The mosaic receptor sorLA/LR11 binds components of the plasminogen-activating system and platelet-derived growth factor-BB similarly to LRP1 (low-density lipoprotein receptor-related protein), but mediates slow internalization of bound ligand

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The type-1 receptor sorLA/LR11, a member of the Vps10p-domain receptor family that also contains domains characterizing members of the LDL (low-density lipoprotein) receptor family, has been shown to induce increased uPAR (urokinase receptor) expression as well as enhanced migration and invasion activities in smooth muscle cells in the presence of PDGF-BB (platelet-derived growth factor-BB). Here we show that sorLA interacts with both components of the plasminogen activating system and PDGF-BB similarly to LRP1 (LDL receptor-related protein/ α_2 -macroglobulin receptor), which is an important clearance receptor with established functions in controlling uPAR expression as well as PDGF-BB signalling. In contrast with LRP1, sorLA does not interact with α_2 -macroglobulin, which is a binding protein for several growth factors, including PDGF-BB. By using LRP1-deficient cells transfected with sorLA, we demonstrate that

sorLA-bound ligand is internalized at a much lower rate than LRP1-bound ligand, and that sorLA is inefficient in regulating cell surface uPAR expression, which depends on rapid internalization of the ternary complex between urokinase-type plasminogen activator, its type-1 inhibitor, and uPAR. Thus, although overlapping with regard to binding profiles, sorLA is substantially less efficient as a clearance receptor than LRP1. We propose that sorLA can divert ligands away from LRP1 and thereby inhibit both their clearance and signalling events mediated by LRP1.

Key words: Vps10p-domain receptor family, endocytosis, receptor-associated protein (RAP), urokinase-type plasminogen activator (uPA), type-1 plasminogen activator inhibitor (PAI-1), platelet-derived growth factor-BB (PDGF-BB).

INTRODUCTION

The mosaic type-1 receptor sorLA/LR11 is a member of the Vps10p domain receptor family, which also contains sortilin and sorCS1–sorCS3 ([1–4] and references therein). The luminal part of sorLA has an N-terminal Vps10p domain, and also contains domains that characterize members of the LDL (low-density lipoprotein) receptor family, including a β -propeller domain and a cluster of 11 LA (LDL receptor class A) repeats, as well as a cluster of fibronectin type III repeats. The cytoplasmic domains of members of the core of the LDL receptor family have specific tyrosine-containing motifs (Asn-Pro-Xaa-Tyr) that are known to interact with adapter proteins (reviewed in [5,6]), whereas the cytoplasmic domain of sorLA has a single, different tyrosine-containing motif (Phe-Ala-Asn-Ser-His-Tyr). Mature sorLA is generated by the furin-mediated cleavage of the pro-receptor, and its cluster of LA repeats binds the chaperone/escort protein RAP (receptor-associated protein) and apolipoprotein E with high affinity, as well as lipoprotein lipase [7]. SorLA is located mainly in paranuclear compartments, but approx. 10 % of the receptors are on the cell surface [7].

SorLA is expressed mainly in the nervous system, but is also found in non-neuronal tissues such as testis, ovary, lymph nodes, distal kidney tubules and, notably, intimal SMCs (smooth muscle

cells) of the aorta, particularly in atheromatous lesions [1,2,8–10]. SorLA may be implicated in the development of atherosclerosis, as overexpression of the receptor in cultured SMCs enhances migration and invasion induced by PDGF-BB (platelet-derived growth factor-BB) [11]. This is accompanied by increased cell surface expression of the receptor (uPAR) for uPA (urokinase-type plasminogen activator), and the mechanisms causing enhanced migration might therefore involve the uPA/uPAR system [11]. These results raised the question of whether sorLA binds components of the plasminogen activating system and PDGF-BB, as shown previously for the LDL receptor core family member LRP1 (LDL receptor-related protein)/ α_2 M (α_2 -macroglobulin) receptor [5,6].

The luminal part of LRP1 has four clusters of LA repeats (numbered from the N-terminus), of which cluster II (eight repeats) and cluster IV (eleven repeats) bind a large number of structurally and functionally diverse ligands [5,6,12,13]. These include the complex between uPA and its type-1 inhibitor PAI-1, and one of the important functions of LRP1 is to provide rapid internalization of cell-surface-bound uPA–PAI-1 complexes [14]. The sequence of events includes high-affinity binding of pro-uPA (single-chain uPA) to the glycolipid-anchored uPAR on the cell surface, activation of pro-uPA to two-chain uPA, and quenching of the catalytic activity by formation of a stable complex between the

Abbreviations used: α_2 M, α_2 -macroglobulin; α_2 M*, receptor-active α_2 M; DTSSP, 3,3'-dithiobis(sulphosuccinimidylpropionate); CHO, Chinese hamster ovary; LDL, low density lipoprotein; LA repeat, LDL receptor class A repeat; LRP1, LDL receptor-related protein; NTF, N-terminal fragment of uPA; PAI-1, type-1 plasminogen activator inhibitor; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor- β ; RAP, receptor-associated protein; SMC, smooth muscle cell; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; wt, wild type.

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bound uPA and its primary inhibitor PAI-1. Alternatively, uPA–PAI-1 complex generated in the fluid phase may bind directly to uPAR. LRP1 then interacts with uPAR-bound uPA–PAI-1 and mediates endocytosis of the ternary uPA–PAI-1–uPAR complex, followed by degradation of uPA–PAI-1 in the lysosomes and recycling of most of the released uPAR to the cell surface [14–19]. Due to incomplete LRP1-mediated recycling and increased degradation of uPAR, loss of LRP1 function is under some conditions associated with increased cell surface levels of uPAR and accelerated cell migration [18,20–22]. The inhibitor-induced partial recycling of uPAR is critically dependent on the rapid endocytosis of LRP1, which is mediated largely by a tyrosine-based signal (Tyr-Xaa-Xaa-Leu motif) in its cytoplasmic domain [23].

