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Cellular uptake of saposin (SAP) precursor and lysosomal delivery by the low density lipoprotein receptor-related protein (LRP)

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Sphingolipid activator proteins SAP-A, -B, -C and -D (also called saposins) are generated by proteolytic processing from a 73 kDa precursor and function as obligatory activators of lysosomal enzymes involved in glycosphingolipid metabolism. Although the SAP precursor can be recognized by the mannose-6-phosphate (M-6-P) receptor and shuttled directly from the secretory pathway to the lysosome, a substantial fraction of newly synthesized precursor is secreted from the cell where it may participate in sphingolipid transport and signaling events. Re-uptake of the secreted precursor is mediated by high-affinity cell surface receptors that are apparently distinct from the M-6-P receptor. We found that the low density lipoprotein receptor-related protein (LRP), a multifunctional endocytic receptor that is expressed on most cells, can mediate cellular uptake and lysosomal delivery of SAP precursor. Additional *in vivo* experiments in mice revealed that the mannose receptor system on macrophages also participates in precursor internalization. We conclude that SAP precursor gains entry into cells by at least three independent receptor mechanisms including the M-6-P receptor, the mannose receptor and LRP.

Keywords: endocytosis/lipoprotein receptor-related protein/mannose-6-phosphate/saposin/sphingolipid

Introduction

Prosaposin, referred to here as SAP precursor, is a 73 kDa polypeptide that is made by most cell types. Proteolytic cleavage of SAP precursor in the lysosome releases four small homologous polypeptides of 8–11 kDa termed saposins A–D (Sandhoff *et al.*, 1995; Sandhoff and Kolter, 1996). The saposins function as necessary activator proteins with partly overlapping specificity for the lysosomal degradation of various glycosphingolipids that is catalyzed by exohydrolases including cerebrosidases, ceramidases, sphingomyelinase, galactosidase and arylsulfatase A. Saposins are thought to facilitate the access of the actual enzymes to their substrates by lifting the latter out of the lipid bilayer of the membrane or by directly activating the respective enzymes (Weiler *et al.*, 1995; Sandhoff and Kolter, 1997; Vaccaro *et al.*, 1997).

Genetic abnormalities of varying severity have been described in which the SAP precursor gene has been found to be mutated at several places resulting in disease phenotypes similar to e.g. juvenile metachromatic leukodystrophy (Rafi *et al.*, 1990; Zhang *et al.*, 1990, 1991; Henseler *et al.*, 1996) or the juvenile variant of Gaucher disease (Harzer *et al.*, 1989; Schnabel *et al.*, 1991; Rafi *et al.*, 1993). Two cases of total SAP precursor deficiency have been described in humans resulting in death during fetal development or early childhood (Harzer *et al.*, 1989; Paton *et al.*, 1990, 1992; Schmid *et al.*, 1992; Bradova *et al.*, 1993). SAP precursor-deficient mice, in contrast, exhibit a bimodal phenotypic distribution, in which animals either die shortly after birth or around 4–5 weeks of age (Fujita *et al.*, 1996). PAS positive lysosomal inclusions are prominent in these animals due to their general inability to efficiently degrade glycosphingolipids.

Studies on the biosynthesis of SAP precursor have shown that a major fraction of the newly synthesized polypeptide is secreted from the cell and is then reinternalized by high-affinity cell surface receptors. A fraction of the precursor is directly shuttled to the lysosome from the secretory pathway (Vielhaber *et al.*, 1996). Because SAP precursor contains mannose-6-phosphate (M-6-P) residues, it is likely that the M-6-P receptor is mediating this trafficking event. However, the M-6-P receptor seems to play a minor role in the re-uptake of SAP precursor from the cell surface, as this process is not efficiently inhibited by addition of M-6-P to the medium of cultured cells, and because murine embryonic fibroblasts deficient in both forms of the M-6-P receptor continue to efficiently internalize SAP precursor (Vielhaber *et al.*, 1996).

Besides its well-documented central role in the cellular metabolism of glycosphingolipids, SAP precursor has been postulated to mediate neurotrophic signaling events (O'Brien *et al.*, 1994). Such a function is supported by the high expression level of SAP precursor mRNA in the brain and the abundance of the protein in cerebrospinal fluid (Kishimoto *et al.*, 1992). The existence of redundant mechanisms by which secreted SAP precursor can be removed from the extracellular space thus gains further importance, as these mechanisms would be essential to dampen and turn off the signaling events.

The low density lipoprotein receptor-related protein (LRP) is a multifunctional endocytic cell surface receptor that is expressed on most cell types. Functions for LRP in the cellular uptake of proteases, protease inhibitor complexes, lipoproteins and lipases are well established (Krieger and Herz, 1994; Rohlmann *et al.*, 1998). A paralogue of LRP, megalin, shares overlapping functions with LRP and in addition has been found to mediate the uptake of several vitamin carrier proteins in the kidney,

further underscoring the emerging role of LDL receptor-related genes in cellular transport processes for a variety of functionally diverse ligands (T.E.Willnow, personal communication).

To explore whether LRP might be involved in the cellular re-uptake of secreted SAP precursor and to assess the contribution of such a possible LRP-dependent secretion/recapture pathway for lysosomal glycosphingolipid degradation, we determined the ability of cultured cells to internalize recombinant ^{35}S-labeled SAP precursor in an LRP-dependent manner. Our goal was to compare qualitatively the role of LRP in cellular SAP precursor metabolism with the previously characterized role of the M-6-P receptor in this process, and to determine whether other redundant SAP precursor uptake mechanisms might exist. Our results show that LRP indeed mediates the major fraction of the cellular uptake of SAP precursor. The M-6-P receptor is also involved, but to a much lesser extent. Intravenous injection of labeled rSAP precursor into mice suggests that the macrophage mannose receptor system (Stahl and Schlesinger, 1980) may also be involved in cellular uptake. Taken together our results suggest a secretion/recapture process as an alternative transport pathway by which newly synthesized SAP precursor is transported to the lysosomes and by which secreted SAP precursor can be removed from the extracellular space. This redundant re-uptake process can be mediated by at least three endocytic receptors, the M-6-P receptor, mannose receptor and LRP.

