

1  
2  
3  
4 **Rat Liver Sinusoidal Endothelial Cells (LSECs) express functional Low Density**  
5  
6 **Lipoprotein Receptor-Related Protein-1 (LRP-1)**  
7

8  
9 Cristina Ionica Øie<sup>1</sup>, Rupa Shree Appa<sup>2</sup>, Ida Hilden<sup>2</sup>, Helle Heibroch Petersen<sup>2</sup>, Albrecht  
10  
11 Gruhler<sup>2</sup>, Bård Smedsrød<sup>3</sup>, John-Bjarne Hansen<sup>1</sup>  
12  
13  
14

15  
16 From the <sup>1</sup>Center for Atherothrombotic Research in Tromsø (CART), Department of  
17  
18 Medicine, Institute of Clinical Medicine, University of Tromsø, Tromsø, Norway; the  
19  
20 <sup>2</sup>Biopharmaceutical Research Unit, Novo Nordisk, Måløv, Denmark; and the <sup>3</sup>Vascular  
21  
22 Biology Research Group, Institute of Medical Biology, University of Tromsø, Tromsø,  
23  
24 Norway  
25  
26  
27  
28  
29  
30

31 **Corresponding author:** Cristina Ionica Øie, Center for Atherothrombotic Research in  
32  
33 Tromsø, Institute of Clinical Medicine, University of Tromsø, N-9037 Tromsø, Norway.  
34  
35 Telephone: +4777644686 (cell +4791682978); Fax: +4777645400; e-mail:  
36  
37 [cristina.ionica.oie@uit.no](mailto:cristina.ionica.oie@uit.no)  
38  
39  
40  
41  
42

43 **Word count:** 5355  
44

45 **Number of figures and tables:** 6 / 0  
46  
47

48 **Abbreviations:** RAP, receptor associated protein;  $\alpha_2M^*$ , trypsin-activated  $\alpha_2$ -  
49  
50 Macroglobulin; PC, parenchymal cell; FSA, formaldehyde-treated bovine serum albumin;  
51  
52  $\alpha$ -coll, collagen  $\alpha$ -chains; HA, hyaluronan; RT, room temperature; KC, Kupffer cell  
53  
54

55 **Financial disclosure:** The present study was supported by an independent grant from  
56  
57 Pfizer AS (JBH); Tromsø Research Foundation (BS), and Medical Faculty, University of  
58  
59 Tromsø, Norway (JBH, BS)  
60  
61  
62  
63  
64  
65

**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  $\alpha_2$ -macroglobulin ( $\alpha_2M^*$ ) 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  $^{125}I$ - $\alpha_2M^*$ , whereas 5% and 11% were associated with LSECs. Uptake of RAP and  $\alpha_2M^*$  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  $^{125}I$ - $\alpha_2M^*$  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.

**Word count abstract:** 241

1  
2  
3  
4 **Keywords:** receptor-mediated endocytosis, blood clearance, RAP,  $\alpha_2$ -macroglobulin,  
5  
6  
7 lipid homeostasis.  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## 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 ( $\alpha$ -chain) containing its binding domains, and a 85 kDa subunit ( $\beta$ -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

1  
2  
3  
4 family's biochemistry and to investigate potential specific interaction of ligands with  
5  
6 LRP-1 and LRP-1 related proteins *in vivo* and *in vitro* [46].  
7

8  
9  $\alpha_2$ -Macroglobulin ( $\alpha_2$ M) [1] is a major human blood glycoprotein able to trap and  
10  
11 inactivate a large variety of proteinases, and inhibit fibrinolysis by reducing plasminogen  
12  
13 to kallikrein [41]. The  $\alpha_2$ M-proteinase complex, the "activated"  $\alpha_2$ M ( $\alpha_2$ M\*), becomes a  
14  
15 specific ligand for the LRP-1, and is eliminated from the circulation by LRP-mediated  
16  
17 endocytosis and subsequently degraded [41].  $\alpha_2$ M was recently found to be a noninvasive  
18  
19 serological biomarker that could predict the stage of liver fibrosis [20]  
20  
21

22  
23 The expression and function of the LRP-1 in liver was so far studied in PCs or PC-like  
24  
25 cell lines [2, 6, 7, 42, 43]. Using RAP and  $\alpha_2$ M\* as potent ligands for LRP-1, we  
26  
27 investigated whether this receptor is also expressed in LSECs.  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## 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).  $\alpha_2$ -Macroglobulin ( $\alpha_2$ M) from bovine plasma was from Roche Diagnostics Norge AS (Oslo, Norway). Carrier free  $\text{Na}^{125}\text{I}$  was from Perkin-Elmer Norge AS (Oslo, Norway), and 1,3,4,6-tetrachloro-3 $\alpha$ , 6 $\alpha$ -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 (Pasing, 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  $\alpha$ -chains ( $\alpha$ -coll) were obtained by incubation at 60°C for 60 min of native triple helical collagen (Vitrogen, Palo Alto, California).

### ***Labeling Procedures***

RAP and  $\alpha_2$ M (50  $\mu$ g) in PBS were directly labeled with Na<sup>125</sup>I employing Iodogen as oxidizing agent [27]. Specific radioactivities were between 5 - 8 x 10<sup>6</sup> cpm/ $\mu$ g.  $\alpha_2$ M-trypsin complexes ( $\alpha_2$ M\*) were prepared as described [16]. Briefly,  $\alpha_2$ M or <sup>125</sup>I- $\alpha_2$ M 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<sub>2</sub>CO<sub>3</sub> (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 <sup>125</sup>I-RAP and <sup>125</sup>I- $\alpha_2$ M\* (0.5  $\mu$ g) were determined as described [38]. Total blood volume was calculated [44].

### ***Hepatocellular Distribution***

Ten min after i.v. administration of 5  $\mu$ g of <sup>125</sup>I-RAP or <sup>125</sup>I- $\alpha_2$ M\*, 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***

1  
2  
3  
4 PCs, LSECs and KCs were prepared by collagenase perfusion of the liver, low speed  
5  
6 differential centrifugation and Percoll gradient sedimentation [39], followed by magnetic  
7  
8 cell separation (MACS) for isolation of KCs and LSECs [11], using biotin anti-rat CD11b  
9  
10 and biotin anti-rat ICAM-1 (Cedarlane®, Ontario, Canada) / streptavidin-conjugated  
11  
12 magnetic beads, respectively (Miltenyi, Inc.), over two MS<sup>+</sup> MiniMACS separation  
13  
14 columns according to the manufacturer. The purity of the PCs (> 95%) was easily  
15  
16 assessed by inspection in the light microscope, as these cells are much larger in diameter  
17  
18 as compared to other liver cells. The purity of LSECs cultures and the degree of LSECs  
19  
20 contamination in the KCs cultures was assessed by immunostaining using Stabilin2  
21  
22 antibody [17, 28]. LSEC preparations were between 95-98% pure. The degree of LSEC  
23  
24 contamination in KCs was less than 10%.

### 31 32 33 ***Kinetics and specificity of endocytosis***

34  
35 Cultures of PCs ( $0.25 \times 10^6$ ) and LSECs ( $0.5 \times 10^6$ ) were established on fibronectin  
36  
37 coated 24-well plates (Becton Dickinson, Plymouth, UK), and KCs ( $0.35 \times 10^6$ ) on non-  
38  
39 coated plastic in 48-well plates. Endocytosis kinetics of <sup>125</sup>I-RAP (4.27 ng), and <sup>125</sup>I-  
40  
41  $\alpha_2M^*$  (10 ng), were studied at 37°C for various time periods. Endocytosis specificity was  
42  
43 studied by incubating LSECs for 2 h at 37°C with trace amounts of <sup>125</sup>I-RAP or <sup>125</sup>I-  
44  
45  $\alpha_2M^*$  alone (control), or with non-labeled (100  $\mu$ g/ml) RAP,  $\alpha_2M^*$ , FSA, Mannan,  $\alpha$ -  
46  
47 coll or HA. Degradation was determined in the spent media by measuring the amount of  
48  
49 acid soluble radioactivity after addition of TCA and centrifugation. Cell-associated ligand  
50  
51 was measured in cells solubilized in 1% SDS. The amount of the non-specific binding  
52  
53 and free <sup>125</sup>I in the cell free wells was subtracted.  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



### ***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, Kjølsås, Norway) was used for mounting. Pictures were taken using a Zeiss Axiovert microscope (x63 objective) and LSM 510 software (Karl Zeiss Microimaging GmbH, Göttingen, Germany).

