Rat Liver Sinusoidal Endothelial Cells (LSECs) express functional Low Density Lipoprotein Receptor-Related Protein-1 (LRP-1)

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Abbreviations: RAP, receptor associated protein; α_2M*, trypsin-activated α_2-Macroglobulin; PC, parenchymal cell; FSA, formaldehyde-treated bovine serum albumin; α-coll, collagen α-chains; HA, hyaluronan; RT, room temperature; KC, Kupffer cell

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ABSTRACT

Background and Aims: The low density lipoprotein receptor-related protein-1 (LRP-1) is a large, multifunctional endocytic receptor from the LDL receptor family, highly expressed in liver parenchymal cells (PCs), neurons, activated astrocytes and fibroblasts. The aim of the study was to investigate if liver sinusoidal endothelial cells (LSECs), highly specialized scavenger cells, express LRP-1. **Methods**: To address this question, experiments were performed *in vivo* and *in vitro* to determine if receptor associated protein (RAP) and trypsin-activated α_2-macroglobulin (α_2M*) were endocytosed in LSECs. **Results**: Both ligands were cleared from the circulation mainly by the liver. Hepatocellular distribution of intravenously administered ligands assessed after magnetic bead cell separation using LSEC- and KC-specific antibodies showed that PCs contained 93% and 82% of liver-associated ^{125}I-RAP and ^{125}I-α_2M*, whereas 5% and 11% were associated with LSECs. Uptake of RAP and α_2M* in the different liver cell population *in vitro* was specific and followed by degradation. The uptake of ^{125}I-RAP was not inhibited by ligands to known endocytosis receptors in LSECs, while uptake of ^{125}I-α_2M* was significantly inhibited by RAP, suggesting the involvement of LRP-1. Immunofluorescence using LRP-1 antibody showed positive staining in LSECs. Ligand blot analyses using total cell proteins and ^{125}I-RAP followed by mass spectrometry further confirmed and identified LRP-1 in LSECs. **Conclusion:** LSECs express functional LRP-1. An important implication of our findings is that LSECs contribute to the rapid removal of blood borne ligands for LRP-1 and may thus play a role in lipid homeostasis.

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Keywords: receptor-mediated endocytosis, blood clearance, RAP, α_2-macroglobulin, lipid homeostasis.

Introduction

The liver represents a major cleaning station of the body, daily eliminating large amounts of circulating macromolecules. Liver sinusoidal endothelial cells (LSECs) are a specialized type of endothelium, lining the liver sinusoids, that use receptor-mediated endocytosis to take up and degrade numerous physiological and non-physiological soluble macromolecules and colloids from the blood [35]. In addition, through their fenestrations, LSECs act as a dynamic filter facilitating the exchange of substrates between blood and hepatocytes [47].

Low density lipoprotein receptor-related protein-1 (LRP-1), a member of the LDL receptor family, is a large, multifunctional endocytic receptor expressed in liver parenchymal cells (PCs), neurons, activated astrocytes and fibroblasts [45]. LRP-1 is synthesized as a single polypeptide chain cleaved in the trans-Golgi to form a heterodimer of two noncovalently bound proteins, a 515 kDa subunit (α-chain) containing its binding domains, and a 85 kDa subunit (β-chain) containing the membrane-spanning region and cytoplasmic tail [18]. LRP-1 is known to interact and mediate endocytosis of more than 40 unrelated ligands ranging from proteins involved in lipoprotein metabolism, viruses to protease/protease inhibitor complexes cytokines, and growth factors [19].

LRP-1 and other members of the LDL receptor family associate with a specific chaperone, receptor-associated protein (RAP). RAP binds LRP-1 at multiple sites to block its interaction with ligands during biosynthesis and traffic to the cell surface [4]. Exogenous administration of RAP acts as a receptor antagonist for all ligands of the LDL receptor family, thus rendering RAP a valuable tool to study the LDL receptor gene

family's biochemistry and to investigate potential specific interaction of ligands with LRP-1 and LRP-1 related proteins *in vivo* and *in vitro* [46].

α_2-Macroglobulin (α_2M) [1] is a major human blood glycoprotein able to trap and inactivate a large variety of proteinases, and inhibit fibrinolysis by reducing plasminogen to kallikrein [41]. The α_2M-proteinase complex, the "activated" α_2M (α_2M*), becomes a specific ligand for the LRP-1, and is eliminated from the circulation by LRP-mediated endocytosis and subsequently degraded [41]. α_2M was recently found to be a noninvasive serological biomarker that could predict the stage of liver fibrosis [20]

The expression and function of the LRP-1 in liver was so far studied in PCs or PC-like cell lines [2, 6, 7, 42, 43]. Using RAP and α_2M* as potent ligands for LRP-1, we investigated whether this receptor is also expressed in LSECs.

Materials and Methods

Animals

Male Sprague-Dawley rats (~ 250g) were from Scanbur BK AB (Sollentuna, Sweden). All experimental protocols were approved by the Norwegian Animal Research Authority in accordance with the Norwegian Animal Experimental and Scientific Purposes Act of 1986.

Chemicals

Receptor associated protein (RAP) was expressed and purified from *Escherichia coli* strain BL21(DE3) (Invitrogen, Taastrup, Denmark) [8](supplementary material). Binding to LRP-1 (BioMac, Leipzig, Germany) was tested by Surface Plasmon Resonance (Biacore 3000 Biosensor instrument, GE Healthcare). α_2-Macroglobulin (α_2M) from bovine plasma was from Roche Diagnostics Norge AS (Olso, Norway). Carrier free $Na^{125}I$ was from Perkin-Elmer Norge AS (Oslo, Norway), and 1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril (Iodogen) and tetramethylrhodamine isothiocyanate (TRITC) were from Pierce Chemical Co., (Rockford, Illinois). Collagenase P was from Worthington Biochemical Corporation (Lakewood, New Jersey). RPMI 1640, penicillin and streptomycin were from PAA (Pashing, Austria). Bovine serum albumin (BSA), mannan, trypsin, soybean trypsin inhibitor, CHAPS and Hepes were from Sigma Co (St. Louis, Missouri), protease inhibitor from Roche (Copenhagen, Denmark), SDS-PAGE gels, nitrocellulose filters, Dynabeads M-280 tosylactivated and MultiMark® Multi-Colored Protein Standard were from Invitrogen. PD-10 columns and Percoll were from Amersham Biotech (Uppsala, Sweden). Formaldehyde-treated bovine serum albumin

(FSA) was prepared as described [31]. High molecular weight hyaluronan (HA) was from Pharmacia (Uppsala, Sweden). Collagen α-chains (α-coll) were obtained by incubation at 60°C for 60 min of native triple helical collagen (Vitrogen, Palo Alto, California).