Recent results in mice with specific inactivation of LRP1 in vascular SMCs have demonstrated that this receptor plays a pivotal atheroprotective role and prevents overexpression of PDGFR (PDGF receptor- β) [24]. Although the mechanism remains unknown, it may include the previously reported binding of PDGF-BB to LRP1, as well as PDGFR-dependent tyrosine phosphorylation of its cytoplasmic domain [25,26]. In addition, LRP1 mediates the inhibition of SMC migration induced by apolipoprotein E, possibly by interfering with the PDGFR signalling cascade [27]. Finally, LRP1 may provide clearance of PDGF-BB either via direct binding and rapid internalization of the growth factor or via binding and internalization of α_2M^* (receptor-active α_2M) with bound growth factor [24–26,28].

To elucidate the functions of sorLA, we analysed its binding of LRP1 ligands, which are important in the LRP1-mediated control of cell migration, and we determined the rate of endocytosis of sorLA relative to that of LRP1 by measuring the rate of internalization of the common ligand RAP into CHO (Chinese hamster ovary) cell lines. We show that sorLA binds components of the plasminogen activating system, as well as PDGF-BB, almost identically to LRP1. By contrast, sorLA does not bind α_2M^* . In addition, ligand bound to sorLA is internalized at a much lower rate than when bound to LRP1; as a result, sorLA is unable to convey the efficient internalization of the uPA–PAI-1 complex and recycling of uPAR, and may be inefficient in providing clearance of PDGF-BB. We suggest that a high level of sorLA expression may divert ligands away from LRP1 and thereby counteract the clearance of, and possibly signalling mediated by, this receptor.

MATERIALS AND METHODS

Reagents and antibodies

Human recombinant RAP was produced as described previously [14] and iodinated using chloramine-T as the oxidizing agent. Other proteins were iodinated similarly with approx. 0.3 mol of iodine/mol of protein. Recombinant human pro-uPA (single-chain uPA; aa 1–411), two-chain active uPA, an N-terminally truncated variant of uPA (low-molecular-mass uPA) comprising aa 137–411, and the N-terminal fragment of uPA (NTF; aa 1–136) were prepared and purified as described previously [29]. tPA (tissue-type plasminogen activator) was from Boehringer-Ingelheim. Human PAI-1 in active and latent forms was prepared as described in [29]. To prepare uPA–PAI-1 complexes, active PAI-1 and uPA were incubated overnight at 4 °C, and complexes were isolated by sequential immunoaffinity chromatography using immobilized monoclonal anti-uPA and anti-PAI-1 antibodies [29]; similar procedures were used to prepare tPA–PAI-1 complexes [30]. To prepare ^{125}I -labelled uPA–PAI-1 complexes, active uPA was iodinated and incubated with excess PAI-1 followed by sequential immunoaffinity chromatography. Recombinant soluble human

uPAR [uPAR-(1–277)] was a gift from Gunilla Højer-Hansen (Finsen Laboratory, Copenhagen, Denmark). Human PDGF-BB was purchased from R & D Systems. α_2M was prepared as described previously and converted into α_2M^* by treatment with 200 mM methylamine for 2 h at 20 °C [31]. *Pseudomonas* exotoxin A was kindly donated by David Fitzgerald (National Institutes of Health, Bethesda, MD, U.S.A.) [32].

LRP1 was purified from human placenta by affinity chromatography using Sepharose-immobilized α_2M^* [31]. The luminal part of mature human sorLA (aa 54–2107), its Vps10p domain (aa 54–731), the luminal part of sorCS1 (aa 78–1067) and the luminal part of sortilin, which consists of a Vps10p domain only (aa 45–725), were produced in CHO cells and purified by affinity chromatography as described [4,7,33]. The soluble receptors were stored at concentrations of 200–400 μ g/ml. Soluble sorLA was used to generate anti-sorLA antibody, and the Ig fraction was purified from goat serum using Protein A–Sepharose.

Cell transfection and culture

The LRP-null CHO cell line (clone 13-5-1) was kindly provided by David Fitzgerald [32] and, like wt (wild type) CHO cells, cultured in serum-free HyQ-CCM5 CHO medium (HyClone, Logan, UT, U.S.A.) unless otherwise stated. The LRP-null cells were transfected with full-length sorLA in the pcDNA 3.1/Zeo(+) vector using FuGENE 6 (Roche Molecular Biochemicals), as described [7]. Stable transfectants were selected in medium containing 300 μ g/ml Zeocin (Invitrogen), and clones expressing sorLA were identified by Western blotting of cell lysates [7] followed by subcloning to ensure uniform expression of sorLA. Three subclones, including clone 13.9 which was used throughout, showed essentially the same expression of sorLA, and the same binding and internalization of ^{125}I -labelled RAP. For quantification of cell surface expression, cells were treated with the impermeable reagent sulfo-*N*-hydroxysuccinimidobiotin (Pierce), and biotinylated cell surface proteins were precipitated with streptavidin-coupled Sepharose. The fractions of streptavidin-bound and unbound sorLA were detected by Western blotting and quantified using a FUJIFILM LAS-1000 luminescence image analyser [4]. For quantification of secretion/shedding of a soluble form of sorLA from the cells stably transfected with full-length sorLA, the cells were washed and incubated for various times at 37 °C followed by Western blotting of sorLA in lysates and in media concentrated by using Centricon-30 (Millipore).

Ligand binding and internalization

Cells were cultured for 24 h in 1.9 cm² wells (5×10^4 /well for binding of RAP; 1.5×10^5 /well for binding of NTF and α_2M^*), washed, and incubated with ^{125}I -labelled ligand at 4 °C in 124 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 2.5 mM NaH₂PO₄, 25 mM Hepes and 1 % (w/v) BSA, pH 7.4 (Krebs/Hepes buffer). Following four washes in ice-cold buffer, the cells were lysed in 1 M NaOH, and binding was determined as radioactivity in the lysate. In several experiments, cells with ligand bound at 4 °C were rapidly exposed to 37 °C warm medium and transferred immediately to a water bath. The medium was removed at various times, followed by treatment of the cells with ice-cold acid solution (0.2 M acetic acid, 100 mM NaCl, pH 2.6) for 2×10 min, and the acid-releasable radioactivity was taken as a measure of surface-bound ligand, whereas the fraction remaining in the cells was considered to be internalized. Degradation of labelled ligand, either directly in cell incubation medium or following release of ligand pre-bound at 4 °C, was measured as the fraction of radioactivity soluble in 12 % (v/v) trichloroacetic acid.