### ***Ligand blotting with <sup>125</sup>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<sub>2</sub>, 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

1  
2  
3  
4 5% BSA and incubated overnight at 4°C with <sup>125</sup>I-RAP before phosphorimaging (Fuji  
5  
6  
7 BAS5000).

### 11 *Identification of LRP-1 by mass spectrometry*

14 A band of > 500 kDa was excised from the silver-stained gel, reduced with DTT,  
15  
16 alkylated with iodoacetamide and digested with trypsin [36]. Nano-LC-ESI-MSMS was  
17  
18 performed (see supplementary materials and methods). Peak lists of MSMS spectra were  
19  
20 generated with DTASuperCharge (<http://msquant.sourceforge.net/>) and searched with  
21  
22 MASCOT against the mammalian part of the IPI\_rat database.  
23  
24  
25  
26  
27

### 28 *LRP mRNA levels in PCs, LSECs and KCs*

31 Total RNA was extracted from isolated PCs, Kupffer and LSECs using total RNA  
32  
33 isolation kit from Macherey-Nagel (Duren, Germany). Approximately 2-10 million  
34  
35 plated cells were lysed (PCs were in suspension). The quantity and quality of RNA was  
36  
37 determined by Nanodrop (ThermoFisher Scientific, Wilmington, DE, USA) and by an  
38  
39 electrophoretic bioanalyzer Agilent 2100 using the Pico 6000 Assay (Agilent  
40  
41 Technologies, Santa Clara, CA, USA). The 260/280 ratios in all samples were 2.1 and  
42  
43 18S/28S ratios were between 1.1-1.4. The yields were 43,1 µg/ml, 15,8 µg/ml and 3647,0  
44  
45 µg/ml for LSECs, KCs and PCs, respectively. Less than 2 µg of RNA for each cell type  
46  
47 was added to the cDNA reaction using MuLV Reverse transcriptase, random decamers,  
48  
49 dNTP mix and placental RNase inhibitor (Ambion, Austin, Texas, USA). RT-PCR was  
50  
51 performed with AmpliTaq Gold, Taqman Universal PCR master mix, primer/probe kit  
52  
53 m1 LRP1 and GAPDH (all from Applied Biosystems, Foster City, CA, USA). Expression  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 levels of LRP were determined by a relative comparison  $\Delta\Delta\text{CT}$  method where the  $\Delta\text{Ct}$   
5  
6 for GAPDH and LRP was compared for each cell type and then related to that of PCs.  
7  
8  
9

### 10 11 *Statistical Analysis*

12  
13  
14 SPSS package for Windows version 15.0 (SPSS Inc., Chicago, Ill, USA) was used for  
15  
16 statistical analyses. Two-sided  $p$  values less than 0.05 were considered significant. Half-  
17  
18 life data was analyzed using GraphPad Prism 4 (GraphPad Software, Inc. La Jolla, CA,  
19  
20  
21 USA).  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## Results

### *Anatomical distribution in vivo of intravenously injected $^{125}\text{I}$ -RAP and $^{125}\text{I}$ - $\alpha_2\text{M}^*$*

The anatomical distribution was investigated 10 min after lateral tail vein administration of  $^{125}\text{I}$ -RAP or  $^{125}\text{I}$ - $\alpha_2\text{M}^*$ . 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  $\alpha_2\text{M}^*$  uptake,  $^{125}\text{I}$ -RAP and  $^{125}\text{I}$ - $\alpha_2\text{M}^*$  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%  $\alpha_2\text{M}^*$  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  $\alpha_2\text{M}^*$  uptake, while the CD11b positive cells (KCs), for 1.6 ± 0.2% and 6.8 ± 0.7%, respectively (Figure 2).

### *In vitro studies*

1  
2  
3  
4 Endocytosis kinetics was studied to compare the uptake of  $^{125}\text{I}$ -RAP and  $^{125}\text{I}$ - $\alpha_2\text{M}^*$  in  
5  
6 cultures of PCs, LSECs and KCs. The endocytosis in PCs increased almost linearly over  
7  
8 time, with  $7.6\pm 1.0$  ng RAP being endocytosed and  $5.7\pm 0.4$  ng degraded after 4 h of  
9  
10 incubation (Figure 3-left panel). Degradation products were detected in the medium after  
11  
12 approx. 20 min, after which degradation proceeded at the same rate as cellular uptake.  
13  
14 Similar kinetics were observed in KCs, but the total amount of endocytosed RAP at the  
15  
16 end of the incubation time was 4 times lower than in PCs. The uptake in LSECs reached a  
17  
18 plateau, with  $1.4\pm 0.4$  ng RAP being endocytosed, and  $0.6\pm 0.2$  ng degraded after 2 h. The  
19  
20 kinetics of endocytosis of  $^{125}\text{I}$ - $\alpha_2\text{M}^*$  in PCs and LSECs were also similar, with 3 fold  
21  
22 more uptake in PCs as compared to the uptake in LSECs (Figure 3-right panel). However,  
23  
24 in the KCs, it was only after 4 h of incubation when degradation products could be  
25  
26 measured in the supernatant, while at shorter time points, the  $\alpha_2\text{M}^*$  was only found  
27  
28 bound to the cells.  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38

39 Specificity of endocytosis of the two ligands was studied in primary cultures of LSECs.  
40  
41 Excess amounts of non-labeled RAP inhibited the endocytosis of trace amounts of  $^{125}\text{I}$ -  
42  
43 RAP by  $88.0\pm 0.1\%$ , suggesting that the uptake of RAP is receptor mediated (Figure 4A).  
44  
45 To gain further insights into the receptor mediated endocytosis, excess amounts of  
46  
47 unlabelled ligands for known candidate receptors were added to primary cultures of  
48  
49 LSECs together with  $^{125}\text{I}$ -RAP. So far, three major receptors for endocytosis have been  
50  
51 identified on LSECs, i.e. the hyaluronan/scavenger receptor (Stabilin2), the  
52  
53 mannose/collagen  $\alpha$ -chain receptor (MANN/COLLA-R), and the Fc $\gamma$ -receptor IIb [22,  
54  
55 25, 26, 28]. Various macromolecules known to bind to these LSECs endocytosis  
56  
57 receptors were tested for potential inhibition of RAP uptake. The presence of high  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 concentrations of formaldehyde treated serum albumin (FSA) or hyaluronan (HA)  
5  
6 (ligands for Stabilin2), mannan or collagen  $\alpha$ -chains ( $\alpha$ -coll) (ligands for the  
7  
8 MANN/COLLA-R) had no effect on the endocytosis and degradation of RAP. Inhibition  
9  
10 of the Fc $\gamma$ -receptor IIb was not tested, as this receptor recognizes only the Fc domain of  
11  
12 IgG-immune complexes [23].  
13  
14  
15  
16  
17  
18

19 Excess amounts of unlabeled  $\alpha_2$ M\* inhibited the endocytosis of  $^{125}$ I- $\alpha_2$ M\* by 73.0%  
20  
21 (p<0.001) (Figure 4B). The presence of RAP had a significant inhibitory effect on both  
22  
23 the cell associated and degraded  $^{125}$ I- $\alpha_2$ M\*, and it inhibited the total uptake by 50.1%  
24  
25 (p<0.001), suggesting the involvement of the LRP-1 in uptake.  
26  
27  
28  
29  
30  
31

### 32 ***Immunofluorescence staining for LRP-1***

33  
34 We used immunostaining to further investigate whether the LRP-1 receptor is expressed  
35  
36 on LSECs. Paraformaldehyde fixed cultures of LSECs were immunostained using mouse  
37  
38 monoclonal LRP-1 antibody recognizing the  $\beta$ -chain (5A6). The cells stained positively  
39  
40 for LRP-1, as visualized by confocal microscopy using goat AlexaFluor488 anti-mouse  
41  
42 antibody (green) (Figure 5). To determine if the LRP-1 positive cells are LSECs, the cells  
43  
44 were preincubated with TRITC-FSA (red), a ligand that is exclusively taken up by the  
45  
46 LSECs in the liver [13]. No staining was observed when the cells were treated with the  
47  
48 secondary antibody only or with control mouse IgG (not shown).  
49  
50  
51  
52  
53  
54  
55