Labeling Procedures

RAP and α_2M (50 μg) in PBS were directly labeled with $Na^{125}I$ employing Iodogen as oxidizing agent [27]. Specific radioactivities were between 5 - 8 x 10^6 cpm/μg. α_2M-trypsin complexes (α_2M*) were prepared as described [16]. Briefly, α_2M or ^{125}I-α_2M was incubated with 2 fold molar trypsin for 5 min at RT, followed by addition of soybean trypsin inhibitor, 5 fold molar more than trypsin. The complex formation was tested by gelatin zymography. FSA in Na_2CO_3 (0.1 M, pH 9.5) was incubated with TRITC at a ligand/dye weight ratio of 5:1, at 4°C overnight and then dialyzed against PBS.

Anatomical Distribution

Anatomical distribution of intravenously (i.v.) administered ^{125}I-RAP and ^{125}I-α_2M* (0.5 μg) were determined as described [38]. Total blood volume was calculated [44].

Hepatocellular Distribution

Ten min after i.v. administration of 5 μg of ^{125}I-RAP or ^{125}I-α_2M*, different liver cells were purified as described bellow. The uptake per cell population in the total liver was calculated based on the rat liver ratio KCs:LSECs:PCs=1:2.5:7.7 [33].

Isolation of PCs, LSECs and KCs

PCs, LSECs and KCs were prepared by collagenase perfusion of the liver, low speed differential centrifugation and Percoll gradient sedimentation [39], followed by magnetic cell separation (MACS) for isolation of KCs and LSECs [11], using biotin anti-rat CD11b and biotin anti-rat ICAM-1 (Cedarlane®, Ontario, Canada) / streptavidin-conjugated magnetic beads, respectively (Miltenyi, Inc.), over two MS^+ MiniMACS separation columns according to the manufacturer. The purity of the PCs (> 95%) was easily assessed by inspection in the light microscope, as these cells are much larger in diameter as compared to other liver cells. The purity of LSECs cultures and the degree of LSECs contamination in the KCs cultures was assessed by immunostaing using Stabilin2 antibody [17, 28]. LSEC preparations were between 95-98% pure. The degree of LSEC contamination in KCs was less than 10%.

Kinetics and specificity of endocytosis

Cultures of PCs (0.25×10^6) and LSECs (0.5×10^6) were established on fibronectin coated 24-well plates (Becton Dickinson, Playmount, UK), and KCs (0.35×10^6) on non-coated plastic in 48-well plates. Endocytosis kinetics of ^{125}I-RAP (4.27 ng), and ^{125}I-α_2M* (10 ng), were studied at 37°C for various time periods. Endocytosis specificity was studied by incubating LSECs for 2 h at 37°C with trace amounts of ^{125}I-RAP or ^{125}I-α_2M* alone (control), or with non-labeled (100 μg/ml) RAP, α_2M*, FSA, Mannan, α-coll or HA. Degradation was determined in the spent media by measuring the amount of acid soluble radioactivity after addition of TCA and centrifugation. Cell-associated ligand was measured in cells solubilized in 1% SDS. The amount of the non-specific binding and free ^{125}I in the cell free wells was subtracted.

Immunofluorescence staining for LRP-1

LSECs on fibronectin coated coverslips were incubated with TRITC-FSA for 1h at 37°C. Unbound TRITC-FSA was removed by washing and incubation continued for another hour. The cells were fixed for 10 min at RT with 4% paraformaldehyde. After permeabilizing the cell membranes with 0.1% TritonX-100 for 3 min, non-specific binding sites were blocked by incubating with 1% BSA. Mouse monoclonal LRP-1 antibody (5A6) (Progen, Heidelberg, Germany) or control mouse IgG (Abcam, Cambridge, UK), 5 μg/ml in PBS 1% BSA, were incubated with the cells for 1h at RT, followed by incubation with AlexaFluor488 goat anti-mouse IgG (Invitrogen, Taastrup, Denmark). Cell nuclei were stained with a solution of 1:1000 DRAQ5 in PBS (Biostatus Ltd, Leicestershire, UK). DakoCytomation Fluorescent Medium (Dako Norge, Kjelsås, Norway) was used for mounting. Pictures were taken using a Zeiss Axiovert microscope (x63 objective) and LSM 510 software (Karl Zeiss Microimaging GmbH, Göttingen, Gemany).

Ligand blotting with ^{125}I-RAP

Total cell protein of LSECs, PCs and human glioblastoma cells (U87) (LGC Standards AB, Borås, Sweden) were extracted with a buffer consisting of 20 mM Hepes pH 7.4, 124 mM NaCl, 10 mM $CaCl_2$, 1% CHAPS, 1mM PMSF and protease inhibitor cocktail for 3h at 4°C. The samples were diluted, divided into two, and incubated overnight at 4°C with Dynabeads coupled with BSA or RAP. The Dynabeads were washed and bound proteins eluted and separated by SDS-PAGE (7% Tris-Acetate) under non-reducing conditions. Protein bands were visualized by silver staining (SilverQuest, Invitrogen, Taastrup, Denmark). The proteins were transferred to nitrocellulose filters, blocked with

5% BSA and incubated overnight at 4°C with ^{125}I-RAP before phosphorimaging (Fuji BAS5000).