Results

To obtain a reproducible source of SAP precursor we amplified the complete cDNA from reverse-transcribed RNA prepared from human fibroblasts. To facilitate detection and purification of the recombinant SAP precursor a myc-tag and a hexahistidine sequence were added to the C-terminal end of the precursor by cloning the cDNA without the stop codon into the pcDNA3.1 expression vector. In this vector, expression of the recombinant protein is driven by the strong cytomegalovirus promoter (Figure 1A). To obtain pure ^{35}S-labeled rSAP precursor, 293 cells were transfected with the expression construct and pulse-labeled with Translabel. Essentially pure ^{35}S-labeled rSAP precursor was obtained by Ni-column chromatography. Figure 1B shows an autoradiograph of an SDS gel on which the medium (lane 1), the column flowthrough (lane 2) and the eluted purified rSAP precursor (lane 3) were analyzed.

To test whether LRP did indeed mediate the cellular uptake of SAP precursor, we first incubated cultured murine embryonic fibroblasts with [^{35}S]rSAP precursor (Figure 2, lane 7). Murine embryonic fibroblasts express LRP abundantly (Willnow and Herz, 1994). Cells were incubated in the absence (Figure 2, lanes 1, 3 and 5) or presence (Figure 2, lanes 2, 4 and 6) of 100 μM chloroquine, a weak base which inhibits lysosomal proteolysis. Cells were also incubated with 1 mg/ml control IgG (Figure 2, lanes 1 and 2) or anti-LRP IgG (Figure 2, lanes 3 and 4). In the absence of chloroquine and anti-LRP IgG (Figure 2, lanes 1 and 5), the fibroblasts took up the ^{35}S-labeled protein and processed it to the mature SAPs (indicated by the respective arrows). Chloroquine inhibited

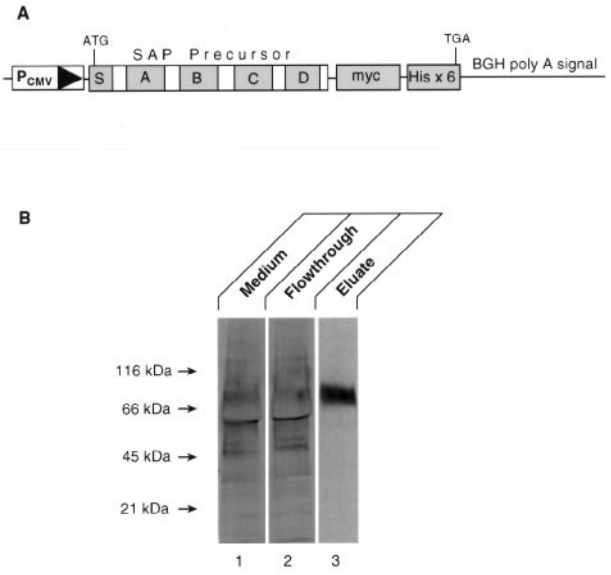

Fig. 1. Cloning and biosynthesis of ^{35}S-labeled rSAP precursor. (**A**) Schematic representation of the expression plasmid for the rSAP precursor fusion protein. The gene for the SAP precursor was cloned under the control of a CMV promoter and fused to a myc and a hexahistidine epitope. The region for the signal sequence (S) and the region for the individual saposins (A, B, C and D) are identified. (**B**) Purification of ^{35}S-labeled rSAP precursor by Ni-column chromatography. 293 cells were transfected with the rSAP precursor expression plasmid and 24 h later, proteins were metabolically labeled with ^{35}S. Samples (15 000 c.p.m.) of the medium (lane 1), the column flow through (lane 2) and the imidazole eluate (lane 3) were separated on 4–15% SDS–PAGE, transferred to nitrocellulose and exposed to film. Lanes 1 and 2 are overexposed in relation to lane 3 to allow comparison of the relative amounts of radioactive protein products. Identity of the recombinant protein was confirmed by immunoblotting with anti-myc antibodies (not shown).

the processing, but not the uptake of the labeled precursor by the cells (Figure 2, lanes 2 and 6). In contrast, addition of anti-LRP IgG to the cultured cells strongly inhibited the cellular uptake of rSAP precursor in the absence (Figure 2, lane 3) or presence (Figure 2, lane 4) of chloroquine. Control IgG had no effect on the cellular uptake or processing. Radioactivity taken up by the cells over the incubation period was quantitated by phosphorimager analysis and the total amount of cell-associated rSAP precursor at the end of the experiment was calculated (Figure 2B). Although the amounts of rSAP precursor that can be produced in our eukaryotic expression system preclude the determination of accurate binding kinetics under ligand saturation conditions, the results of this analysis nevertheless show that LRP-mediated uptake of rSAP precursor is physiologically significant and comparable to other established ligands, e.g. tissue-type plasminogen activator (Orth *et al.*, 1992).

To confirm the role of LRP in the cellular uptake of SAP precursor we made use of murine embryonic fibroblasts in which LRP had been destroyed by homologous recombination (MEF-2 cells). The ability of these cells to take up various ligands was compared with that of wild type embryonic fibroblasts (MEF-1). We also examined the ability of a universal inhibitor of ligand binding to LRP, the receptor-associated protein RAP, to block cellular uptake of four different radiolabeled ligands, [^{125}I]α_2-macroglobulin (Figure 3A), [^{35}S]rSAP precursor (Figure