### 56 ***LRP-1 expression in liver cells***

57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 The expression of LRP in the different liver cells was assessed by extraction of mRNA  
5 and RT-PCR. We found that after normalizing for an internal mRNA control GAPDH  
6  
7 (which showed minimum variation in samples), the LRP-1 expression levels in PCs were  
8  
9 6 times higher than in LSECs and KCs, with LSECs and KCs expressing approximately  
10  
11 similar levels. To further confirm that LRP-1 is expressed in LSECs, total cell protein  
12  
13 was incubated with Dynabeads coupled to RAP, to pull down the receptor/s binding  
14  
15 RAP. Bound proteins were eluted from the beads, and run in parallel on two SDS-PAGE  
16  
17 gels under non-reducing conditions. The proteins on the first gel were visualized by silver  
18  
19 staining (Figure 6.A). Dynabeads coupled to BSA were used as negative control showing  
20  
21 that LRP-1 does not bind unspecifically to the beads. PCs and U87 cell line were used as  
22  
23 positive controls, as both cell types are known to express LRP-1 [5, 34]. A band of > 500  
24  
25 kDa appeared on the silver stained gel from cell extracts of all three cell types (Figure  
26  
27 6.A). The proteins from the second gel were transferred to nitrocellulose filters and  
28  
29 incubated with <sup>125</sup>I-RAP before phosphorimaging. The results presented in Figure 6.B  
30  
31 show that <sup>125</sup>I-RAP bound to a single protein from the cell extracts of LSECs. The  
32  
33 respective band corresponded to a similar band from the U87 and PCs extracts. A  
34  
35 sensitive and specific high-performance liquid chromatography-tandem mass  
36  
37 spectrometry using electrospray ionization (LC-ESI-MSMS) was chosen to study the  
38  
39 band excised from the silver-stained gel. Rat LRP-1 (IPI00369995) was identified with a  
40  
41 Mascot score of 134. Identified peptides are listed in Table 1 (supplementary material).  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## Discussion

RAP, a potent inhibitor of all known ligand interactions with LRP-1, and  $\alpha_2\text{M}^*$ , 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  $\alpha_2\text{M}^*$ . Liver cell separation showed that intravenously administered  $^{125}\text{I}$ -RAP and  $^{125}\text{I}$ - $\alpha_2\text{M}^*$  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  $^{125}\text{I}$ -RAP was not inhibited by ligands to known endocytosis receptors in LSECs, while uptake of  $^{125}\text{I}$ - $\alpha_2\text{M}^*$  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  $^{125}\text{I}$ -RAP followed by mass spectrometry further confirmed and identified LRP-1 in LSECs.

Several studies have shown that  $\alpha_2\text{M}$ -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



1  
2  
3  
4 in accordance with the previous findings, with the liver being the main site of uptake of  
5  
6  $^{125}\text{I}$ -RAP and  $^{125}\text{I}$ - $\alpha_2\text{M}^*$ .  
7  
8  
9

10  
11 It has been previously reported that the liver PCs are the main site of uptake of  $\alpha_2\text{M}^*$  [10,  
12  
13 15]. The authors used autoradiography as a method for identifying the anatomical site of  
14  
15 uptake. This technique, however, makes it difficult to distinguish between KCs and  
16  
17 LSECs. Since at that time (1985) it was generally believed that KC = liver  
18  
19 reticuloendothelial system (RES), it is fully understandable that the authors did not  
20  
21 suggest the possibility that LSECs could be an alternative site of uptake in addition to the  
22  
23 KCs. It was not until 1990 that LSEC was proposed, based on a solid body of evidence,  
24  
25 to be a very important part of the liver RES [40]. In our study, we have used an antibody-  
26  
27 based purification method that allowed us to achieve higher purities and more defined  
28  
29 cell populations. Our results show that LSECs also contributed to the elimination of RAP  
30  
31 and  $\alpha_2\text{M}^*$  from the circulation, albeit to a lesser extent than PCs.  
32  
33  
34  
35  
36  
37  
38  
39  
40

41 The most likely receptor candidate, LRP-1, has previously been shown to be expressed in  
42  
43 the liver by PCs only [24, 34]. The following findings in the present study point to the  
44  
45 presence and a functional role for LRP-1 in LSECs. First, excessive amounts of unlabeled  
46  
47 RAP efficiently inhibited the uptake and degradation of  $^{125}\text{I}$ -RAP and  $^{125}\text{I}$ - $\alpha_2\text{M}^*$  in  
48  
49 primary cultures of LSECs. Second, excess amounts of ligands to known scavenger  
50  
51 receptors in LSECs failed to inhibit the uptake of RAP.  
52  
53  
54  
55  
56  
57

58 To obtain direct evidence for identification of LRP-1 in LSECs and thereby rule out  
59  
60 possible influence of marginal contamination of PCs, the cell cultures were pre-incubated  
61  
62  
63  
64  
65

1  
2  
3  
4 with TRITC-FSA, a ligand that is known to be taken up only by the LSECs in the liver  
5  
6 [12]. Immunofluorescence staining of the cells with TRITC-FSA and LRP-1 antibody  
7  
8 revealed double staining and provided strong evidence for the expression of LRP-1 in  
9  
10 LSECs. Further isolation of a RAP-binding protein of MW > 500 kDa from LSEC  
11  
12 extracts, with subsequent mass spectrometry, confirmed the expression of LRP-1 in  
13  
14 LSEC.  
15  
16  
17  
18  
19  
20

21 Our time course studies of RAP and  $\alpha_2M^*$  endocytosis in LSECs and KCs revealed  
22  
23 binding and degradation of the ligands, suggesting a functional role of LRP-1 in these  
24  
25 cells. However, the functional capacity of LRP-1 appeared to be lower in LSECs and  
26  
27 KCs than in PCs (Figure 3). LRP-1 mRNA expression was detected in all three liver cell  
28  
29 types, and relative LRP-1 expression level was 6 times greater in PCs than in LSECs and  
30  
31 KCs. If the relative protein expression levels mirror the RNA levels, this may explain the  
32  
33 differences between RAP and  $\alpha_2M^*$  uptake observed in the in vitro PC and non-PC cell  
34  
35 assays. Another interesting finding was that the uptake capacity for RAP was higher in all  
36  
37 three cell types as compared to that for  $\alpha_2M^*$ . This may be due to involvement of other  
38  
39 receptor(s) that recognize RAP. Such receptor could be the LDL receptor [30] which is  
40  
41 known to be expressed on PCs and is able to bind to RAP, although with less affinity than  
42  
43 LRP-1 [3, 24].  
44  
45  
46  
47  
48  
49  
50

51  
52  
53 In conclusion, we demonstrate for the first time that the expression of functional LRP-1  
54  
55 in liver is not restricted only to PCs, it is also found in LSECs, and to a lesser extent in  
56  
57 KCs. Characterized by a very high endocytic activity [37], LSECs would contribute  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 significantly to the elimination of the various LRP-1 ligands from the circulation in  
5  
6 normal, healthy conditions.  
7

8  
9 However, in pathological conditions, such as severe fatty liver diseases, as well as in  
10  
11 aging [14, 21, 29], significant changes occur in the hepatic sinusoids (e.g. fibrosis,  
12  
13 narrowing of the sinusoids, reduced blood flow and capillarization of the liver sinusoids).  
14

15  
16 These changes are hallmarked structurally by a progressive loss in fenestrae in LSECs,  
17  
18 concomitant with the development of a basal lamina and deposition of collagen in the  
19  
20 space of Disse. Given the role of fenestration in the transfer of lipoproteins from the  
21  
22 blood to hepatocytes, it is likely that defenestration will impair lipoprotein/chylomicron  
23  
24 remnants clearance by the hepatocytes. In addition, the transport in the opposite direction  
25  
26 of some lipoproteins manufactured by hepatocytes will be impaired. Further studies are  
27  
28 needed to investigate the endocytic function of LSECs via the LRP-1 in pathological  
29  
30 conditions.  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## Acknowledgment

We thank Prof. Jan Olof Winberg and laboratory engineer Eli Berg from the Biochemistry Research Group, for their help with the trypsin activation of  $\alpha_2$ M and the zymography testing of the complex. We thank Dr. Peter McCourt for reviewing the manuscript. The authors who have taken part in this study declared that they do not have anything to disclose regarding funding from industry or conflict of interest with respect to this manuscript.