Identification of LRP-1 by mass spectrometry

A band of > 500 kDa was excised from the silver-stained gel, reduced with DTT, alkylated with iodoacetamide and digested with trypsin [36]. Nano-LC-ESI-MSMS was performed (see supplementary materials and methods). Peak lists of MSMS spectra were generated with DTASuperCharge (http://msquant.sourceforge.net/) and searched with MASCOT against the mammalian part of the IPI_rat database.

LRP mRNA levels in PCs, LSECs and KCs

Total RNA was extracted from isolated PCs, Kupffer and LSECs using total RNA isolation kit from Macherey-Nagel (Duren, Germany). Approximately 2-10 million plated cells were lysed (PCs were in suspension). The quantity and quality of RNA was determined by Nanodrop (ThermoFisher Scientific, Wilmington, DE, USA) and by an electropheretic bioanalyzer Agilent 2100 using the Pico 6000 Assay (Agilent Technologies, Santa Clara, CA, USA). The 260/280 ratios in all samples were 2.1 and 18S/28S ratios were between 1.1-1.4. The yields were 43,1 µg/ml, 15,8 µg/ml and 3647,0 µg/ml for LSECs, KCs and PCs, respectively. Less than 2 µg of RNA for each tell type was added to the cDNA reaction using MuLV Reverse transcriptase, random decamers, dNTP mix and placental RNAse inhibitor (Ambion, Austin,Texas , USA). RT-PCR was performed with AmpliTaq Gold, Taqman Universal PCR master mix, primer/probe kit m1 LRP1and GAPDH (all from Applied Biosystems, Foster City, CA, USA). Expression

levels of LRP were determined by a relative comparison ΔΔCT method where the ΔCt for GAPDH and LRP was compared for each cell type and then related to that of PCs.

Statistical Analysis

SPSS package for Windows version 15.0 (SPSS Inc., Chicago, Ill, USA) was used for statistical analyses. Two-sided *p* values less than 0.05 were considered significant. Half-life data was analyzed using GraphPad Prism 4 (GraphPad Software, Inc. La Jolla, CA, USA).

Results

*Anatomical distribution in vivo of intravenously injected ^{125}I-RAP and ^{125}I-α_2M**

The anatomical distribution was investigated 10 min after lateral tail vein administration of ^{125}I-RAP or ^{125}I-α_2M*. The blood was removed by systemic perfusion through the heart and 15 organs were surgically excised and measured for radioactivity. In accordance with previous studies, we found that the liver is the main site of uptake (Figure 1), and that only very small amounts of radioactivity were recovered in other organs (not shown).

Hepatocellular distribution

To determine which liver cells are responsible for RAP and α_2M* uptake, ^{125}I-RAP and ^{125}I-α_2M* were i.v injected into rats followed by liver perfusion with collagenase and isolation of PCs, LSECs and KCs by means of Percoll gradient and an antibody-based magnetic separation method (MACS). All steps were performed at 4°C to prevent cellular loss of internalized ligand due to degradation. In the whole liver, 93.1±1% of RAP and 82.2±6% α_2M* was found associated with the PCs (Figure 2). The LSECs and KCs were further purified using specific antibodies [11]. The ICAM-1 positive cells (LSECs) were found to be responsible for 5.3 ± 1.3% of RAP uptake and 11.1 ± 6.7% of α_2M* uptake, while the CD11b positive cells (KCs), for 1.6 ± 0.2% and 6.8 ± 0.7%, respectively (Figure 2).

In vitro studies

Endocytosis kinetics was studied to compare the uptake of ^{125}I-RAP and ^{125}I-α_2M* in cultures of PCs, LSECs and KCs. The endocytosis in PCs increased almost linearly over time, with 7.6±1.0 ng RAP being endocytosed and 5.7±0.4 ng degraded after 4 h of incubation (Figure 3-left panel). Degradation products were detected in the medium after approx. 20 min, after which degradation proceeded at the same rate as cellular uptake. Similar kinetics were observed in KCs, but the total amount of endocytosed RAP at the end of the incubation time was 4 times lower than in PCs. The uptake in LSECs reached a plateau, with 1.4±0.4 ng RAP being endocytosed, and 0.6±0.2 ng degraded after 2 h. The kinetics of endocytosis of ^{125}I-α_2M* in PCs and LSECs were also similar, with 3 fold more uptake in PCs as compared to the uptake in LSECs (Figure 3-right panel). However, in the KCs, it was only after 4 h of incubation when degradation products could be measured in the supernatant, while at shorter time points, the α_2M* was only found bound to the cells.

Specificity of endocytosis of the two ligands was studied in primary cultures of LSECs. Excess amounts of non-labeled RAP inhibited the endocytosis of trace amounts of ^{125}I-RAP by 88.0±0.1%, suggesting that the uptake of RAP is receptor mediated (Figure 4A). To gain further insights into the receptor mediated endocytosis, excess amounts of unlabelled ligands for known candidate receptors were added to primary cultures of LSECs together with ^{125}I-RAP. So far, three major receptors for endocytosis have been identified on LSECs, i.e. the hyaluronan/scavenger receptor (Stabilin2), the mannose/collagen α-chain receptor (MANN/COLLA-R), and the Fcγ-receptor IIb [22, 25, 26, 28]. Various macromolecules known to bind to these LSECs endocytosis receptors were tested for potential inhibition of RAP uptake. The presence of high

concentrations of formaldehyde treated serum albumin (FSA) or hyaluronan (HA) (ligands for Stabilin2), mannan or collagen α-chains (α-coll) (ligands for the MANN/COLLA-R) had no effect on the endocytosis and degradation of RAP. Inhibition of the Fcγ-receptor IIb was not tested, as this receptor recognizes only the Fc domain of IgG-immune complexes [23].

Excess amounts of unlabeled α_2M* inhibited the endocytosis of ^{125}I-α_2M* by 73.0% ($p<0.001$) (Figure 4B). The presence of RAP had a significant inhibitory effect on both the cell associated and degraded ^{125}I-α_2M*, and it inhibited the total uptake by 50.1% ($p<0.001$), suggesting the involvement of the LRP-1 in uptake.