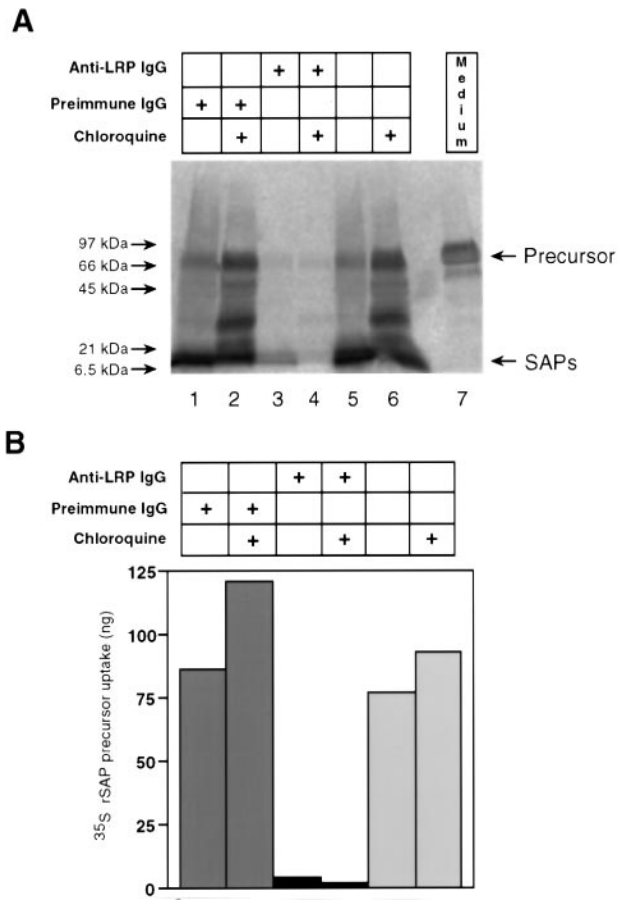

Fig. 2. Uptake of rSAP precursor is abolished in the presence of antibodies raised against LRP. (**A**) Primary mouse fibroblasts (1×10^6/dish) were incubated with 2 μg ^{35}S-labeled rSAP precursor (1×10^6 c.p.m., lane 7) for 14 h with the following additions: 100 μM chloroquine (lane 2, 4 and 6), 1 mg/ml preimmune IgG (lane 1 and 2), 1 mg/ml anti-LRP IgG (lane 3 and 4) or no additions (lane 5 and 6). Proteins were separated by 4–15% SDS–PAGE, transferred to nitrocellulose and exposed to film. (**B**) Results shown in (A) were quantitated by phosphoimager analysis and absolute amounts (ng/10^6 cells) of cell-associated rSAP precursor at the end of the incubation period were calculated.

3B), [^{125}I]anti-myc IgG (Figure 3C), and [^{125}I]LDL (Figure 3D) in the two cell types. MEF-1 cells efficiently took up [^{125}I]α_2M (Figure 3A, lane 1), as analyzed by autoradiography of cell lysates separated by SDS–PAGE. Cellular uptake was blocked by GST–RAP, a recombinant fusion protein of glutathione *S*-transferase (GST) with RAP (Figure 3A, lane 2), but not by GST alone (Figure 3A, lane 3). Addition of chloroquine to the incubation medium increased the intracellular accumulation of [^{125}I]α_2M by preventing its lysosomal degradation (Figure 3A, lane 4). In no case did the LRP-deficient MEF-2 cells take up any [^{125}I]α_2M (Figure 3A, lanes 5–8). Medium containing labeled ligand was loaded in Figure 3A, lane 9, in all panels.

When [^{35}S]rSAP precursor was added to MEF-1 (Figure 3B, lanes 1–4) and MEF-2 (lanes 5–8) cells, a similar result was obtained. In the absence of GST–RAP, MEF-1 cells took up and processed rSAP precursor (Figure 3B, lane 1). Addition of GST–RAP to the incubation medium substantially inhibited the uptake (Figure 3B, lane 2). GST had no effect (Figure 3B, lane 3). As shown before (Figure 2, lanes 2 and 6), chloroquine led to the accumulation of unprocessed precursor (Figure 3B, lane 4). Under all conditions, MEF-2 cells were almost completely unable to take up [^{35}S]rSAP precursor (Figure 3B, lanes 5–8). Only a minute residual accumulation of processed SAPs could be observed (Figure 3B, lanes 5–7) which was not affected by GST–RAP (Figure 3B, lane 6). However, chloroquine prevented this accumulation (Figure 3B, lane 8), indicating that the small amount of rSAP precursor that entered the LRP-deficient MEF-2 cells entered the cells through the endocytic/lysosomal pathway.

The LRP-dependent uptake of rSAP precursor by cultured murine embryonic fibroblasts was further confirmed by an indirect antibody uptake experiment (Figure 3C). A monoclonal antibody directed against the myc epitope in the rSAP precursor was labeled with ^{125}I, incubated with unlabeled rSAP precursor and added to MEF-1 and MEF-2 cells as described for [^{125}I]α_2M in Figure 3A and for [^{35}S]rSAP precursor in Figure 3B. Only MEF-1 cells (Figure 3C, lanes 1–4), but not the LRP-deficient MEF-2 cells (Figure 3C, lanes 5–8) took up the ^{125}I-labeled IgG. Following endocytic uptake by MEF-1 cells, the labeled antibody was rapidly degraded (Figure 3C, lanes 1 and 3). No residual degradation fragments could be seen when GST–RAP was present (Figure 3C, lane 2), demonstrating that endocytic uptake of the antibody was blocked by RAP. Chloroquine prevented degradation of the antibody in the MEF-1 cells, indicating that degradation occurs in the lysosome.

To demonstrate that MEF-2 cells are able to endocytose ligands from the culture medium, we incubated MEF-1 and MEF-2 cells with ^{125}I-labeled LDL, a specific ligand for the LDL receptor that does not interact with LRP. MEF-1 and MEF-2 cells took up [^{125}I]LDL (Figure 3D), but degraded it very rapidly so that no intermediate degradation fragments were detected inside the cells under these conditions (Figure 3D, lanes 1–3 and 5–7). However, chloroquine prevented the lysosomal degradation of the endocytosed LDL and similar amounts accumulated in MEF-1 (Figure 3D, lane 4) and MEF-2 (Figure 3D, lane 8) cells indicating that MEF-2 cells can endocytose extracellular ligands normally.

Our experiments in which we used antibodies directed against LRP, the universal inhibitor of LRP, GST–RAP, and genetically defined LRP-expressing (MEF-1) and -deficient (MEF-2) cells strongly suggest that the predominant route of cellular uptake of SAP precursor by fibroblasts involves an LRP-dependent mechanism. To investigate whether this finding also held true for cell types other than fibroblasts or whether different mechanisms could also mediate SAP precursor uptake in other tissues, we tested several differentiated cell lines for LRP expression (Figure 4A) and for their ability to take up [^{35}S]rSAP precursor (Figure 4B). In addition to MEF-1 cells (Figure 4A, lane 1) and MEF-2 cells (Figure 4A, lane 2), membrane lysates from five other established cell lines were subjected to immunoblot analysis with an LRP-specific antibody. These include the human fibroblast line 398 (Figure 4A, lane 3), the rat pheochromocytoma cell line PC12 (Figure 4A, lane 4), rat hepatoma FTO2B cells (Figure 4A, lane 5), the mouse Sertoli cell line TM4 (Figure 4A, lane 6) and the murine neuroblastoma derived C-46 cells (Figure 4A, lane 7). With the exception of the