**REFERENCES**

- [1] Barrett AJ, Starkey PM. The interaction of alpha 2-macroglobulin with proteinases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. *Biochem J* 1973;133(4): 709-724.
- [2] Beisiegel U, Weber W, Ihrke G, Herz J, Stanley KK. The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. *Nature* 1989;341(6238): 162-164.
- [3] Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science* 1986;232(4746): 34-47.
- [4] Bu G, Geuze HJ, Strous GJ, Schwartz AL. 39 kDa receptor-associated protein is an ER resident protein and molecular chaperone for LDL receptor-related protein. *Embo J* 1995;14(10): 2269-2280.
- [5] Bu G, Maksymovitch EA, Geuze H, Schwartz AL. Subcellular localization and endocytic function of low density lipoprotein receptor-related protein in human glioblastoma cells. *J Biol Chem* 1994;269(47): 29874-29882.
- [6] Bu G, Maksymovitch EA, Schwartz AL. Receptor-mediated endocytosis of tissue-type plasminogen activator by low density lipoprotein receptor-related protein on human hepatoma HepG2 cells. *J Biol Chem* 1993;268(17): 13002-13009.
- [7] Bu G, Williams S, Strickland DK, Schwartz AL. Low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor is an hepatic receptor for tissue-type plasminogen activator. *Proc Natl Acad Sci U S A* 1992;89(16): 7427-7431.
- [8] Christensen JH, Hansen PK, Lillelund O, Thogersen HC. Sequence-specific binding of the N-terminal three-finger fragment of Xenopus transcription factor IIIA to the internal control region of a 5S RNA gene. *FEBS Lett* 1991;281(1-2): 181-184.
- [9] Damiao AO, Sipahi AM, Albuquerque MR, Laudanna AA, Quintao EC. Chylomicron metabolism in experimental cirrhosis and cholestasis. *Res Exp Med (Berl)* 1993;193(2): 89-95.
- [10] Davidsen O, Christensen EI, Gliemann J. The plasma clearance of human alpha 2-macroglobulin-trypsin complex in the rat is mainly accounted for by uptake into hepatocytes. *Biochim Biophys Acta* 1985;846(1): 85-92.
- [11] Do H, Healey JF, Waller EK, Lollar P. Expression of factor VIII by murine liver sinusoidal endothelial cells. *J Biol Chem* 1999;274(28): 19587-19592.
- [12] Elvevold K, Simon-Santamaria J, Hasvold H, McCourt P, Smedsrod B, Sorensen KK. Liver sinusoidal endothelial cells depend on mannose receptor-mediated recruitment of lysosomal enzymes for normal degradation capacity. *Hepatology* 2008;48(6): 2007-2015.
- [13] Elvevold KH, Nedredal GI, Revhaug A, Smedsrod B. Scavenger properties of cultivated pig liver endothelial cells. *Comp Hepatol* 2004;3(1): 4.
- [14] Farrell GC, Teoh NC, McCuskey RS. Hepatic microcirculation in fatty liver disease. *Anat Rec (Hoboken)* 2008;291(6): 684-692.
- [15] Feldman SR, Rosenberg MR, Ney KA, Michalopoulos G, Pizzo SV. Binding of alpha 2-macroglobulin to hepatocytes: mechanism of in vivo clearance. *Biochem Biophys Res Commun* 1985;128(2): 795-802.
- [16] Gliemann J, Larsen TR, Sottrup-Jensen L. Cell association and degradation of alpha 2-macroglobulin-trypsin complexes in hepatocytes and adipocytes. *Biochim Biophys Acta* 1983;756(2): 230-237.

- 1  
2  
3  
4 [17] Hansen B, Longati P, Elvevold K, Nedredal GI, Schledzewski K, Olsen R, et al.  
5 Stabilin-1 and stabilin-2 are both directed into the early endocytic pathway in hepatic  
6 sinusoidal endothelium via interactions with clathrin/AP-2, independent of ligand  
7 binding. *Exp Cell Res* 2005;303(1): 160-173.
- 8 [18] Herz J, Kowal RC, Goldstein JL, Brown MS. Proteolytic processing of the 600 kd  
9 low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi  
10 compartment. *Embo J* 1990;9(6): 1769-1776.
- 11 [19] Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. *J*  
12 *Clin Invest* 2001;108(6): 779-784.
- 13 [20] Ho AS, Cheng CC, Lee SC, Liu ML, Lee JY, Wang WM, et al. Novel biomarkers  
14 predict liver fibrosis in hepatitis C patients: alpha 2 macroglobulin, vitamin D binding  
15 protein and apolipoprotein AI. *J Biomed Sci* 2010;17: 58.
- 16 [21] Ito Y, Sorensen KK, Bethea NW, Svistounov D, McCuskey MK, Smedsrod BH,  
17 et al. Age-related changes in the hepatic microcirculation in mice. *Exp Gerontol*  
18 2007;42(8): 789-797.
- 19 [22] Lovdal T, Andersen E, Brech A, Berg T. Fc receptor mediated endocytosis of  
20 small soluble immunoglobulin G immune complexes in Kupffer and endothelial cells  
21 from rat liver. *J Cell Sci* 2000;113 ( Pt 18): 3255-3266.
- 22 [23] Lovdal T, Berg T. Transcription of Fc(gamma) receptors in different rat liver  
23 cells. *Cell Biol Int* 2001;25(8): 821-824.
- 24 [24] Lund H, Takahashi K, Hamilton RL, Havel RJ. Lipoprotein binding and  
25 endosomal itinerary of the low density lipoprotein receptor-related protein in rat liver.  
26 *Proc Natl Acad Sci U S A* 1989;86(23): 9318-9322.
- 27 [25] Magnusson S, Berg T. Extremely rapid endocytosis mediated by the mannose  
28 receptor of sinusoidal endothelial rat liver cells. *Biochem J* 1989;257(3): 651-656.
- 29 [26] Malovic I, Sorensen KK, Elvevold KH, Nedredal GI, Paulsen S, Erofeev AV, et  
30 al. The mannose receptor on murine liver sinusoidal endothelial cells is the main  
31 denatured collagen clearance receptor. *Hepatology* 2007;45(6): 1454-1461.
- 32 [27] Markwell MA. A new solid-state reagent to iodinate proteins. I. Conditions for the  
33 efficient labeling of antiserum. *Anal Biochem* 1982;125(2): 427-432.
- 34 [28] McCourt PA, Smedsrod BH, Melkko J, Johansson S. Characterization of a  
35 hyaluronan receptor on rat sinusoidal liver endothelial cells and its functional relationship  
36 to scavenger receptors. *Hepatology* 1999;30(5): 1276-1286.
- 37 [29] McLean AJ, Cogger VC, Chong GC, Warren A, Markus AM, Dahlstrom JE, et al.  
38 Age-related pseudocapillarization of the human liver. *J Pathol* 2003;200(1): 112-117.
- 39 [30] Medh JD, Fry GL, Bowen SL, Pladet MW, Strickland DK, Chappell DA. The 39-  
40 kDa receptor-associated protein modulates lipoprotein catabolism by binding to LDL  
41 receptors. *J Biol Chem* 1995;270(2): 536-540.
- 42 [31] Mego JL, Bertini F, McQueen JD. The use of formaldehyde-treated <sup>131</sup>I-  
43 albumin in the study of digestive vacuoles and some properties of these particles from  
44 mouse liver. *J Cell Biol* 1967;32(3): 699-707.
- 45 [32] Ohlsson K. Elimination of <sup>125</sup>I-trypsin alpha-macroglobulin complexes from  
46 blood by reticuloendothelial cells in dogs. *Acta Physiol Scand* 1971;81(2): 269-272.
- 47 [33] Pertoft H, Smedsrod B. Separation and characterization of liver cells. In: Pretlow  
48 TG, Pretlow TPNY, eds *Cell Separation Methods and Selected Applications Volume 4*  
49 *Academic Press* 1987: 1-24.
- 50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4 [34] Schmoelzl S, Benn SJ, Laithwaite JE, Greenwood SJ, Marshall WS, Munday NA,  
5 et al. Expression of hepatocyte low-density lipoprotein receptor-related protein is post-  
6 transcriptionally regulated by extracellular matrix. *Lab Invest* 1998;78(11): 1405-1413.  
7 [35] Seternes T, Sorensen K, Smedsrod B. Scavenger endothelial cells of vertebrates: a  
8 nonperipheral leukocyte system for high-capacity elimination of waste macromolecules.  
9 *Proc Natl Acad Sci U S A* 2002;99(11): 7594-7597.  
10 [36] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of  
11 proteins silver-stained polyacrylamide gels. *Anal Chem* 1996;68(5): 850-858.  
12 [37] Smedsrod B. Clearance function of scavenger endothelial cells. *Comp Hepatol*  
13 2004;3 Suppl 1: S22.  
14 [38] Smedsrod B, Johansson S, Pertoft H. Studies in vivo and in vitro on the uptake  
15 and degradation of soluble collagen alpha 1(I) chains in rat liver endothelial and Kupffer  
16 cells. *Biochem J* 1985;228(2): 415-424.  
17 [39] Smedsrod B, Pertoft H. Preparation of pure hepatocytes and reticuloendothelial  
18 cells in high yield from a single rat liver by means of Percoll centrifugation and selective  
19 adherence. *J Leukoc Biol* 1985;38(2): 213-230.  
20 [40] Smedsrod B, Pertoft H, Gustafson S, Laurent TC. Scavenger functions of the liver  
21 endothelial cell. *Biochem J* 1990;266(2): 313-327.  
22 [41] Sottrup-Jensen L. Alpha-macroglobulins: structure, shape, and mechanism of  
23 proteinase complex formation. *J Biol Chem* 1989;264(20): 11539-11542.  
24 [42] Tamaki C, Ohtsuki S, Iwatsubo T, Hashimoto T, Yamada K, Yabuki C, et al.  
25 Major involvement of low-density lipoprotein receptor-related protein 1 in the clearance  
26 of plasma free amyloid beta-peptide by the liver. *Pharm Res* 2006;23(7): 1407-1416.  
27 [43] Warshawsky I, Bu G, Schwartz AL. 39-kD protein inhibits tissue-type  
28 plasminogen activator clearance in vivo. *J Clin Invest* 1993;92(2): 937-944.  
29 [44] Waynforth HB, Flecknell PA. *Experimental and Surgical Technique in the Rat*.  
30 San Diego, CA; Academic; 1992(Second Edition): 342.  
31 [45] Willnow TE. The low-density lipoprotein receptor gene family: multiple roles in  
32 lipid metabolism. *J Mol Med* 1999;77(3): 306-315.  
33 [46] Willnow TE. Receptor-associated protein (RAP): a specialized chaperone for  
34 endocytic receptors. *Biol Chem* 1998;379(8-9): 1025-1031.  
35 [47] Wisse E. An electron microscopic study of the fenestrated endothelial lining of rat  
36 liver sinusoids. *J Ultrastruct Res* 1970;31(1): 125-150.  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## FIGURE LEGENDS