Immunofluorescence staining for LRP-1

We used immunostaining to further investigate whether the LRP-1 receptor is expressed on LSECs. Paraformaldehyde fixed cultures of LSECs were immunostained using mouse monoclonal LRP-1 antibody recognizing the β-chain (5A6). The cells stained positively for LRP-1, as visualized by confocal microscopy using goat AlexaFluor488 anti-mouse antibody (green) (Figue 5). To determine if the LRP-1 positive cells are LSECs, the cells were preincubated with TRITC-FSA (red), a ligand that is exclusively taken up by the LSECs in the liver [13]. No staining was observed when the cells were treated with the secondary antibody only or with control mouse IgG (not shown).

LRP-1 expression in liver cells

The expression of LRP in the different liver cells was assessed by extraction of mRNA and RT-PCR. We found that after normalizing for an internal mRNA control GAPDH (which showed minimum variation in samples), the LRP-1 expression levels in PCs were 6 times higher than in LSECs and KCs, with LSECs and KCs expressing approximately similar levels. To further confirm that LRP-1 is expressed in LSECs, total cell protein was incubated with Dynabeads coupled to RAP, to pull down the receptor/s binding RAP. Bound proteins were eluted from the beads, and run in parallel on two SDS-PAGE gels under non-reducing conditions. The proteins on the first gel were visualized by silver staining (Figure 6.A). Dynabeads coupled to BSA were used as negative control showing that LRP-1 does not bind unspecifically to the beads. PCs and U87 cell line were used as positive controls, as both cell types are known to express LRP-1 [5, 34]. A band of > 500 kDa appeared on the silver stained gel from cell extracts of all three cell types (Figure 6.A). The proteins from the second gel were transferred to nitrocellulose filters and incubated with ^{125}I-RAP before phosphorimaging. The results presented in Figure 6.B show that ^{125}I-RAP bound to a single protein from the cell extracts of LSECs. The respective band corresponded to a similar band from the U87 and PCs extracts. A sensitive and specific high-performance liquid chromatography-tandem mass spectrometry using electrospray ionization (LC-ESI-MSMS) was chosen to study the band excised from the silver-stained gel. Rat LRP-1 (IPI00369995) was identified with a Mascot score of 134. Identified peptides are listed in Table 1 (supplementary material).

Discussion

RAP, a potent inhibitor of all known ligand interactions with LRP-1, and α_2M*, a specific ligand for the LRP-1 receptor, were used in this study to investigate whether LRP-1 is expressed in liver cells other than PCs. In agreement with previous studies we found that the liver is the principal elimination site of intravenously administered RAP and α_2M*. Liver cell separation showed that intravenously administered ^{125}I-RAP and ^{125}I-α_2M* accumulated mainly in PCs (93% and 82/% of liver uptake, respectively), while 5% and 11% were associated with LSECs, respectively. Endocytosis by LSECs of both ligands *in vitro* was receptor mediated, and succeeded by degradation. The uptake of ^{125}I-RAP was not inhibited by ligands to known endocytosis receptors in LSECs, while uptake of ^{125}I-α_2M* was significantly inhibited by excess of non-labeled RAP, suggesting the involvement of LRP-1. An immunofluorescence assay using a monoclonal LRP-1 antibody showed positive staining in LSECs. Ligand blot analyses using total cell protein and ^{125}I-RAP followed by mass spectrometry further confirmed and identified LRP-1 in LSECs.

Several studies have shown that α_2M-protease complexes have a circulatory half-life of 2-4 min (for review, see[41]), and RAP has a half-life of 0.5 min [43]. Using whole body autoradiography, it has been reported that the liver was the main site for uptake of both ligands [32, 43]. In this study we investigated the anatomical distribution by measuring the radioactivity in 15 organs, 10 min after intravenous injection. This method gives a better quantification of the distribution since the blood is washed out of the organs by systemic perfusion, and the radioactivity is measured in the total organ. Our results were

in accordance with the previous findings, with the liver being the main site of uptake of ^{125}I-RAP and ^{125}I-α_2M*.

It has been previously reported that the liver PCs are the main site of uptake of α_2M* [10, 15]. The authors used autoradiography as a method for identifying the anatomical site of uptake. This technique, however, makes it difficult to distinguish between KCs and LSECs. Since at that time (1985) it was generally believed that KC = liver reticuloendothelial system (RES), it is fully understandable that the authors did not suggest the possibility that LSECs could be an alternative site of uptake in addition to the KCs. It was not until 1990 that LSEC was proposed, based on a solid body of evidence, to be a very important part of the liver RES [40]. In our study, we have used an antibody-based purification method that allowed us to achieve higher purities and more defined cell populations. Our results show that LSECs also contributed to the elimination of RAP and α_2M* from the circulation, albeit to a lesser extent than PCs.

The most likely receptor candidate, LRP-1, has previously been shown to be expressed in the liver by PCs only [24, 34]. The following findings in the present study point to the presence and a functional role for LRP-1 in LSECs. First, excessive amounts of unlabeled RAP efficiently inhibited the uptake and degradation of ^{125}I-RAP and ^{125}I-α_2M* in primary cultures of LSECs. Second, excess amounts of ligands to known scavenger receptors in LSECs failed to inhibit the uptake of RAP.

To obtain direct evidence for identification of LRP-1 in LSECs and thereby rule out possible influence of marginal contamination of PCs, the cell cultures were pre-incubated

with TRITC-FSA, a ligand that is known to be taken up only by the LSECs in the liver [12]. Immunofluorescence staining of the cells with TRITC-FSA and LRP-1 antibody revealed double staining and provided strong evidence for the expression of LRP-1 in LSECs. Further isolation of a RAP-binding protein of MW > 500 kDa from LSEC extracts, with subsequent mass spectrometry, confirmed the expression of LRP-1 in LSEC.