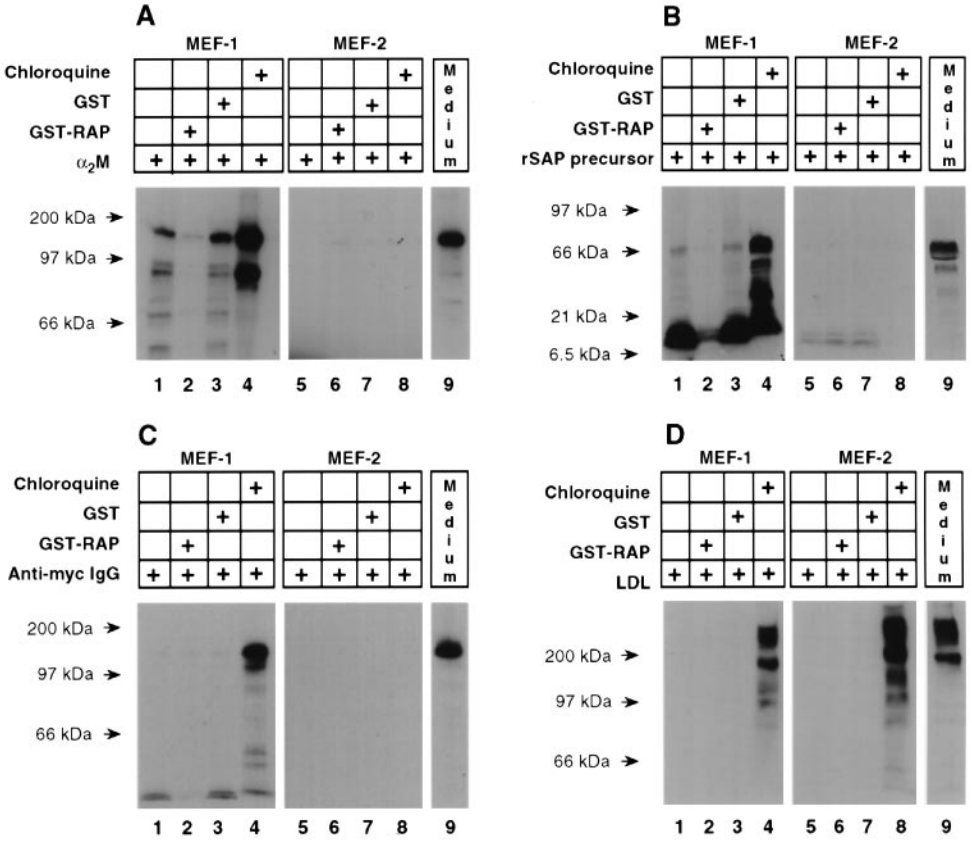

Fig. 3. Uptake of radiolabeled proteins by wild type (MEF-1) and LRP$^{-/-}$ (MEF-2) primary mouse fibroblasts. Wild type fibroblasts (lanes 1–4) and LRP$^{-/-}$ fibroblasts (lanes 5–8) (Willnow and Herz, 1994) were incubated with (**A**) 2 μg ^{125}I-labeled α_2-macroglobulin (1×10^7 c.p.m.); (**B**) 2 μg ^{35}S-labeled rSAP precursor (1×10^6 c.p.m.); (**C**) 2 μg rSAP precursor, 2 μg ^{125}I-labeled anti-myc IgG (1×10^7 c.p.m.); or (**D**) 2 μg ^{125}I-labeled LDL with the following additions: 30 μg/ml GST–RAP (lanes 2 and 6), 30 μg/ml GST (lanes 3 and 7), 100 μM chloroquine (lanes 4 and 8). Cells were harvested after 14 h, proteins were separated by 4–15% SDS–PAGE, transferred to nitrocellulose and exposed to film.

genetically LRP-deficient MEF-2 cells and the C-46 line, all other cell lines expressed LRP, albeit at different levels. Consistent with our finding in embryonic fibroblasts that the major portion of cellular uptake of rSAP precursor is LRP-dependent, only the LRP expressing cells, but not the LRP-deficient C-46 cell line (Figure 4B, lanes 9 and 19) were able to take up detectable amounts of [^{35}S]rSAP precursor from the extracellular medium in the absence (Figure 4B, lanes 1, 3, 5 and 7) or presence (Figure 4B, lanes 11, 13, 15 and 17) of chloroquine, respectively. In all cases this uptake was competed by GST–RAP, indicating that LRP was involved in the process. However, despite a relatively low level of LRP expression, TM4 cells efficiently took up rSAP precursor. GST–RAP completely prevented this uptake, raising the possibility that other RAP-sensitive receptors, e.g. the LDL receptor gene family member LR8 which is abundantly expressed by Sertoli cells (Lindstedt *et al.*, 1997), might also mediate SAP precursor uptake. Furthermore, in PC12 cells RAP competition was incomplete (Figure 4B, lanes 4 and 14), suggesting that LRP-independent pathways to a variable degree can also mediate the endocytosis of SAP precursor in different cell types.

To investigate the mechanisms responsible for the residual LRP-independent endocytic uptake of SAP precursor, we examined the role the M-6-P receptor might play in this process. Most of the soluble lysosomal hydrolases are transported via these receptors (Kornfeld and Mellman, 1989). SAP precursor also bears M-6-P residues and would therefore be a candidate for the transport by these receptors (Vielhaber *et al.*, 1996). MEF-1 cells incubated with [^{35}S]rSAP precursor (Figure 5, lane 1) efficiently took up and processed this ligand to the mature SAPs. In the presence of GST–RAP very little precursor or processed SAPs accumulated in the cells (Figure 5, lane 2), indicating that RAP had prevented the uptake. Addition of 10 mM M-6-P alone to the cultured cells had no discernible effect (Figure 5, lane 3) on the cells' ability to take up and process SAP precursor. However, when the cells were incubated in the presence of GST–RAP together with M-6-P, the residual uptake seen with GST–RAP alone was abolished indicating that LRP works together with M-6-P receptors in mediating the uptake of SAP precursor from the medium.