Ligand blotting and cross-linking experiments

For ligand blotting, the purified soluble receptors were subjected to non-reducing PAGE using 0.1 % SDS and blotted on to PVDF membranes using standard procedures. After blocking for 2 h in buffer containing 2 % (w/v) skimmed milk and 0.05 % Tween 20, and washes in Krebs/Hepes buffer containing 0.5 % BSA, the membranes were incubated in the same buffer with approx. 1×10^5 c.p.m./ml labelled ligand, washed, and processed for autoradiography.

For cross-linking to sorLA on the cell surface, cells were incubated with 125 I-labelled ligand (approx. 2×10^5 c.p.m./ml) in Krebs/Hepes buffer, pH 7.4. After washes, bound proteins were cross-linked using a concentration of 2 mM of the membrane-impermeable and thiol-cleavable cross-linker DTSSP [3,3'-dithio-bis(sulphosuccinimidylpropionate); Pierce] for 30 min at 4 °C in PBS containing 0.6 mM Ca^{2+} . The reaction was quenched with buffer containing 20 mM Tris, and cell lysates were prepared in 1 % (v/v) Triton X-100, 20 mM Tris/HCl and 10 mM EDTA, pH 8.0. Centrifuged lysates supplemented with 4 vol. of PBS were incubated with GammaBind Sepharose beads (Amersham Biosciences) pretreated with 10 μ g of goat anti-sorLA Ig. Finally, immunoprecipitated ligand was analysed by SDS/PAGE under non-reducing (not shown) and reducing conditions followed by autoradiography.

Solid-phase binding assay and surface plasmon resonance analysis

Microtitre wells (Polysorp; Nunc, Roskilde, Denmark) were coated overnight with 50 μ l of PDGF-BB (0.1 μ M) in Tris-buffered saline, pH 8.5, and blocked in the same buffer containing 5 % (w/v) BSA. 125 I-labelled sorLA-(54–2107) (approx. 1 nM) was then added to the wells in the absence or presence of inhibitor and, following incubation overnight at 4 °C, the microtitre wells were washed, and bound radioactivity was determined.

Surface plasmon resonance measurements were performed on a BIAcore 2000 instrument (Biacore, Uppsala, Sweden) equipped with CM5 sensor chips activated as described in [33]. LRP1, sorLA and sortilin were immobilized to densities of 27, 54 and 60 fmol/mm² respectively, and samples for binding (40 μ l) were injected at 5 μ l/min at 25 °C in 10 mM Hepes, 150 mM NaCl, 1.5 mM CaCl_2 , 1 mM EGTA and 0.005 % Tween 20, pH 7.4. Binding was expressed in relative response units as the response obtained from the flow cell containing immobilized receptor minus the response obtained when using an activated but uncoupled chip. The overall K_d values were determined by BIAevaluation 3.0 software using a Langmuir 1:1 binding model and simultaneous fitting to all curves in the concentration range considered (global fitting).

RESULTS

Characterization of sorLA transfectants

To measure binding and internalization mediated by sorLA, LRP-null CHO cells were transfected with the full-length receptor, and subclones were selected to provide stable and uniform expression. Figure 1(A) shows that the transfectants expressed sorLA, in contrast with the parental LRP-null cells and wt CHO cells, and that approx. 8 % of the receptors were expressed on the cell surface (Figure 1B), in agreement with previous results in transfected wt CHO cells [7]. The relatively low mobility of purified soluble sorLA (Figure 1A) has been noted before, and may be due to differential glycosylation [7]. Figure 1(C) shows, using cross-linking

Figure 1 Characterization of LRP-null CHO cells transfected with sorLA

(A) Purified soluble sorLA [sorLA-(54–2107)] and cell lysates were resolved by reducing SDS/PAGE (4–16 % acrylamide) and subjected to Western blotting. Lane 1, soluble sorLA; lane 2, lysate of LRP-null CHO cells transfected with full-length sorLA; lanes 3 and 4, lysates of parental LRP-null CHO cells and of wt CHO cells respectively. (B) Western blots of biotinylated and non-biotinylated sorLA in lysates of transfectants. The cells were surface-biotinylated and lysed, and biotinylated material was recovered on streptavidin–Sepharose. Lane 1, biotinylated sorLA, representing 18.8 % of the bead fraction. Lane 2, non-biotinylated sorLA in the same lysate, representing 1.8 % of the amount not bound to the beads. Scanning densitometry revealed that 8.1 % of sorLA had been accessible to surface biotinylation (6.7–9.1 % in three separate experiments). Lane 3, absence of biotinylated material on Sepharose without streptavidin. Lane 4, recovery of sorLA in the non-bound fraction. Lysate incubated once with streptavidin–Sepharose did not contain biotinylated sorLA (results not shown). (C) Immunoprecipitation of RAP cross-linked to sorLA expressed on the cell surface. The sorLA transfectants and parental LRP-null cells were incubated for 2 h at 4 °C with 125 I-RAP (10^5 c.p.m./ml) with or without unlabelled RAP, washed, and treated with the thiol-cleavable cross-linker DTSSP. Labelled RAP bound to sorLA was immunoprecipitated from lysates with goat anti-sorLA Ig using GammaBind beads followed by reducing SDS/PAGE (8–16 % acrylamide) and autoradiography. Lane 1, LRP-null cells; lane 2, sorLA transfectants; lane 3, sorLA transfectants plus 200 nM unlabelled RAP.

and immunoprecipitation with anti-sorLA antibody, that 125 I-labelled RAP bound to sorLA on the cell surface.

Because a soluble form of sorLA is secreted by various cell types ([34] and references therein), we assessed secretion in the transfectants. The results of four experiments (not shown) demonstrated that 3–5 % of the total amount of sorLA in the cells was released into the medium after 1 h at 37 °C, whereas no release could be detected after 3 h at 4 °C. The subsequent experiments with cells were performed at 4 °C and, after washings, for short time periods at 37 °C; the amount of sorLA in the medium was negligible under these conditions.