Our time course studies of RAP and α_2M* endocytosis in LSECs and KCs revealed binding and degradation of the ligands, suggesting a functional role of LRP-1 in these cells. However, the functional capacity of LRP-1 appeared to be lower in LSECs and KCs than in PCs (Figure 3). LRP-1 mRNA expression was detected in all three liver cell types, and relative LRP-1 expression level was 6 times greater in PCs than in LSECs and KCs. If the relative protein expression levels mirror the RNA levels, this may explain the differences between RAP and α_2M* uptake observed in the in vitro PC and non-PC cell assays. Another interesting finding was that the uptake capacity for RAP was higher in all three cell types as compared to that for α_2M*. This may be due to involvement of other receptor(s) that recognize RAP. Such receptor could be the LDL receptor [30] which is known to be expressed on PCs and is able to bind to RAP, although with less affinity than LRP-1 [3, 24].

In conclusion, we demonstrate for the first time that the expression of functional LRP-1 in liver is not restricted only to PCs, it is also found in LSECs, and to a lesser extent in KCs. Characterized by a very high endocytic activity [37], LSECs would contribute

significantly to the elimination of the various LRP-1 ligands from the circulation in normal, healthy conditions.

However, in pathological conditions, such as severe fatty liver diseases, as well as in aging [14, 21, 29], significant changes occur in the hepatic sinusoids (e.g. fibrosis, narrowing of the sinusoids, reduced blood flow and capillarization of the liver sinusoids). These changes are hallmarked structurally by a progressive loss in fenestrae in LSECs, concomitant with the development of a basal lamina and deposition of collagen in the space of Disse. Given the role of fenestration in the transfer of lipoproteins from the blood to hepatocytes, it is likely that defenestration will impair lipoprotein/chylomicron remnants clearance by the hepatocytes. In addition, the transport in the opposite direction of some lipoproteins manufactured by hepatocytes will be impaired. Further studies are needed to investigate the endocytic function of LSECs via the LRP-1 in pathological conditions.

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FIGURE LEGENDS

Fig. 1. Anatomical distribution

^{125}I-RAP (white bars) or ^{125}I-α_2M* (0.5 μg) (gray bars) in 0.5 ml physiological saline were i.v. injected in the tail vein, and radioactivity in 15 organs free of blood was measured 10 min after injection. The results are presented in radioactivity – counts per minute (cpm) recovered within each organ. Radioactivity in tissues and organs other than those shown in the figure was less than 10^4 cpm. Bars are means ± SD for 3 animals.

Fig. 2. Hepatocellular distribution

Ten min after i.v. injection of 5 μg ^{125}I-RAP (white bars) or ^{125}I-α_2M* (gray bars), the liver cells were isolated by collagenase perfusion, Percoll gradient and MACS using specific antibodies for LSECs and KCs. The content of radioactivity is presented as percent per total cell population in liver, given that the total number of KCs, LSECs and PCs in rat liver relate to each other as 1:2.5:7.7 (20). Bars are means ± SD of 3 animals.

Fig. 3. Kinetics of endocytosis of ^{125}I-RAP (left panel) and ^{125}I-α_2M* (right panel) in primary cultures of PCs, LSECs and KCs

Trace amounts of radio-labeled ligands (approx. 4.27 ng ^{125}I-RAP and 10 ng ^{125}I-α_2M*) were added to cultures of 0.25 x 10^6 PCs, 0.5 x 10^6 LSECs and 0.3 x 10^6 KCs. Cell-associated (∇) and degraded (acid-soluble) (□) ligands were determined after various periods of incubation at 37^{o}C as described in the Materials and Methods. Total endocytosed ligand (○) represents the sum of cell-associated and acid-soluble radioactivity. The amount of the non-specific binding and free ^{125}I in the cell free wells

was subtracted. The results are presented as ng ligand/10^6 cells. Bars are means ± SD of 3 experiments.

Fig. 4. Specificity of endocytosis of ^{125}I-RAP (A) and ^{125}I-α_2M* (B) in LSECs

Monolayer cultures were incubated for 2 h at 37°C with approx. 4.27 ng ^{125}I-RAP or10 ng ^{125}I-α_2M* alone (Control) or together with 100 μg/ml of unlabelled RAP, FSA, Mannan, α-coll or HA. Results are presented as percentage of Control. Gray bars represent cell-associated ligand (SDS soluble). White bars represent degraded ligand (TCA soluble). Control values were 19 ± 2% and 5 ± 0.2% of total added ^{125}I-RAP and ^{125}I-α_2M*, respectively. Bars are means ± SEM for 3 to 7 experiments.

Fig. 5. Immunofluorescence staining for LRP-1

LSECs on fibronectin coated glass coverslips were incubated for 1 h at 37°C with TRITC-FSA (red), fixed with 4% paraformaldehyde and cell membranes permeabilized with 0.1% Triton X-100. Indirect immunostaining was performed using mouse monoclonal anti-LRP-1 and AlexaFluor 488 goat anti-mouse IgG (green). Cell nuclei were stained with DRAQ5 (blue).

Fig. 6. Ligand blotting

Total cell proteins of LSECs, PCs and human glioblastoma cell line (U87), were incubated with Dynabeads coupled with RAP or BSA overnight. The Dynabeads were washed and RAP-binding proteins separated by SDS-PAGE (7% Tris-Acetate), under non-reducing conditions. A band of > 500 kDa appeared on the silver stained gel from

cell extracts of all three cell types (A). Lane designation at top of the gel indicates the cellular source of samples that were loaded. Prestained molecular mass markers is indicated in kDa. The proteins were transferred to nitrocellulose filters, blocked with BSA, the blot incubated with ^{125}I-RAP and subjected to autoradiography (B).