### Fig. 1. Anatomical distribution

$^{125}\text{I}$ -RAP (white bars) or  $^{125}\text{I}$ - $\alpha_2\text{M}^*$  (0.5  $\mu\text{g}$ ) (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  $\pm$  SD for 3 animals.

### Fig. 2. Hepatocellular distribution

Ten min after i.v. injection of 5  $\mu\text{g}$   $^{125}\text{I}$ -RAP (white bars) or  $^{125}\text{I}$ - $\alpha_2\text{M}^*$  (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  $\pm$  SD of 3 animals.

### Fig. 3. Kinetics of endocytosis of $^{125}\text{I}$ -RAP (left panel) and $^{125}\text{I}$ - $\alpha_2\text{M}^*$ (right panel) in primary cultures of PCs, LSECs and KCs

Trace amounts of radio-labeled ligands (approx. 4.27 ng  $^{125}\text{I}$ -RAP and 10 ng  $^{125}\text{I}$ - $\alpha_2\text{M}^*$ ) were added to cultures of  $0.25 \times 10^6$  PCs,  $0.5 \times 10^6$  LSECs and  $0.3 \times 10^6$  KCs. Cell-associated ( $\nabla$ ) and degraded (acid-soluble) ( $\square$ ) ligands were determined after various periods of incubation at  $37^\circ\text{C}$  as described in the Materials and Methods. Total endocytosed ligand ( $\circ$ ) represents the sum of cell-associated and acid-soluble radioactivity. The amount of the non-specific binding and free  $^{125}\text{I}$  in the cell free wells



1  
2  
3  
4 was subtracted. The results are presented as ng ligand/ $10^6$  cells. Bars are means  $\pm$  SD of 3  
5  
6 experiments.  
7  
8  
9

10  
11 Fig. 4. Specificity of endocytosis of  $^{125}\text{I}$ -RAP (A) and  $^{125}\text{I}$ - $\alpha_2\text{M}^*$  (B) in LSECs

12  
13  
14 Monolayer cultures were incubated for 2 h at  $37^\circ\text{C}$  with approx. 4.27 ng  $^{125}\text{I}$ -RAP or 10  
15  
16 ng  $^{125}\text{I}$ - $\alpha_2\text{M}^*$  alone (Control) or together with 100  $\mu\text{g}/\text{ml}$  of unlabelled RAP, FSA,  
17  
18 Mannan,  $\alpha$ -coll or HA. Results are presented as percentage of Control. Gray bars  
19  
20 represent cell-associated ligand (SDS soluble). White bars represent degraded ligand  
21  
22 (TCA soluble). Control values were  $19 \pm 2\%$  and  $5 \pm 0.2\%$  of total added  $^{125}\text{I}$ -RAP and  
23  
24  $^{125}\text{I}$ - $\alpha_2\text{M}^*$ , respectively. Bars are means  $\pm$  SEM for 3 to 7 experiments.  
25  
26  
27  
28  
29  
30  
31

32 Fig. 5. Immunofluorescence staining for LRP-1

33  
34 LSECs on fibronectin coated glass coverslips were incubated for 1 h at  $37^\circ\text{C}$  with  
35  
36 TRITC-FSA (red), fixed with 4% paraformaldehyde and cell membranes permeabilized  
37  
38 with 0.1% Triton X-100. Indirect immunostaining was performed using mouse  
39  
40 monoclonal anti-LRP-1 and AlexaFluor 488 goat anti-mouse IgG (green). Cell nuclei  
41  
42 were stained with DRAQ5 (blue).  
43  
44  
45  
46  
47  
48

49 Fig. 6. Ligand blotting

50  
51 Total cell proteins of LSECs, PCs and human glioblastoma cell line (U87), were  
52  
53 incubated with Dynabeads coupled with RAP or BSA overnight. The Dynabeads were  
54  
55 washed and RAP-binding proteins separated by SDS-PAGE (7% Tris-Acetate), under  
56  
57 non-reducing conditions. A band of  $> 500$  kDa appeared on the silver stained gel from  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 cell extracts of all three cell types (A). Lane designation at top of the gel indicates the  
5  
6 cellular source of samples that were loaded. Prestained molecular mass markers is  
7  
8 indicated in kDa. The proteins were transferred to nitrocellulose filters, blocked with  
9  
10 BSA, the blot incubated with  $^{125}\text{I}$ -RAP and subjected to autoradiography (B).  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Figure 1