Binding of 125 I-RAP (approx. 40 pM) was determined at 4 °C in sorLA transfectants, wt CHO cells expressing endogenous LRP1, and LRP-null cells. After 3 h, the transfectants bound about six times more and the wt CHO cells about three times more than the LRP-null cells (39.1 ± 1.9 %, 19.6 ± 1.1 % and 5.9 ± 0.4 % of the added tracer respectively; means $\pm$ S.D., $n = 3$). Cell-associated radioactivity measured in the presence of 1 μ M unlabelled RAP (0.8 % of the added tracer) was considered non-specific and subtracted from all measurements. The residual binding of 125 I-RAP to LRP-null cells is in agreement with previous results [23]. The affinities of sorLA and LRP1 for binding of RAP were similar, with half-maximal inhibition of 125 I-RAP binding at approx. 0.5 nM unlabelled RAP (results not shown). In all subsequent experiments using 125 I-RAP, incubations of sorLA transfectants, wt CHO cells and LRP-null cells were performed in parallel, and binding to sorLA or LRP1 was determined by subtracting radioactivity associated with the LRP-null cells from radioactivity associated with the transfectants and wt CHO cells.

SorLA is endocytosed slowly compared with LRP1

In order to measure the rate of endocytosis, cells preincubated with 125 I-RAP at 4 °C were washed and re-incubated at 37 °C, and the fractions representing cell-surface-associated (i.e. acid-released), internalized (i.e. not acid-released) and dissociated 125 I-RAP were determined at different times up to 8 min. Degradation of 125 I-RAP

Figure 2 Internalization of RAP mediated by sorLA and LRP1

SorLA transfectants, parental LRP-null cells and wt CHO cells were incubated with ^{125}I -RAP for 3 h at 4 °C, washed, transferred to 37 °C warm medium and incubated for the times indicated. The medium was removed and assayed for dissociated radioactivity (▼), and the cells were immediately placed on ice followed by acid treatment to determine the released (i.e. surface-associated; ○) and non-released (i.e. internalized; ●) fractions. Radioactivity associated with the sorLA transfectants and wt CHO cells, corrected for the minor radioactivity associated with LRP-null cells at each time point, was considered initially bound to sorLA (A) or LRP1 (B). The results are expressed as a percentage of ^{125}I -RAP bound to sorLA or LRP at time zero (i.e. without incubation at 37 °C), and the points represent mean values of four replicates.

was negligible within this time frame (results not shown). Figure 2(A) shows that RAP was internalized at a comparatively low rate when bound to sorLA, whereas binding to LRP1 resulted in very fast internalization (Figure 2B), as demonstrated previously [35]. Whereas essentially all RAP bound to LRP1 was internalized rapidly, a substantial fraction of RAP that bound to sorLA dissociated into the medium. Because dissociation of RAP from sorLA and LRP1 was similar at 4 °C (not shown), the results suggest that the higher rate of dissociation from sorLA is the result of the lower rate of internalization. In the displayed experiment, 24% of the RAP bound initially to sorLA was internalized by 4 min, whereas LRP1 internalized 72% of the ligand in 1 min. The rate constant of internalization was therefore approx. $6.0\% \cdot \text{min}^{-1}$ for sorLA, and at least $72\% \cdot \text{min}^{-1}$ for LRP1; similar results were obtained in four additional experiments (sorLA, $6.4 \pm 1.9\% \cdot \text{min}^{-1}$; LRP1, $77.5 \pm 9.3\% \cdot \text{min}^{-1}$; means $\pm$ S.D.).

In some experiments, cells with ^{125}I -RAP pre-bound at 4 °C were incubated for prolonged periods at 37 °C, and the results showed that most of the ligand internalized via sorLA was eventually degraded to trichloroacetic acid-soluble fragments by 3 h. However, due to the relatively low rate and extent of internalization, possibly including shedding of the receptor, sorLA mediated degradation of only 22–31% ($n=4$) of the initially bound

Figure 3 Binding of the uPA-PAI-1 complex to electroblotted sorLA

Purified soluble sorLA, LRP1 and soluble sorCS1 were subjected to 4–16% PAGE using 0.1% SDS and electroblotted on to PVDF membranes. The membranes were incubated with 2×10^6 c.p.m./ml ^{125}I -labelled uPA-PAI-1 complex, washed, and subjected to autoradiography. Lane 1, 0.5 µg of sorLA; lane 2, 0.5 µg of LRP1; lane 3, 4 µg of sorCS1.

RAP after 3 h, compared with 82–89% degradation mediated by LRP1.

The results demonstrate a marked difference between sorLA and LRP1 in the kinetics of endocytosis as determined with RAP. We next analysed binding of components of the plasminogen activating system.

Similar binding of components of the plasminogen activating system to sorLA and LRP1

The uPA-PAI-1 complex binds primarily to the glycolipid-anchored uPAR on the cell surface with very high affinity (K_d approx. 50 pM) and, when expressed, LRP1 then binds the uPA-PAI-1 moiety and conveys rapid internalization of the ternary uPA-PAI-1-uPAR complex. Initial experiments with ^{125}I -labelled uPA-PAI-1 complex showed that the sorLA transfectants, the parental LRP-null cells and wt CHO cells bound nearly the same amount of tracer at 4 °C (results not shown), as expected in view of the high level of cell surface uPAR in CHO cells and the ability of hamster uPAR to bind human uPA with high affinity [18,36,37]. Binding of the uPA-PAI-1 complex was abolished by 100 nM of the uPAR-binding NTF, whereas 600 nM RAP had little effect (results not shown). This demonstrates that most uPA-PAI-1 complex binds to uPAR on the cells, and that only a minor part binds directly to endocytic receptors, in agreement with previous results in monocytes expressing both uPAR and LRP1 [14].

