell surface could help the cells to migrate more rapidly along a gradient of Ox-LDL, thereby ingesting the modified lipoprotein and other inflammatory agents which reside in an atherosclerotic plaque. This would shorten the exposure time of the SRA-expressing cells and neighboring cells to inflammatory signals and might explain why SRA1 knockout mice in a APOE3 Leiden transgenic mouse background develop more severe lesions when fed an atherogenic diet than APOE3 Leiden mice fed identically [2]. It could also explain the observation of de Winther and coworkers [5] that SRA1 overexpression in LDL receptor-deficient transgenic mice fed a high fat diet actually reduces atherosclerosis.

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