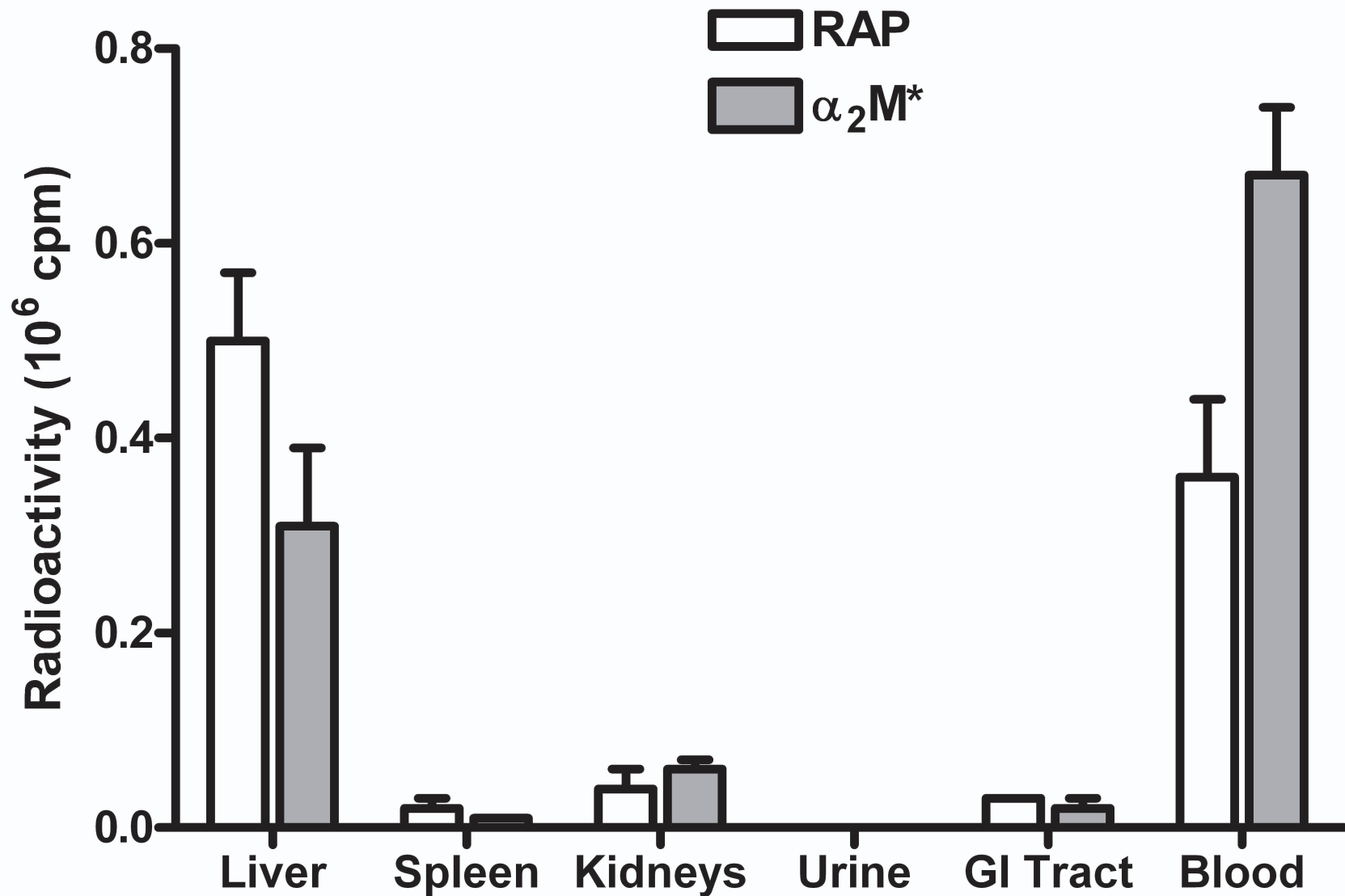
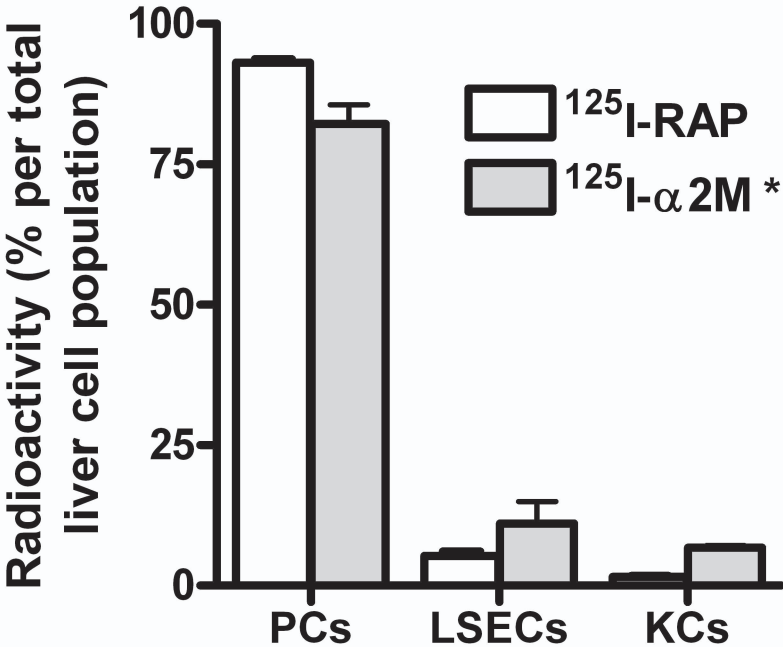


Figure 2



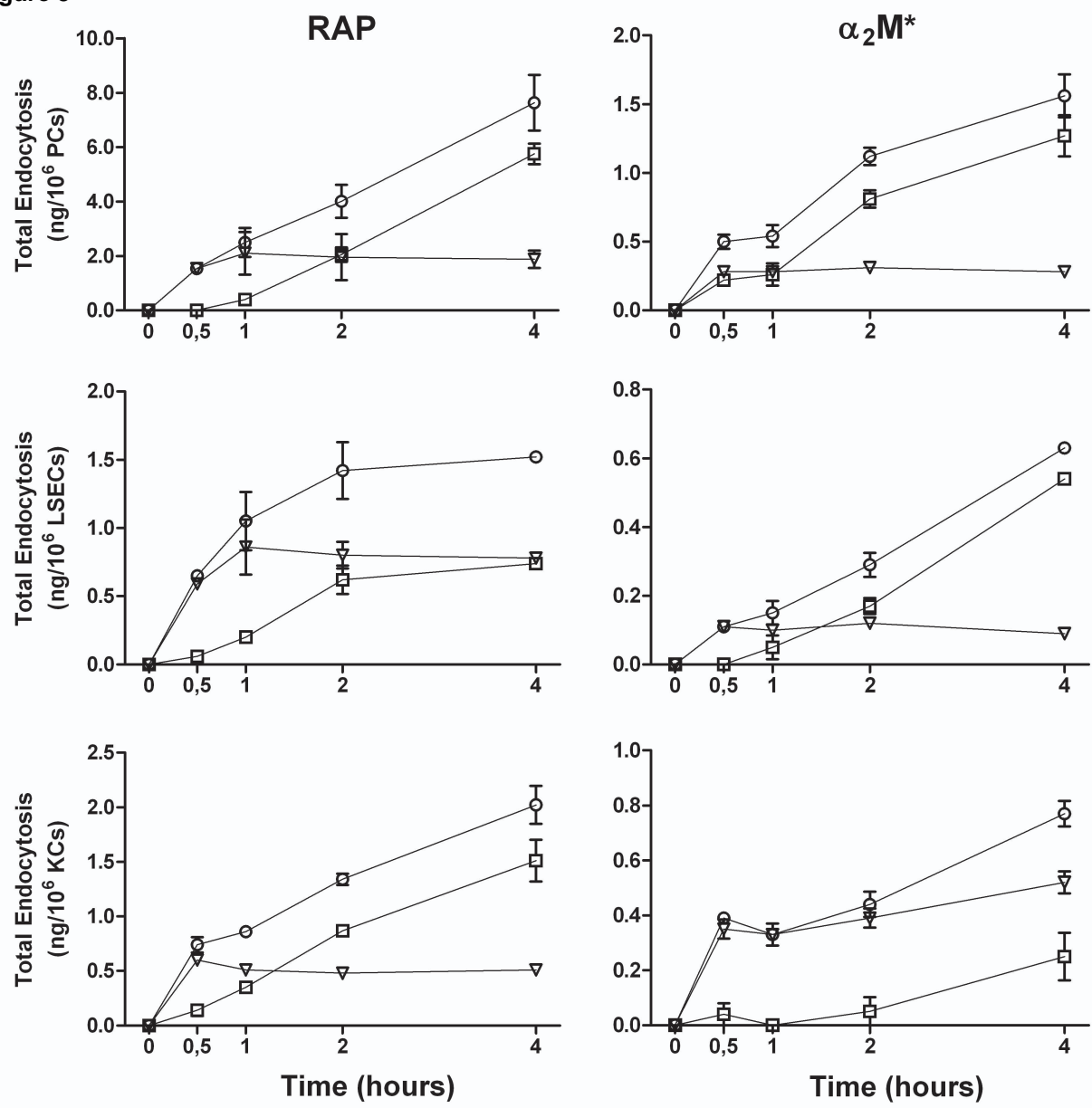
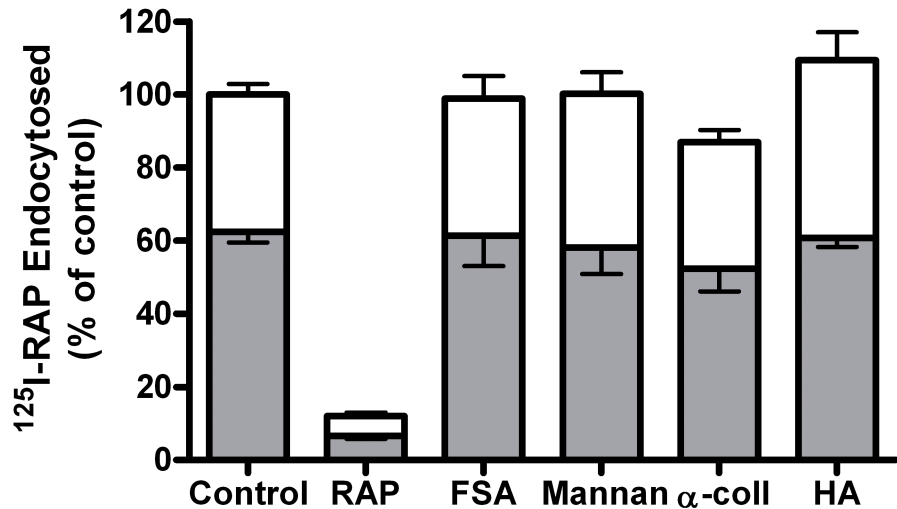
**Figure 3**