As binding of the uPA-PAI-1 complex directly to sorLA and LRP1 could not be measured in CHO cells, we used purified receptors for further analysis. Figure 3 shows that ^{125}I -labelled uPA-PAI-1 bound to electroblotted purified sorLA as well as to LRP1, but not to the Vps10p domain receptor family member sorCS1 (control), which is also unable to bind RAP [4]. Characterization of the interactions by plasmon resonance analysis revealed similar binding of uPA-PAI-1 to sorLA and LRP1 (Figures 4A and 4B). RAP (500 nM) and EDTA (10 mM) abolished binding of the complex to both receptors, and heparin (10 units/ml) and apolipoprotein E3 (500 nM; not shown) inhibited binding by approx. 60%. Separate experiments showed that 10 nM RAP was sufficient to block binding of 60 nM uPA-PAI-1 complex to both sorLA and LRP1, with half-maximal inhibition at 1.5–2.0 nM RAP, strongly suggesting that the complex interacts with RAP binding domains on the receptors. Figures 4(C) and 4(D) demonstrate closely similar affinities for binding of the complex to sorLA and LRP1, with overall K_d values of approx. 2 nM.

To evaluate a possible contribution of the sorLA Vps10p domain, which binds RAP with low affinity [7], the purified domain

Figure 4 Affinity of binding of the uPA-PAI-1 complex to sorLA and LRP1

Binding was measured by surface plasmon analysis using chips with 54 fmol/mm² immobilized sorLA (**A, C**) and 27 fmol/mm² LRP1 (**B, D**). The chips showed nearly equal binding of 500 nM RAP (950 and 980 response units by 600 s; not shown), and the calculated binding capacities were 0.44 mol of RAP/mol of sorLA and 0.91 mol of RAP/mol of LRP1. (**A, B**) The chips were superfused with 100 nM uPA-PAI-1 with or without inhibitors at 100 s, followed by buffer alone at 600 s. The response of 500 nM RAP alone has been subtracted. (**C, D**) The chips were superfused with 5–200 nM uPA-PAI-1. The calculated K_d values and capacities for binding of complex were as follows: sorLA, 2.3 nM and 0.26 mol of uPA-PAI-1/mol of receptor; LRP1, 1.9 nM and 0.35 of mol uPA-PAI-1/mol of receptor.

was immobilized and superfused with 200 nM uPA-PAI-1 complex. Low but significant binding was apparent (approx. 60 response units at 600 s; calculated overall K_d approx. 1 μ M). This prompted us to analyse the Vps10p domain of the endocytic receptor sortilin (i.e. the luminal part of the receptor [38]), which is the only structure other than clusters of LA repeats that is known to bind RAP with high affinity [33]. The result (not shown) demonstrated RAP-inhibitable binding of the uPA-PAI-1 complex to sortilin with a K_d of approx. 40 nM. This emphasizes further the relationship between binding of RAP and of the uPA-PAI-1 complex, and extends previous observations that some ligands are shared by sortilin and LRP1 [39]. On the other hand, the minor contribution of the Vps10p domain of sorLA strongly suggests that binding of the uPA-PAI-1 complex occurs almost entirely to its LA repeat cluster.

To analyse the regions in the uPA-PAI-1 complex involved in binding to sorLA, experiments analogous to those shown in Figures 4(C) and 4(D) were performed to estimate K_d values for binding of the compounds listed in Table 1. Like LRP1, sorLA bound both PAI-1 (active, latent or in complex with tPA) and uPA, suggesting that both the uPA and PAI-1 moieties of the complex participate in making contact with the receptor. Pro-uPA (single-chain uPA) bound equally well to sorLA and LRP1, and 500 nM pro-uPA inhibited binding of the uPA-PAI-1 complex to both receptors by approx. 60 % (results not shown), in accordance with blocking of those sites on the receptors that interact with the uPA moiety. Although binding of NTF could not be measured, the A-chain of uPA might be involved in addition to the B-chain, since a complex of PAI-1 with low-molecular-mass uPA, which contains only the B-chain, showed reduced affinity. Binding of

Table 1 Affinities for binding of components of the uPA-PAI-1 complex to sorLA and LRP1

The experiments were performed similarly to those shown in Figures 4(C) and 4(D). LMM uPA denotes low-molecular-mass uPA. The K_d values for binding of ligands to sorLA and LRP1 are given as mean values and ranges from three experiments.

Ligand	K_d (nM)	
	SorLA	LRP1
uPA-PAI-1	2.3 (2.1–2.5)	2.1 (1.9–2.8)
Active PAI-1	23 (20–32)	24 (15–25)
Latent PAI-1	41 (24–100)	33 (14–40)
uPA	12 (10–13)	7 (6–10)
Pro-uPA	5 (5–6)	5 (3–7)
LMM uPA	~1000 (500–1500)	~1000 (500–1500)
(LMM uPA)-PAI-1	33 (33–44)	42 (37–47)
NTF	> 5000	> 5000
tPA-PAI-1	23 (21–42)	12 (8–21)

all compounds listed in Table 1 was blocked by RAP, and none of them bound significantly to purified sorLA Vps10p domain (K_d values > 2 μ M). The results are in general accordance with previously reported inhibition of binding of ¹²⁵I-labelled uPA-PAI-1 to LRP1 immobilized in microtitre wells [15], and strongly suggest that the same regions of the complex are involved in binding to sorLA and LRP1.

To explore further the mode of binding, we tested the effect of soluble uPAR on the binding of pro-uPA to sorLA. uPAR binds to the N-terminal growth factor domain of pro-uPA and inhibits

Figure 5 Inhibition of binding of pro-uPA to sorLA by soluble uPAR

The chip with immobilized sorLA was superfused with 50 nM pro-uPA, 50 nM pro-uPA plus 1 μ M soluble uPAR, or 1 μ M soluble uPAR alone.