Figure 1

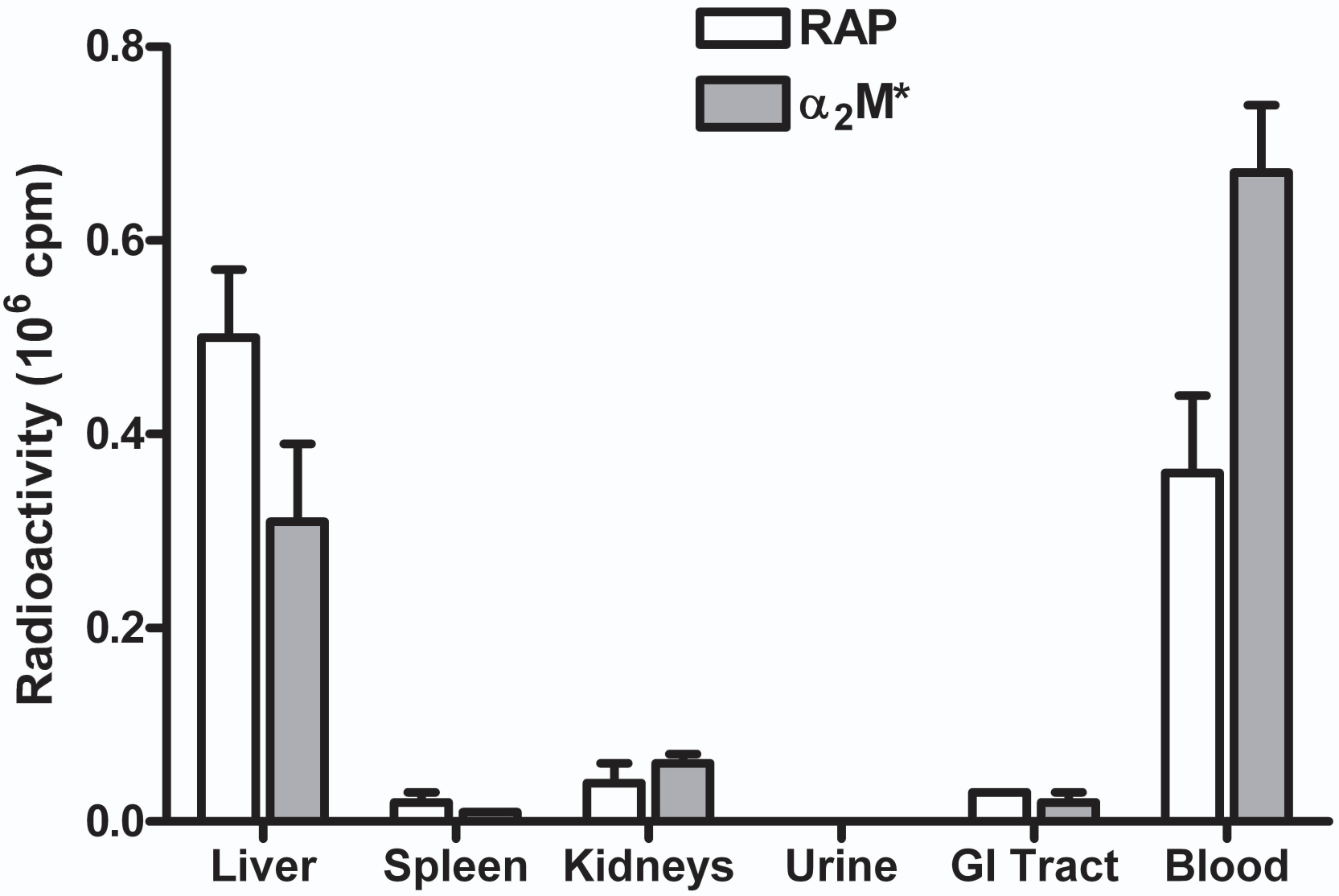

RAP
α2M*
Radioactivity (10^6 cpm)
0.8
0.6
0.4
0.2
0.0
Liver
Spleen
Kidneys
Urine
GI Tract
Blood

Figure 2

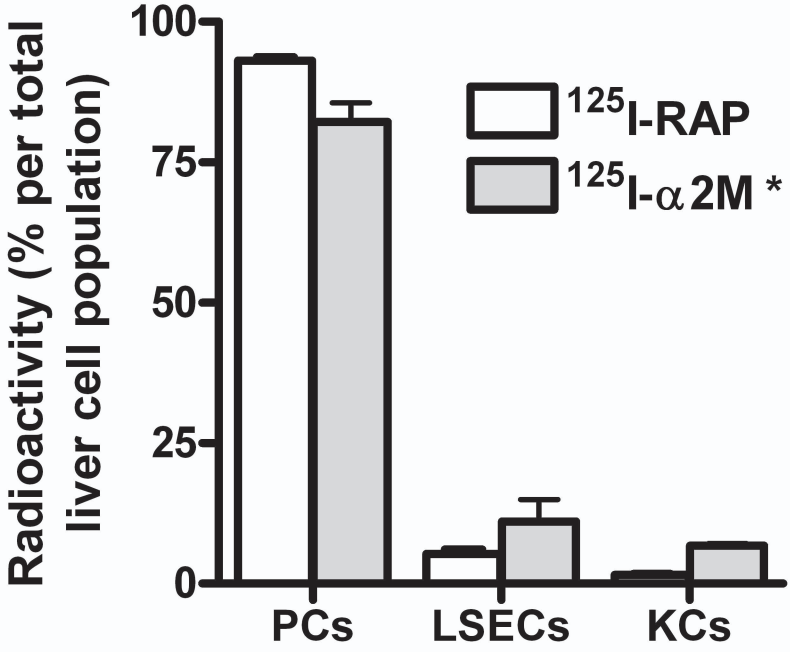

Radioactivity (% per total liver cell population)
100
75
50
25
0
125I-RAP
125I-α2M *
PCs
LSECs
KCs