Figure 4

**A**



**B**

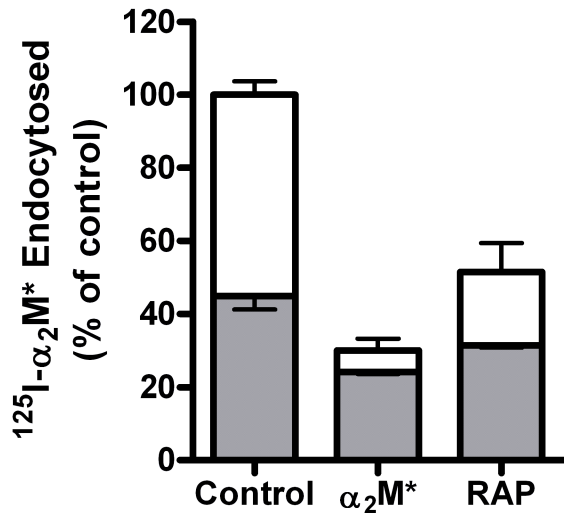
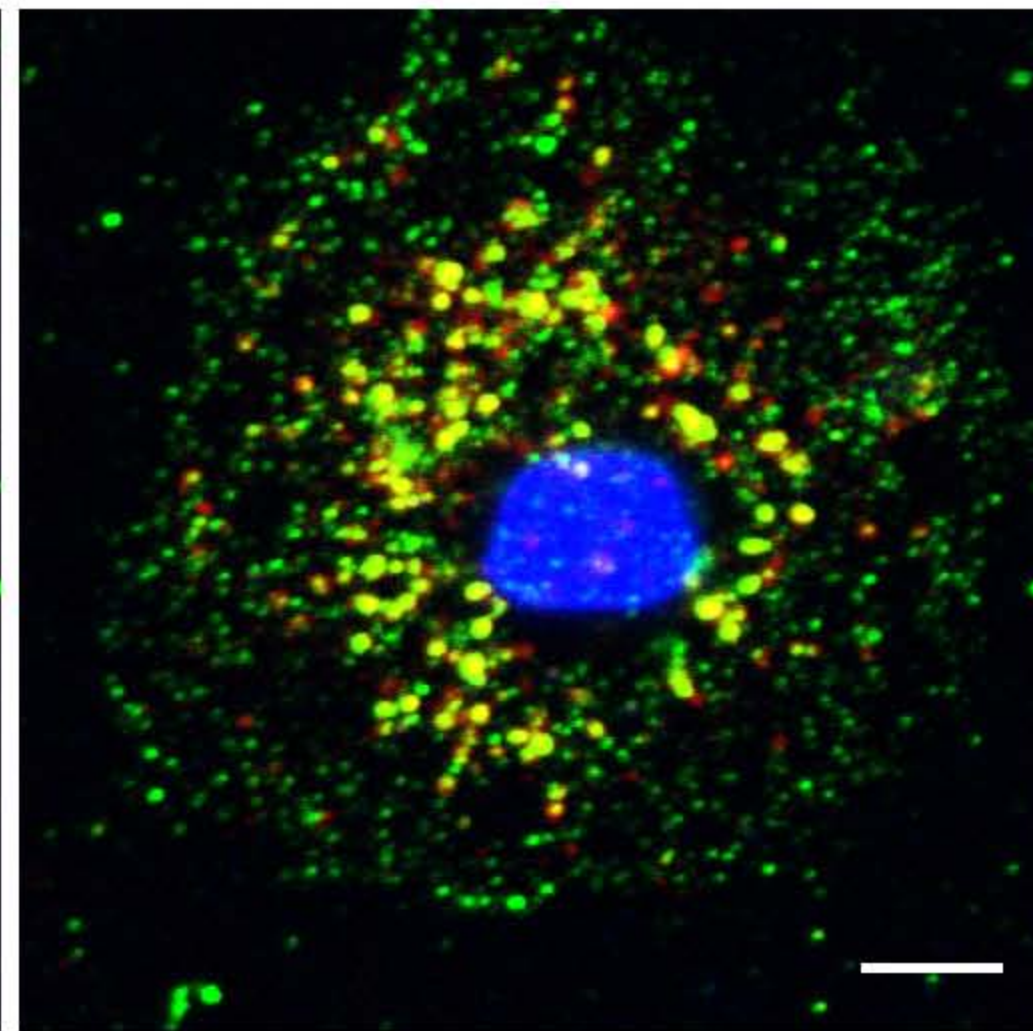
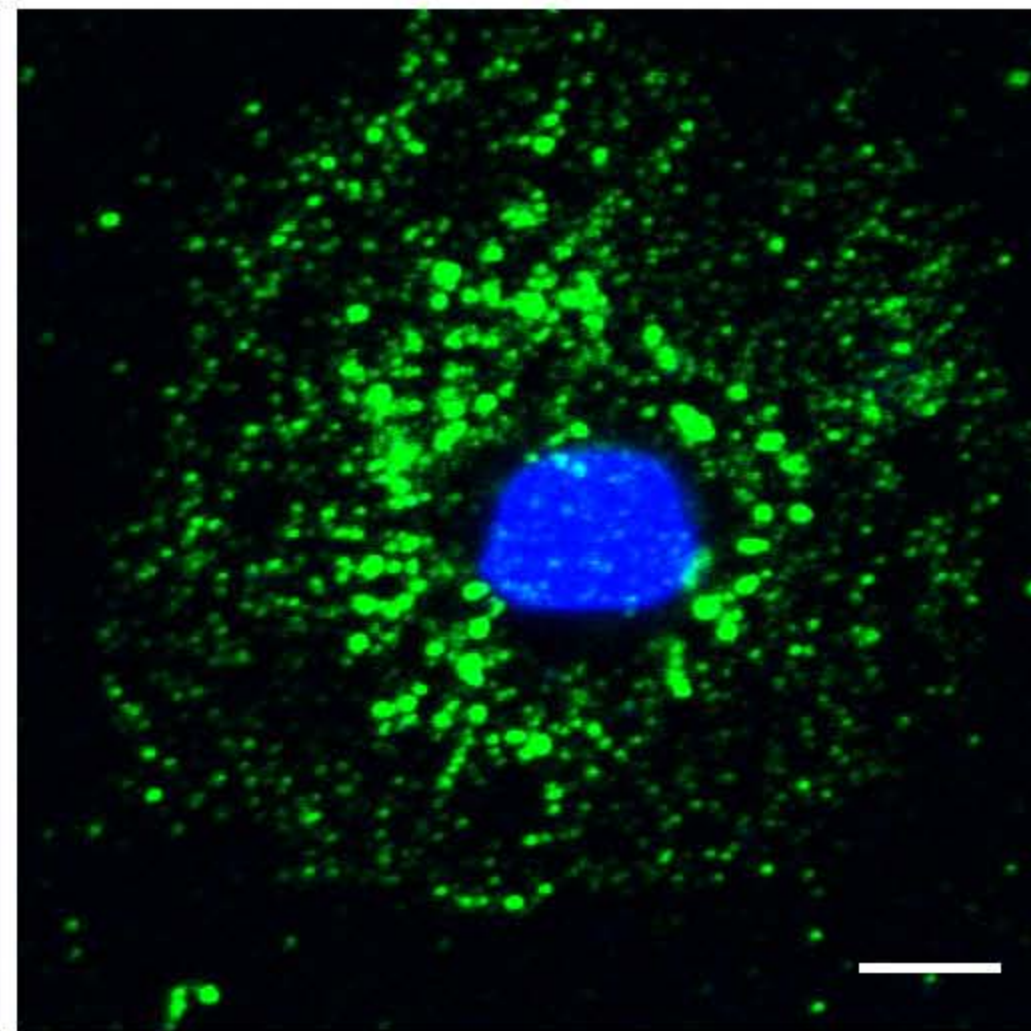
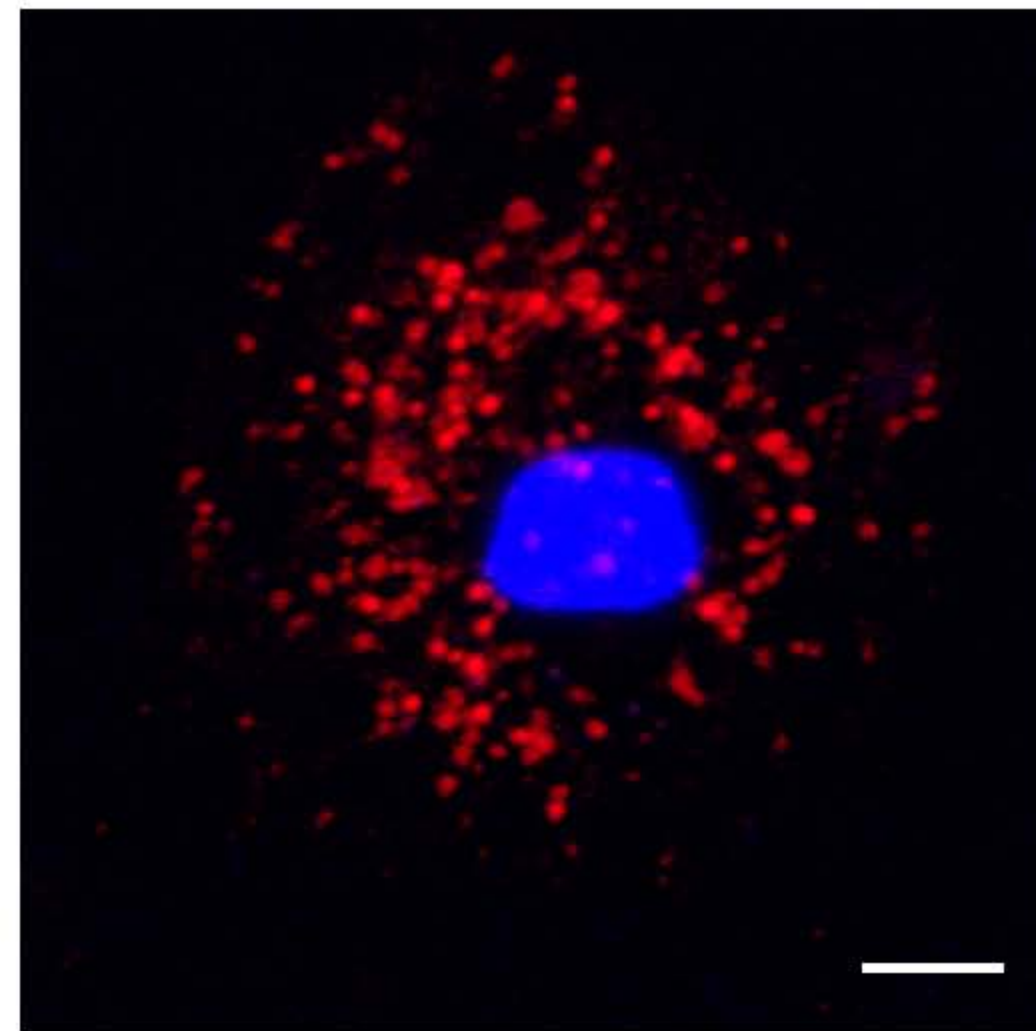