Our experiments so far had been conducted with rSAP precursor which had been produced by overexpression in 293 cells. A concern about this recombinant material was that, due to the massive degree of overexpression obtained in this mammalian expression system, the number of M-6-P residues on the recombinant protein might be reduced. This in turn could result in our underestimating the role of the M-6-P receptor pathway for the cellular uptake of SAP precursor. In contrast, the ability of LRP to bind the precursor would not be expected to be influenced by such differences in M-6-P content. To control for this possibility, we decided to examine the

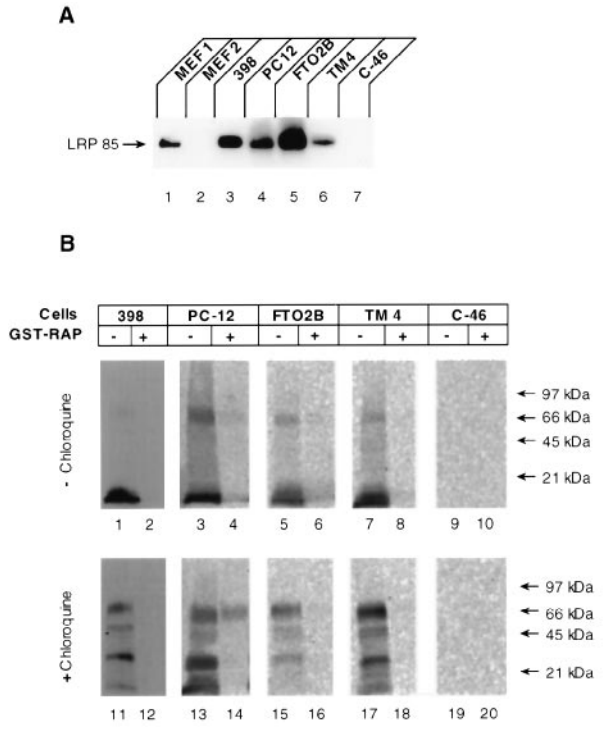

Fig. 4. Correlation between cellular LRP expression and uptake of rSAP precursor. (**A**) Western blotting of LRP-85. 100 μg of proteins from wild type (lane 1) and LRP$^{-/-}$ (lane 2) primary mouse fibroblasts (Willnow and Herz, 1994), human fibroblasts 398 (lane 3), PC12 cells (lane 4), the rat hepatoma cell line FTO2B (lane 5), the mouse sertoli cell line TM4 (lane 6), and the neuroblastoma cell line C-46 (lane 7) were separated by 4–15% SDS–PAGE, transferred to nitrocellulose and immunoblotting was performed using an antibody directed against LRP-85. (**B**) Cells were incubated for 14 h with 2 μg ^{35}S-labeled rSAP precursor (1×10^6 c.p.m.) and the following additions: 30 μg/ml GST (odd numbered lanes), 30 μg/ml GST–RAP (even numbered lanes) and with 100 μM chloroquine (lane 11–20). Cells were harvested after 14 h, proteins were separated by 4–15% SDS–PAGE, transferred to nitrocellulose and analyzed on a phosphoimager. In all cases, addition of GST–RAP reduced cellular uptake by ⩾90%.

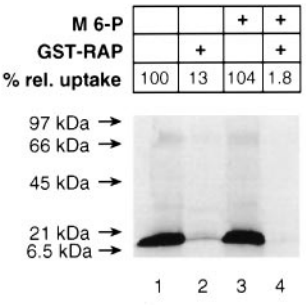

Fig. 5. Residual uptake of rSAP precursor in the presence of GST–RAP is abolished by addition of M-6-P. Primary wild type mouse fibroblasts were incubated for 14 h with 2 μg ^{35}S-labeled rSAP precursor (1×10^6 c.p.m.) and the following additions: no additions (lane 1), 30 μg/ml GST–RAP (lane 2) 10 mM M-6-P (lane 3), and 10 mM M-6-P and 30 μg/ml GST–RAP (lane 4). Cells were harvested, proteins were separated by 4–15% SDS–PAGE, transferred to nitrocellulose and exposed to film. Relative cellular uptake was quantitated and is shown in relation to uptake in the absence of additions (lane 1, 100%).

uptake of native [^{35}S]SAP precursor produced in human fibroblasts in the presence and absence of RAP and M-6-P (Figure 6). Human skin fibroblasts were pulse-labeled with Translabel and chased for 14 h. The relative amounts

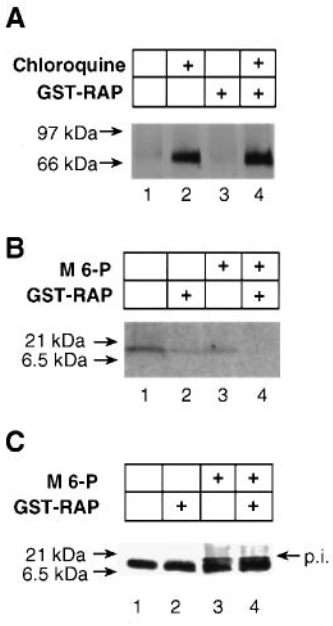

Fig. 6. Biosynthesis and uptake of native SAP precursor by human fibroblasts. (**A**) Proteins produced by human fibroblasts were metabolically labeled in the presence of 100 μM chloroquine (lanes 2 and 4) or 30 μg/ml GST–RAP (lanes 3 and 4) and immunoprecipitation of secreted proteins from the medium was performed using antibodies directed against SAP-A. (**B**) Human fibroblasts were incubated for 14 h in dialyzed medium derived from the experiment described in (A) (lane 2) containing ^{35}S-labeled secreted proteins and the indicated additions. (**C**) Human fibroblasts were metabolically labeled and chased for 14 h in the presence of 30 μg/ml GST–RAP (lanes 2 and 4) or 10 mM M-6-P (lanes 3 and 4). Cells were harvested, lysed and immunoprecipitation was performed using antibodies directed against SAP-A.

of [^{35}S]SAP precursor in the culture medium in the presence or absence of GST–RAP and chloroquine was estimated by immunoprecipitation using an anti-SAP-A antibody. As had been previously reported (Vielhaber *et al.*, 1996), inclusion of chloroquine (Figure 6A, lanes 2 and 4) in the culture medium substantially increased the cellular secretion of native endogenous [^{35}S]SAP precursor. GST–RAP alone did not affect the amount of native [^{35}S]SAP precursor that accumulated in the culture medium (Figure 6A, lane 3) suggesting that rapid LRP-mediated re-uptake is not a major factor determining the steady state concentration of secreted SAP precursor in the extracellular environment. [^{35}S]SAP precursor containing medium was dialyzed extensively to remove any traces of chloroquine and then added to cultured human fibroblasts in the presence or absence of GST–RAP and/or M-6-P (Figure 6B). In the absence of either inhibitor of cellular SAP precursor uptake, mature SAPs accumulated in the cells (Figure 6B, lane 1). GST–RAP substantially reduced this accumulation (Figure 6B, lane 2). In contrast to the uptake of rSAP precursor by murine fibroblasts (Figure 5), M-6-P noticeably reduced the accumulation of native SAPs in the human cells (Figure 6B, lane 3) indicating that the M-6-P content of the recombinant material may in fact be somewhat reduced as a result of inefficient phosphate addition or processing due to the massive overexpression in the 293 cells. Alternatively, the C-terminal tags could affect receptor recognition or phosphate addition. As had been the case in the murine

cells, however, GST–RAP together with M-6-P completely blocked the uptake of SAP precursor (Figure 6B, lane 4).