Figure 3

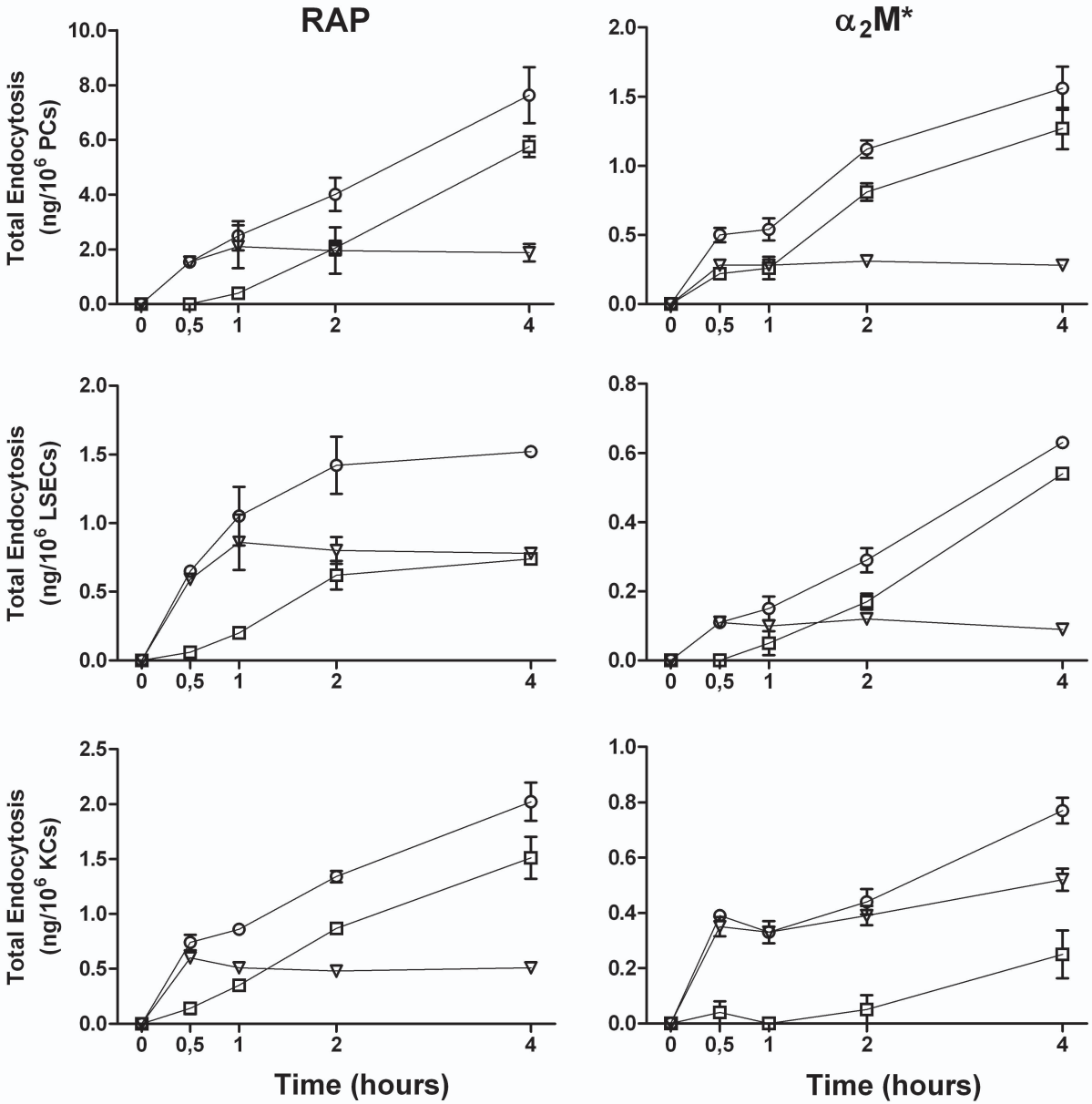

RAP
α2M*
Total Endocytosis (ng/10^6 PCs)
Total Endocytosis (ng/10^6 LSECs)
Total Endocytosis (ng/10^6 KCs)
Time (hours)
Time (hours)

Figure 4

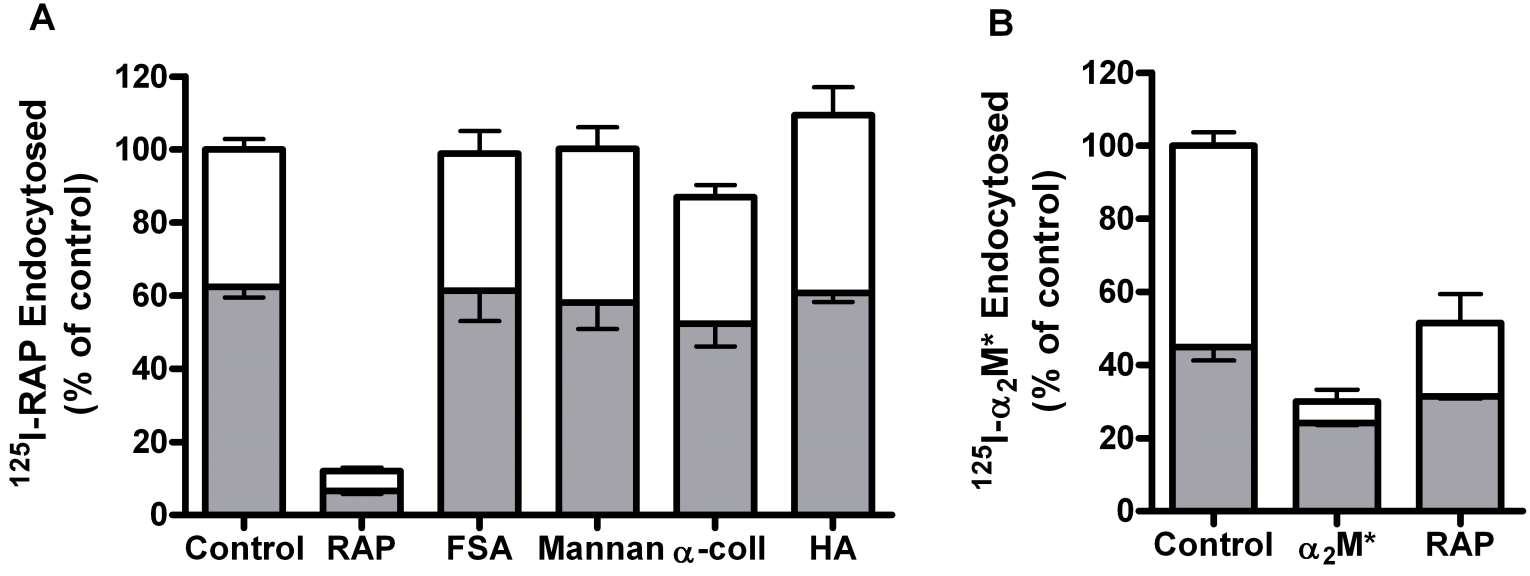

A
^{125}I-RAP Endocytosed
(% of control)
120
100
80
60
40
20
0
Control
RAP
FSA
Mannan
α-coll
HA
B
^{125}I-α$_2$M* Endocytosed
(% of control)
120
100
80
60
40
20
0
Control
α$_2$M*
RAP