Figure 6 Binding of tPA to sorLA independent of binding of the uPA-PAI-1 complex

The chip with immobilized sorLA was superfused with 1 μ M tPA or buffer and, at 700 s, with 100 nM uPA-PAI-1 in the presence of 1 μ M tPA or with 100 nM uPA-PAI-1 alone.

its interaction with LRP1, thereby protecting the pro-uPA-uPAR complex from premature internalization before cleavage of pro-uPA to active uPA and the formation of a ternary complex with PAI-1 has occurred [15]. Figure 5 shows that soluble uPAR (1 μ M) inhibited binding of 50 nM pro-uPA to sorLA by more than 90%, and that soluble uPAR alone bound poorly to the receptor, if at all. On the other hand, uPAR caused only a slight decrease in binding of uPA-PAI-1 (results not shown), presumably because most binding regions in the complex remain available after binding of uPAR. Although data are displayed only for sorLA, essentially identical results were obtained in parallel experiments with LRP1.

We tested tPA because it binds to LRP1 but is very inefficient in inhibiting binding of the uPA-PAI-1 complex and pro-uPA to this receptor ($K_i > 1 \mu$ M) [15]. Initial experiments (results not shown) demonstrated that sorLA (but not its Vps10p domain) and LRP1 both bound tPA with a K_d of approx. 80 nM. Figure 6 shows that pre-binding of a saturating concentration of tPA (1 μ M) to sorLA did not inhibit binding of 100 nM uPA-PAI-1. Conversely, 200 nM uPA-PAI-1 complex and 1 μ M pro-uPA did not inhibit binding of 100 nM tPA to sorLA (results not shown); similar results were obtained with LRP1. Separate experiments demonstrated that RAP is a relatively poor inhibitor of tPA binding

Figure 7 Binding of uPA-PAI-1 and the ternary uPA-PAI-1-uPAR complex to sorLA on the cell surface

(A) SorLA transfectants and LRP-null cells were incubated for 2 h at 4 $^{\circ}$ C with 125 I-uPA-PAI-1 (2×10^5 c.p.m./ml) in the absence or presence of 300 nM unlabelled RAP, washed, and treated with DTSSP. Cross-linked complex was immunoprecipitated from lysates with anti-sorLA Ig followed by reducing SDS/PAGE and autoradiography. Lane 1, 125 I-uPA-PAI-1 tracer alone, demonstrating radioactivity predominantly in the B-chain of uPA covalently bound to PAI-1; lane 2, LRP-null cells incubated with 125 I-uPA-PAI-1; lane 3, sorLA transfectants incubated with 125 I-uPA-PAI-1; lane 4, sorLA transfectants incubated with 125 I-uPA-PAI-1 in the presence of 0.5 μ M RAP. (B) SorLA transfectants (lanes 1 and 2) and LRP-null cells (lane 3) were incubated with 125 I-labelled soluble uPAR (6×10^5 c.p.m./ml) in the absence or presence of 4 nM unlabelled uPA-PAI-1 complex and treated with DTSSP. Cross-linked complex was immunoprecipitated and subjected to reducing SDS/PAGE and autoradiography. Lane 1, no addition, demonstrating absence of direct binding of 125 I-uPAR to sorLA transfectants; lane 2, sorLA transfectants incubated with 125 I-uPAR plus 4 nM uPA-PAI-1; lane 3, absence of binding of 125 I-uPAR plus uPA-PAI-1 to LRP-null cells.

to both sorLA and LRP1 (approx. 60% inhibition with 5 μ M RAP).

These results demonstrated striking similarities between sorLA and LRP1 in binding of several components of the plasminogen activating system, and the subsequent experiments were designed to explore effects of the interaction of uPA-PAI-1 with the two receptors in cells.

SorLA is inefficient in regulating the cell surface expression of uPAR

Since most uPA-PAI-1 complex bound to uPAR on the cell surface, we wanted to ascertain that some bound to sorLA. The transfectants were therefore incubated with 125 I-labelled complex at 4 $^{\circ}$ C followed by cross-linking with DTSSP, immunoprecipitation by anti-sorLA antibody, and autoradiography of radioactivity resolved by reducing SDS/PAGE. Figure 7(A) shows that the labelled complex bound to the sorLA transfectants (lane 3) in a RAP-inhibitable manner (lane 4), but not to the parental LRP-null cells (lane 2). To show that the uPA-PAI-1 complex can also interact with sorLA on cells when bound to uPAR, the sorLA transfectants were incubated with 125 I-labelled soluble uPAR with or without unlabelled uPA-PAI-1 complex, followed by cross-linking and immunoprecipitation. Figure 7(B) shows binding of labelled uPAR to the transfectants only in the presence of uPA-PAI-1 complex (lane 2 compared with lane 1), and not to the parental cells (lane 3), demonstrating that the ternary complex binds to sorLA, as shown previously for LRP1 and LRP1B mini-receptors [18].

Experiments were then performed to see if sorLA had any effect on the expression of uPAR on the cell surface. Initial experiments (results not shown) demonstrated that whereas wt CHO cells expressing LRP1 rapidly internalized 125 I-labelled uPA-PAI-1 complex, in accordance with previous results [17,18], we could not detect internalization in the sorLA transfectants above the low level observed in the parental LRP-null cells. We then used 125 I-labelled NTF to monitor whether sorLA can mediate the

Figure 8 Effect of the uPA-PAI-1 complex on cell surface uPAR expression

(A) SorLA transfectants (hatched bars), LRP-null cells (open bars) and wt CHO cells (cross-hatched bars) were grown (1.5×10^5 cells/well) in serum-free CHO culture medium for 24 h, washed, and incubated for 30 min at 37 °C in Krebs/Hepes buffer without (no addition) or with 2.0 nM unlabelled uPA-PAI-1 complex to saturate uPAR. Following washes, the cells were incubated in the same buffer without uPA-PAI-1 for 0, 45 and 90 min at 37 °C, and then with 125 I-labelled NTF (60 000 c.p.m./ml) for 2 h at 4 °C. (B) Cells were grown in serum-free culture medium (control) or in Dulbecco's modified Eagle's medium with 10% (v/v) foetal calf serum for 24 h, washed, and incubated for 1 h in Krebs/Hepes buffer at 37 °C, and then with 125 I-NTF for 2 h at 4 °C. (C) Cells grown in serum-free culture medium were incubated for 18 h at 37 °C in Krebs/Hepes buffer without (control) or with 2.0 nM unlabelled uPA-PAI-1 complex, washed, incubated in Krebs/Hepes buffer for 1 h at 37 °C and, after washings, with 125 I-NTF for 2 h at 4 °C. The results are means $\pm$ S.D. of four replicates.

reappearance of functional uPAR on the cell surface after saturation of this receptor with the uPA-PAI-1 complex. Figure 8(A) shows that, following incubation with 2 nM uPA-PAI-1 complex for 30 min to saturate uPAR, most 125 I-NTF binding activity was recovered after 45–90 min in the wt CHO cells, in agreement with previous results [18,40], whereas little recovery was observed in the sorLA transfectants. These results show that sorLA is unable to mediate efficient uPA-PAI-1-induced internalization and recycling of uPAR to the cell surface.