Next we investigated whether en route to the lysosome the SAP precursor follows an obligate secretion/recapture pathway involving LRP and the M-6-P receptor, or whether it can be shuttled to the lysosome via an alternative intracellular route. To address this question we performed a pulse–chase experiment in metabolically labeled human fibroblasts in the presence or absence of GST–RAP and M-6-P in the culture medium. We reasoned that during an obligate exposure to the extracellular space cellularly produced SAP precursor would have to compete with GST–RAP and M-6-P for binding to the receptors that mediate its transport. In this case, processing of SAP precursor to mature SAPs should not occur or be substantially reduced. Our results show that the amount of intracellular immunoprecipitable ^{35}S-labeled SAP (Figure 6C, lane 1) does not appear to be affected by the inclusion of GST–RAP (Figure 6C, lane 2), M-6-P (Figure 6C, lane 3), or both (Figure 6C, lane 4) in the culture medium. However, in the presence of M-6-P (Figure 6C, lanes 3 and 4) SAP processing does not proceed to completion. The accumulation of a processing intermediate (p.i.) suggests that the lysosomes might be depleted of proteases or glycosidases required for SAP precursor processing.

To demonstrate the ability of LRP to mediate the cellular uptake of SAP precursor *in vivo*, we designed a turnover experiment in which we measured the rate of [^{35}S]rSAP precursor removal from the circulation of animals that had or had not been coinjected intravenously with GST–RAP. In contrast to our findings in cultured cells where LRP constituted the predominant pathway for cellular uptake of [^{35}S]SAP precursor, we found in preliminary experiments that the clearance rate of this ligand from plasma was only minimally decreased by GST–RAP coinjection, although the clearance of [^{125}I]α_2-macroglobulin is completely blocked by this approach (Willnow and Herz, 1994; Herz *et al.*, 1995). This finding suggested that LRP-independent pathways are more efficient in clearing SAP precursor in the liver than in the cultured cells we have examined.

Since SAP precursor is heavily glycosylated, we asked whether carbohydrate-specific receptors other than the M-6-P receptor might also mediate the clearance of SAP precursor. The liver is an organ that is known to express a diverse spectrum of endocytic receptors including lectins, such as the asialoglycoprotein receptor on hepatocytes and the mannose receptor on resident macrophages (the Kupffer cells), which recognize specific carbohydrate side-chains on glycosylated proteins. Binding of glycoproteins to the mannose receptor can be efficiently competed by ovalbumin, a heavily glycosylated protein. To test whether the mannose receptor, in concert with LRP, was mediating the bulk of SAP precursor removal from the circulation, we injected [^{35}S]rSAP precursor alone (Figure 7A, □) or together with ovalbumin (○), GST–RAP (■), or both proteins (●) into the circulation of wild type mice. While both ovalbumin or GST–RAP on their own had a small, but reproducible inhibitory effect on the clearance of rSAP precursor from the circulation of the injected mice, injection of both proteins together almost completely blocked the clearance, suggesting that LRP and mannose receptor together mediate the clearance of SAP precursor

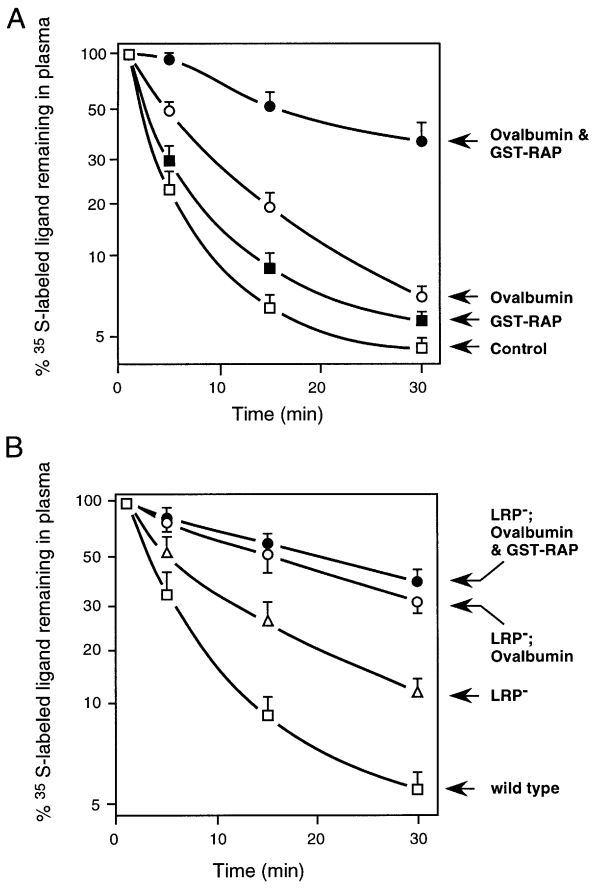

Fig. 7. *In vivo* turnover of ^{35}S-labeled rSAP precursor in mice. Anesthetized wild type mice (**A**) or liver-specific LRP knockout mice (**B**) were intravenously injected with 2 μg ^{35}S-labeled rSAP precursor (1×10^6 c.p.m.) and 10 mg BSA (□), 10 mg ovalbumin (○), 1.3 mg GST–RAP (■) or both 1.3 mg GST–RAP and 10 mg ovalbumin (●). Blood samples were taken after 1, 5, 15 and 30 min and radioactivity remaining in plasma was expressed as the percentage of radioactivity present 1 min after injection.

by the liver. This finding was confirmed by other turnover experiments in which [^{35}S]rSAP precursor was injected into mice in which the LRP gene had been destroyed by tissue-specific knockout techniques in the liver (Figure 7B) (Rohlmann *et al.*, 1996, 1998). While liver LRP-deficient mice cleared [^{35}S]rSAP precursor significantly slower from the circulation (△) than wild type mice (□), coinjection of ovalbumin into liver LRP-deficient mice further decreased the clearance rate (○). As expected, coinjection of GST–RAP and ovalbumin did not significantly further decrease the ability of the knockout animals to clear the ligand (●).