Figure 5

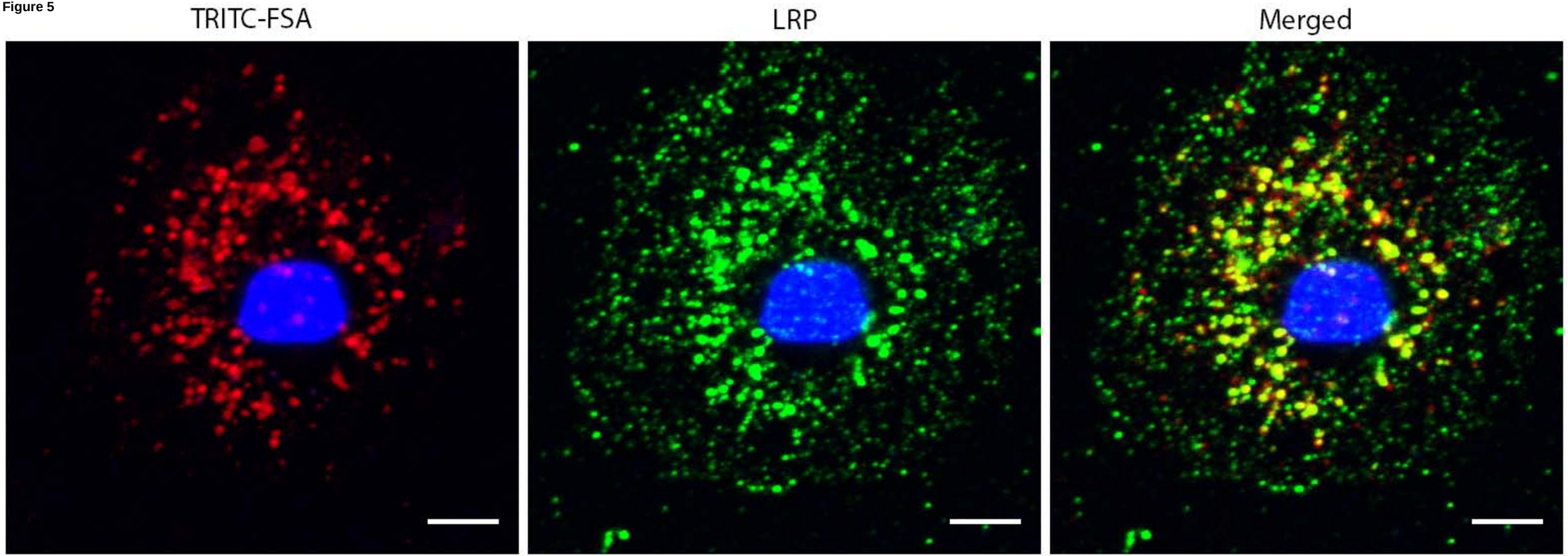
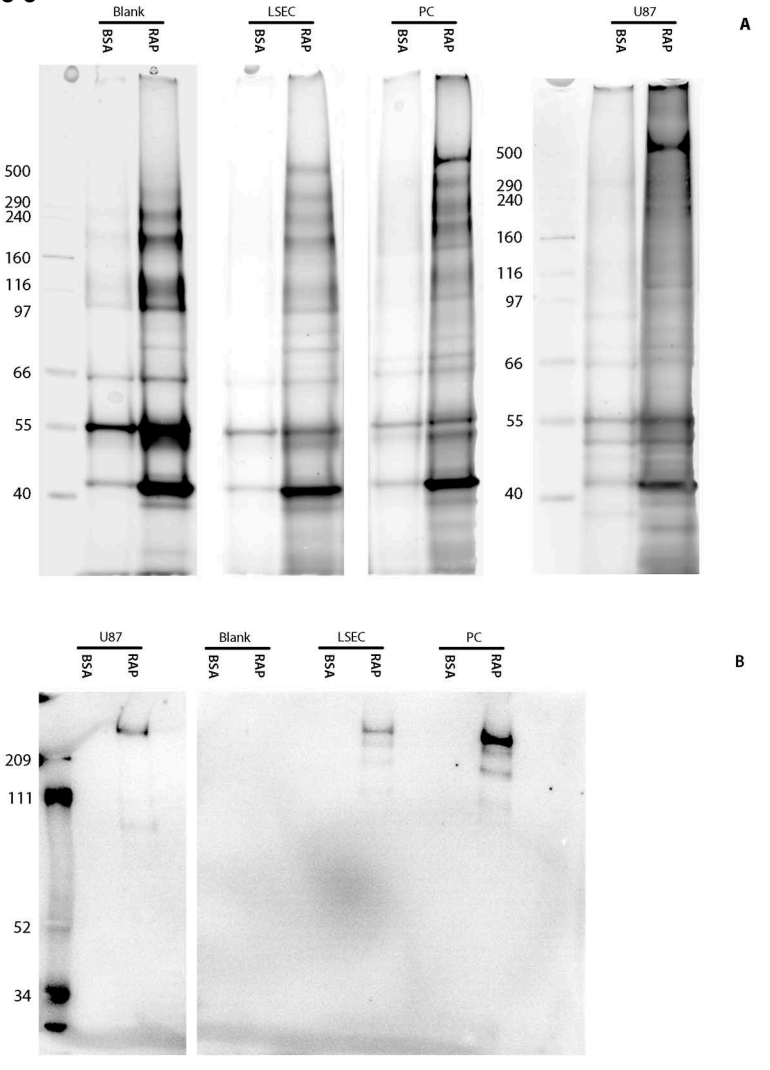


Figure 6


A
Blank
LSEC
PC
U87
BSA
RAP
500
290
240
160
116
97
66
55
40
B
U87
Blank
LSEC
PC
209
111
52
34

Supplementary material - RAP purification

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