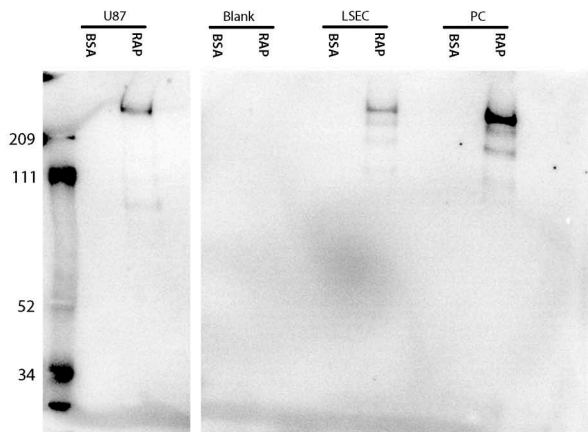
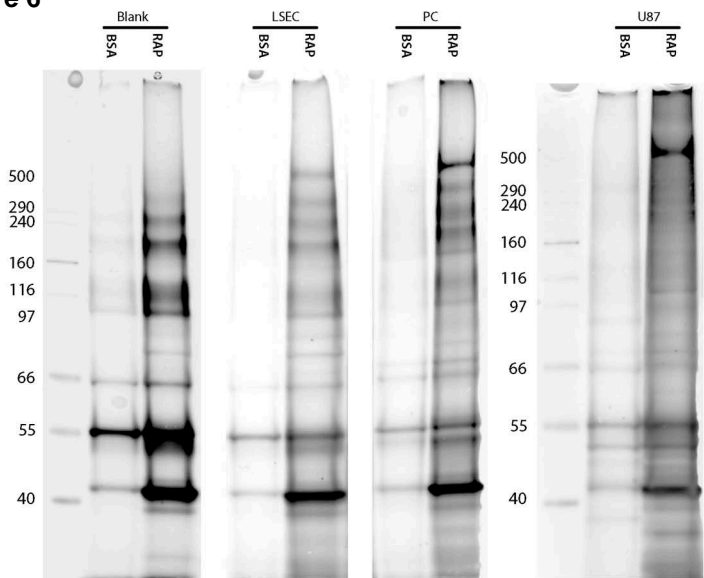
Figure 5

TRITC-FSA

LRP

Merged



**Figure 6**



**Supplementary material - RAP purification**

[Click here to download Supplementary material: Supplementary - RAP purification.doc](#)

**Supplementary material - Mass spectrometry**

**[Click here to download Supplementary material: Supplementary - Mass spectrometry.doc](#)**

**Supplementary material - Identified peptides MS**

[Click here to download Supplementary material: Supplementary - Table 1.doc](#)