Discussion

In this study we have investigated the subcellular pathways by which SAP precursor is transported from the endoplasmic reticulum, the site of biosynthesis, to the lysosomes, where it is processed to the mature SAPs. We found that several independent receptor-mediated pathways promote the transport of SAP precursor to the lysosome (Figure 8). They include the M-6-P receptor, the mannose receptor and LRP. SAP precursor can be transported along a direct intracellular shunt pathway

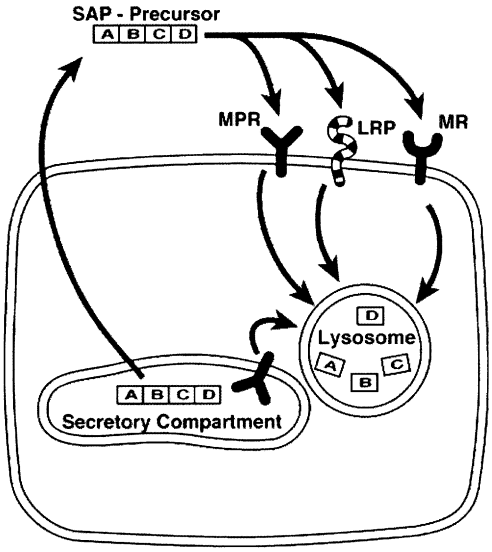

Fig. 8. Schematic representation of possible SAP precursor trafficking in cells. SAP precursor is either transported on an intracellular level toward the lysosomes or secreted. Uptake from the extracellular space by receptor mediated endocytosis is performed by either LRP, the M-6-P receptor (MPR) or the mannose receptor. M-6-P receptor and LRP are ubiquitously expressed, whereas mannose receptor expression is mainly restricted to macrophages.

from the secretory pathway to the lysosome, presumably involving the M-6-P receptor (Vielhaber *et al.*, 1996). Alternatively, it can first be secreted from the cell, followed by endocytic re-uptake (Vielhaber *et al.*, 1996). In cultured cells this secretion/recapture pathway appears to be mediated mainly by LRP and, to a lesser extent, by the M-6-P receptor. *In vivo* the mannose receptor was found to be equally effective in removing intravenously injected SAP precursor from the bloodstream into the liver.

Secretion and receptor-mediated re-uptake of lysosomal enzymes was first described in the now classic experiments conducted by Hickman and Neufeld (1972) who found that conditioned medium from normal cells, but not from cells derived from patients with I-cell disease, could correct the lysosomal α-iduronidase defect in Hurler disease fibroblasts. The reason for this lack of complementation lies in the fact that in I-cell disease the enzyme GlcNAc-1-P transferase, which is required for the addition of phosphate groups to mannose residues on glycoproteins, is defective (Varki *et al.*, 1981). Although SAP precursor has also been found to carry M-6-P residues, it is nevertheless taken up normally by cells even when it is not glycosylated and also by cells that are deficient in both types of M-6-P receptors (Vielhaber *et al.*, 1996). Our results, which show that LRP can mediate the uptake of SAP precursor in a M-6-P-independent manner, provide a molecular explanation for this finding.

Although our *in vivo* clearance experiments suggest a role of the mannose receptor in the process (Figure 7), most types of cultured cells seem to employ only two receptor systems, M-6-P-dependent receptors and LRP, for the uptake of SAP precursor from the extracellular space (Figures 2–5). Cellular uptake of the precursor and accumulation of mature SAPs derived from it in the lysosomes were completely blocked by the functional inactivation of LRP and M-6-P receptors at the level of the cell surface. However, intracellular transport of newly synthesized SAP precursor to the lysosomes was not affected under these conditions and led only to a slight reduction of mature SAPs. This was caused in part by impaired processing of the precursor (indicated by p.i. in Figure 6C), suggesting that the lysosomes were also deficient for a precursor processing enzyme which apparently also depends on a secretion/recapture step for its lysosomal routing. This finding raises the possibility that other soluble lysosomal enzymes like cathepsin D, β-glycosylceramidase and α-glucosidase (Hasilik and Neufeld, 1980; van Dongen *et al.*, 1985) which are routed to the lysosomes in a M-6-P-independent manner also rely on LRP for their transport. The finding that only cellular uptake of SAP precursor and not its intracellular routing is significantly reduced when LRP is inhibited at the cell surface is supported by turnover studies of biosynthetically labeled sphingolipids and fatty acids in LRP-deficient cells (S.Hüttler, unpublished observations). The turnover of most sphingolipids appeared to be normal, while the metabolism of ceramide, fatty acids and glucosylceramide was significantly reduced.

Why does the endocytosis of SAP precursor employ multiple unrelated cell surface receptors for its re-uptake by the cell? A possible explanation might be that SAP precursor has other functions besides giving rise to the enzymatic cofactors SAP-A, -B, -C and -D. SAP precursor has been proposed to function as a signaling molecule with neurotrophic activity (Kotani *et al.*, 1996; Hiraiwa *et al.*, 1997) and as a carrier protein that can mediate the cellular uptake of sphingolipids and gangliosides (Hiraiwa *et al.*, 1992). Amongst other places, SAP precursor is highly expressed in the brain and in cerebrospinal fluid (Hineno *et al.*, 1991) from where efficient LRP-mediated uptake would ensure the rapid attenuation of such signaling events in neurons. Interestingly, a paradigm for an endocytic receptor that also dampens a cellular signaling event is the large cation-independent M-6-P receptor which was shown to remove insulin-like growth factor II in a M-6-P-independent manner from the extracellular space (Morgan *et al.*, 1987). As a possible mediator of sphingolipid transport between cells, SAP precursor would presumably act in a functional role analogous to that of apoproteins in lipoprotein metabolism. The large fraction of newly synthesized SAP precursor that is secreted from cells already under normal conditions suggests that the capacity of the M-6-P receptor is not sufficient for its quantitative transport. Redundant uptake mechanisms for SAP precursor that do not depend on M-6-P residues would also prevent its accumulation in the extracellular space under conditions where extracellular phosphatase activity might otherwise destroy this receptor recognition signal.