Previous results have shown that wt mouse embryonic fibroblasts and HT 1080 fibrosarcoma cells exhibit reduced levels of uPAR on the cell surface compared with cells made deficient in LRP1 [20,41]. This phenomenon might be due to the presence of a uPA-inhibitor complex in the serum-containing growth medium, because the efficiency of uPAR recycling following inhibitor-induced and LRP1-mediated internalization is less than 100%, and a fraction of the internalized uPAR is catabolized [11,17,21], which in turn causes down-regulation of uPAR after prolonged incubations. To test the effect of sorLA, binding of NTF was measured in cells grown in the presence of 10% (v/v) foetal calf serum and compared with that in cells grown in the standard serum-free CHO culture medium. As shown in Figure 8(B), the sorLA transfectants grown in the presence of serum bound nearly the same amount of 125 I-NTF as the parental LRP-null cells,

Figure 9 Binding of PDGF-BB to sorLA

(A) Microtitre wells were coated with PDGF-BB or BSA (50 μ l; 2.5 μ g/ml) and blocked in 5% (w/v) BSA. 125 I-labelled sorLA (5×10^4 c.p.m. in 100 μ l) in the absence or presence of RAP (0.2 or 1.0 μ M) was added to each well and incubated overnight at 4 °C, followed by washes and measurement of bound radioactivity. When coated with 5.0 μ g/ml PDGF-BB, $2.5 \pm 0.13\%$ of 125 I-sorLA bound to the wells (not shown). The results are means $\pm$ S.D. of four replicates. (B) Plasmon resonance analysis of PDGF-BB (1.2–20 nM) binding to sorLA (solid lines) and LRP1 (broken lines; only displayed at 10 and 20 nM). The bottom line shows the absence of binding of 20 nM PDGF-BB in the presence of 20 nM EDTA. The K_d values for binding to sorLA and LRP1 were determined to be 10 and 32 nM respectively.

whereas the wt CHO cells, as expected, showed a marked decrease in binding. In addition, long-term incubation with unlabelled uPA-PAI-1 complex in serum-free medium caused a decrease of NTF binding to wt CHO cells, but not to the sorLA transfectants (Figure 8C). Thus, in spite of efficient binding of the uPA-PAI-1 complex, sorLA is inefficient in regulating the cell surface expression of uPAR.

SorLA binds PDGF-BB, but not α_2 M*

PDGF-BB binds to LRP1 with K_d values estimated previously at 12–17 nM, as determined by plasmon resonance analysis [25]. We determined whether sorLA might bind PDGF-BB, because this growth factor is implicated in the enhanced migration exhibited by SMCs transfected with sorLA [11]. Figure 9(A) shows binding of 125 I-labelled sorLA in microtitre wells coated with PDGF-BB and its partial inhibition by RAP. This result is similar to that obtained previously with 125 I-labelled LRP1 [25]. As shown in Figure 9(B), surface plasmon resonance analysis revealed overall K_d values for binding of PDGF-BB to sorLA and LRP1 of approx. 10 and 32 nM respectively, with the growth factor dissociating more slowly from sorLA than from LRP1. The Vps10p domain of sorLA is unlikely to be involved in the binding reaction, because sorLA propeptide (1 μ M) and neurotensin (10 μ M), which bind to this domain [7], did not inhibit binding of PDGF-BB, and because binding to the purified Vps10p domain of sorLA, as well as to that of sortilin, was negligible (results not shown).

We finally tested binding of $\alpha_2\text{M}^*$ to sorLA, because PDGF-BB may be cleared not only via direct binding to LRP1, but also via binding to $\alpha_2\text{M}$ [28], which in turn is internalized and degraded. Binding of $\alpha_2\text{M}^*$ to sorLA could not be detected by plasmon resonance analysis (results not shown), and whereas wt CHO cells bound ^{125}I -labelled $\alpha_2\text{M}^*$ ($8.4 \pm 0.4\%$ of the added tracer; mean $\pm$ S.D. of four separate experiments) in a reaction completely inhibited by 400 nM $\alpha_2\text{M}^*$ or RAP, the sorLA transfectants and parental LRP-null cells showed no binding above background levels ($<0.1\%$ of the added tracer). When taken together, the results suggest that sorLA contributes little to the clearance of directly bound PDGF-BB due to its low rate of endocytosis, and not at all via interaction of $\alpha_2\text{M}^*$ with bound growth factor.

DISCUSSION

The present study shows that sorLA binds several ligands similarly to LRP1, but exhibits a comparatively diminished rate of internalization. The slow internalization and inefficiency in regulating the cell surface expression of uPAR is in agreement with the recent results of Zhu et al. [34], who also demonstrated secretion of a soluble form of sorLA in a variety of cell types. The moderate secretion observed in the clone 13.9 transfectants at 37 °C (absent at 4 °C) is unlikely to have influenced the results of the present experiments, which employed washing procedures and incubations at 37 °C for short time periods. However, secretion may be physiologically relevant in some settings, because sorLA is secreted in SMCs from rabbit aortas, and because conditioned medium containing secreted sorLA is reported to increase SMC migration in the presence of PDGF-BB [34]. It is unknown if the sorLA itself, or a ligand bound to the secreted receptor, elicits this effect.