In summary, our studies have defined LRP as a mediator for the M-6-P-independent cellular transport of SAP precursor and have thereby further expanded the role that LRP plays in multiple and functionally diverse biochemical and physiological processes. The involvement of LRP in lipoprotein metabolism and cholesterol transport have been well characterized. The present findings now point out a much broader role for LRP in lipid metabolism which is not limited to lipoproteins and cholesterol transport but also includes the homeostasis of other abundant

components of cellular biological membranes, the glycosphingolipids.

Materials and methods

Materials

Trans^{35}S-label^{TM} (41.77 TBq/mmol), methionine- and cysteine-free Dulbecco's modified Eagle's medium (DMEM) were from ICN. Chloroquine, ovalbumin, M-6-P, Tween 20, fetal calf serum, horse serum and BSA were from Sigma. DMEM and DHG were purchased from Gibco-BRL. MBS Transfection Kit was from Stratagene. Imidazole and pcDNA 3.1Myc-His were from Invitrogen. Anti-Sap-A, anti-SAP-D (Vielhaber *et al.*, 1996) and anti-LRP (Kowal *et al.*, 1989, 1990) antibodies were prepared as described. Secondary antibody (goat anti-rabbit, peroxidase conjugate) was purchased from Pierce.

Construction of human SAP precursor expression vector

PCR on a human liver cDNA library was performed using the primers 5′-TAGAAAGCTTAGGTTCCACACATGGCGTTTGCAATG-3′, which eliminates the stop codon and adds a *Hin*dIII site on the 3′ end of the prosaposin cDNA and 5′-GCCAGGTACCCGCGCTATGTACGCCCT-CTTCCTCC-3′ which adds a Asp718 site on the 5′ end. The PCR product was digested with *Hin*dIII and Asp718, cloned into pcDNA3.1(–)/Myc-His (Invitrogen) and confirmed by sequencing.

Cell culture, transfection, expression and purification of recombinant SAP precursor

Twenty dishes (60 mm) of 293 cells were grown to 70% confluency in DMEM, low glucose, 10% FCS and cotransfected with 5 μg SAP precursor plasmid and 2 μg pVA plasmid/dish using Stratagene MBS Kit. Twenty-four hours later cells were washed with phosphate-buffered saline (PBS), and incubated in methionine- and cysteine-free medium containing 0.2% BSA for 1 h. Cells were then pulsed with 500 μCi/dish ^{35}S-Translabel in 1.5 ml of the same media for 3 h and chased for 14 h by adding 1.5 ml DMEM, 0.2% bovine serum albumen (BSA). The medium from all dishes was collected, loaded onto a 3 ml Ni-column (ProBond, Invitrogen) and recycled three times. The column was washed with 100 ml PBS and the recombinant protein was eluted with 4 ml 300 mM imidazole dissolved in PBS. The eluate was dialyzed against PBS. The concentration of recombinant SAP precursor was estimated at 0.1 mg/ml and specific radioactivity at 500 c.p.m./ng.

Cell culture and ligand uptake experiments

Primary mouse fibroblasts (MEF cells) were grown in DMEM high glucose, 10% FCS; rat pheochromocytoma (PC12) cells in DHG, 10% FCS, 5% horse serum; a mouse Sertoli cell line (TM4) in DHG, 2.5% FCS, 5% horse serum; the rat hepatoma cell line FTO2B in DHG, 5% FCS; the murine neuroblastoma cell line (C-46) in DMEM 10% FCS; human fibroblasts in DMEM, 10% FCS. Human fibroblasts were grown at 35°C, 8.5% CO_2, all other cells were grown at 37°C, 8.5% CO_2 in 6-well plates (Costar) to 90% confluency. Cells were incubated for 14 h in 1.5 ml DMEM, 0.2% BSA containing radiolabeled SAP precursor (2 μg, ~1×10^6 c.p.m.) or iodinated proteins (2 μg, ~1×10^7 c.p.m.). Where indicated M-6-P (final concentration 10 mM), chloroquine (final concentration 100 μM) or GST–RAP (final concentration 30 μg/ml) were added. Cells were washed twice with PBS and lysed in 50 μl PBS, 1% Triton X-100 containing proteinase inhibitors (Complete Mini, Boehringer Mannheim) for 5 min on ice. Nuclei were removed by centrifugation (12 000 *g*, 1 min), proteins were separated on 4–15% polyacrylamide gels (Bio-Rad), transferred to nitrocellulose for 30 min at 10 V using a semi-dry transfer cell (Trans-blot SD, Bio-Rad) and exposed either to autoradiography film (Reflecton, NEN) or analyzed on a phosphorimager (BAS 1000, Fujix).

Preparation and radiolabeling of ligands

LDL was purified from human plasma and iodinated using the iodine monochloride method as described previously (Goldstein *et al.*, 1983). Recombinant GST–RAP was produced as a glutathione S-transferase fusion protein in DH5α bacteria (Herz *et al.*, 1991). Recombinant GST–RAP and methylamine-activated α_2-macroglobulin were iodinated using the Iodogen method (Fraker and Speck, 1978).

Metabolic labeling and immunoprecipitation of human fibroblasts

Human fibroblasts were grown in 60 mm dishes to 95% confluency and pulse-labeled as described above. Where indicated M-6-P (final concentration 10 mM), chloroquine (final concentration 100 μM) or GST–RAP (final concentration 30 μg/ml) were added. Chase was performed by adding 1.5 ml DMEM containing 0.2% BSA for 12 h. Immunoprecipitation of proteins from either the media or the cell lysate was performed as described (Vielhaber *et al.*, 1996).

In vivo turnover experiments

Wild type mice or mice lacking LRP expression in the liver were injected with 2 μg ^{35}S-labeled SAP precursor (1×10^6 c.p.m.) and either 10 mg BSA, 10 mg ovalbumin or 1.3 mg GST–RAP. Blood samples (~100 μl) were collected in EDTA-vials (Microvette CB1000, Sarstedt) at 1, 5, 15 and 30 min after injection and plasma was separated from cells by centrifugation (12 000 *g*, 5 min). Plasma samples (50 μl) were counted and relative radioactivity was expressed as a percentage of radioactivity measured at 1 min.

Western blotting

Cell lysates (100 μg/lane) were separated by SDS–PAGE and proteins were transferred to nitrocellulose as described above. Membranes were blocked in PBS, 0.05% Tween 20, 5% non-fat dry milk for 1 h. All further incubations were done in the same buffer. Blots were incubated with anti-LRP85 antibody at 1:1000 dilution. Bound IgG was detected with peroxidase conjugated goat anti rabbit IgG and ECL (SuperSignal Substrate, Pierce).

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