With regard to binding of the uPA–PAI-1 complex and its components to sorLA, the LA repeat cluster appears to function as a unit similar to clusters II and IV of LRP1. Although the affinity of the uPA–PAI-1 complex for binding to isolated LA clusters of LRP1 has not been determined, previous experiments have shown approx. 3-fold higher affinities of pro-uPA and the tPA–PAI-1 complex for binding to recombinant cluster II than to cluster IV [12]. It may thus be assumed that the uPA–PAI-1 complex binds with highest affinity to cluster II, which is therefore likely to account for most of the binding to the LRP1 holoreceptor. Since sorLA binds the uPA–PAI-1 complex as efficiently as does LRP1, and since binding to its Vps10p domain is minimal, it is likely that the LA repeat cluster of sorLA has a high functional similarity to cluster II of LRP1.

Previous studies have shown that binding of the uPA–PAI-1 complex within cluster II of LRP1 occurs preferentially to the two-domain fragment comprising LA repeats 5 and 6 (the third and fourth LA repeats in cluster II, as cluster I contains two repeats). The surface-exposed Asp and Trp residues at identical positions in the two repeats are necessary, although not sufficient, for binding [42]. In fact, repeat 5 has two consecutive exposed acidic residues that both participate in high-affinity binding [42], in agreement with the observation that exposed basic residues in the PAI-1 moiety are important [43–45]. Among the 11 LA repeats in sorLA, repeats 1, 3, 4 and 8 have Trp and acidic residues at positions identical to those in cluster II of LRP1, and repeat 2 has two consecutive acidic residues and a Tyr instead of a Trp residue, whereas the remaining repeats lack exposed acidic residues. Thus repeats 1–4 seem to fulfil a minimum requirement for participating in binding of the uPA–PAI-1 complex. Concerning the surprising binding of uPA–PAI-1 to sortilin, it should be noted that this receptor, in contrast with sorCS1, has two consecutive acidic

residues (Glu⁶³⁸ and Asp⁶³⁹) that are spaced between cysteine residues like in LA repeats and, if exposed, might participate in the binding reaction.

tPA is thought to bind outside the LA repeat clusters of LRP1 [12], and the poor inhibition by RAP suggests that this may also be the case for binding to sorLA. It is therefore possible that not only the LA repeat cluster of sorLA, but also its β -propeller domain and epidermal growth factor class B-like motif, may share ligands with LRP1. Although the role of the binding of tPA to sorLA remains to be determined, it is of note that tPA promotes hippocampal long-term potentiation via a mechanism that requires binding to cell surface LRP1 [46], and it is possible that alternative binding to sorLA may modulate this effect.

Although several of the numerous established LRP1 ligands may interact with sorLA, it is evident that some of them do not. One example is $\alpha_2\text{M}^*$, which requires simultaneous interaction with clusters I and II for high-affinity binding to LRP1 [47]. Another example is *Pseudomonas* exotoxin A, which enters cells via binding to LRP1 and is approx. 100-fold more toxic to wt CHO cells than to CHO LRP-null cells [32]. We found no binding of the toxin to immobilized sorLA, and toxicity was not different in the sorLA transfectants and LRP-null cells, as determined by measuring the inhibition of protein synthesis (results not shown). Interestingly, this toxin interacts selectively with cluster IV of LRP1 [13], further supporting the notion that cluster II exhibits a high functional similarity to the LA repeat cluster of sorLA.

The low rate of endocytosis of sorLA, as compared with LRP1, probably explains its inefficiency in mediating the recycling and down-regulation of cell surface uPAR. In this regard, sorLA resembles LRP1B, a member of the core of the LDL receptor gene family, which has the same overall domain structure as LRP1 but exhibits a low rate of endocytosis due to a 33-amino-acid inserted sequence in the cytoplasmic tail [18,48]. As judged from results with a mini-receptor comprising cluster IV and the transmembrane and cytoplasmic domains, LRP1B binds the ternary uPA–PAI-1–uPAR complex, but is unable to mediate recycling of uPAR [18]. Irrespective of the mechanisms involved, binding of uPA–PAI-1 complexes to sorLA in competition with LRP1 may interfere with LRP1-regulated uPAR expression and function, possibly including signalling pathways thought to originate from uPAR [49,50].

Previous results have shown that PDGF-BB binds to LRP1 and induces PDGFR- and Src-elicited tyrosine phosphorylation of its cytoplasmic tail, and it was suggested that binding of PDGF-BB dimers could promote co-localization of LRP1 and PDGFR on the cell surface [25]. In addition, it has been reported that LRP1 can form a complex with PDGFR [24]. According to this model, binding of PDGF-BB to LRP1 is part of the mechanism that may prevent overexpression of PDGFR, and alternative binding of the growth factor to sorLA may inhibit this effect, as well as clearance of the growth factor. Because phosphorylation of the LRP1 tail may be an important event in modulating PDGFR signalling [25], we investigated whether PDGF could induce phosphorylation of the sorLA tail. We did not detect phosphorylation of sorLA in the transfectants (results not shown) after incubation with PDGF-BB under conditions that induce PDGFR phosphorylation. However, we were also unable to detect LRP1 phosphorylation in the wt CHO cells, in agreement with the observation that the phosphorylation is cell-type-specific [25], and future studies should determine if PDGF-BB can under some conditions induce phosphorylation of sorLA.

The present results may help to explain the observations [11] that overexpression of sorLA causes increased expression of uPAR as well as increased migration of cultured SMCs in the presence of PDGF-BB. Thus the increased uPAR levels might

be caused by preferential binding of uPA–inhibitor complexes to sorLA, which would reduce the LRP1-mediated down-regulation of uPAR and increase the migratory potential of the cells. In addition, the LRP1-mediated suppression of migration induced by PDGFR might be reduced as a result of PDGF-BB binding to sorLA rather than to LRP1. Concerning the situation *in vivo* with high expression of sorLA in SMCs of atheromatous lesions [10], competition with LRP1 for binding of apolipoprotein E may cause further attenuation of the ability of LRP1 to mediate the suppression of PDGFR signalling [27].

In conclusion, we show that sorLA shares several ligands with LRP1, including uPA–inhibitor complexes and PDGF-BB, but mediates comparatively slow internalization. It is proposed that sorLA, when overexpressed, may divert ligands away from LRP1 and thereby counteract effects mediated by this receptor.

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