



HAL
open science

The low-density lipoprotein receptor-related protein 1 (LRP1) mediates the endocytosis of the cellular prion protein

David R Taylor, Nigel M Hooper

► **To cite this version:**

David R Taylor, Nigel M Hooper. The low-density lipoprotein receptor-related protein 1 (LRP1) mediates the endocytosis of the cellular prion protein. *Biochemical Journal*, 2006, 402 (1), pp.17-23. 10.1042/BJ20061736 . hal-00478699

HAL Id: hal-00478699

<https://hal.science/hal-00478699>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

The low-density lipoprotein receptor-related protein 1 (LRP1) mediates the endocytosis of the cellular prion protein

David R. Taylor and Nigel M. Hooper*

Proteolysis Research Group
Institute of Molecular and Cellular Biology
Faculty of Biological Sciences
and Leeds Institute of Genetics, Health and Therapeutics
University of Leeds
Leeds LS2 9JT
UK

* To whom correspondence should be addressed: tel. +44 113 343 3163; fax. +44 113 343 3167; e-mail: n.m.hooper@leeds.ac.uk

Running title: LRP1 mediates the endocytosis of PrP

Key words: amyloid precursor protein, copper, endocytosis, low-density lipoprotein receptor-related protein-1, prion, receptor associated protein.

Abbreviations used: APP, amyloid precursor protein; GPI, glycosyl-phosphatidylinositol; HSPG, heparin sulphate proteoglycan; KPI, Kunitz-type protease inhibitor; LDL, low-density lipoprotein; LRP, LDL receptor-related protein; PAI-1, plasminogen activator inhibitor-1; PBS, phosphate-buffered saline; PrP^C, cellular form of the prion protein; PrP^{Sc}, infectious form of the prion protein; RAP, receptor associated protein; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor.

Abstract

The cellular prion protein (PrP^C) is located at the surface of neuronal cells in detergent-insoluble lipid rafts, yet is internalised by clathrin-dependent endocytosis. As PrP^C is glycosyl-phosphatidylinositol-anchored, it requires a transmembrane adaptor protein to connect it to the clathrin endocytosis machinery. Using receptor associated protein and siRNA of particular low-density lipoprotein family members, in combination with immunofluorescence microscopy and surface biotinylation assays, we show that the transmembrane low-density lipoprotein receptor-related protein 1 (LRP1) is required for the Cu²⁺-mediated endocytosis of PrP^C in neuronal cells. We show also that another LRP1 ligand that can cause neurodegenerative disease, the Alzheimer's amyloid precursor protein, does not modulate the endocytosis of PrP^C.

Introduction

The prion protein (PrP) is the principal agent responsible for the transmissible spongiform encephalopathies, a group of fatal neurodegenerative diseases including Creutzfeldt-Jakob disease in humans, scrapie in sheep and bovine spongiform encephalopathy in cattle [1]. In these prion diseases the normal cellular form of the prion protein (PrP^C) undergoes a conformational change to the infectious form, PrP^{Sc}. PrP^C is transported through the secretory pathway to the cell surface where it is attached to the outer leaflet of the plasma membrane via a glycosyl-phosphatidylinositol (GPI) anchor and localised in cholesterol- and glycosphingolipid-rich lipid rafts [2, 3]. The N-terminal half of PrP^C contains four octapeptide repeats (PHGG(G/S)WGQ; residues 59-90) that bind Cu²⁺ ions. The physiological importance of Cu²⁺ binding to the octarepeats is evidenced by the finding that exposure of neuronal cells to concentrations (50-100 μM) of Cu²⁺ similar to that in the extracellular spaces of the brain [4, 5] results in the rapid internalisation of PrP^C [6-8]. This metal-dependent endocytosis of PrP^C was abrogated by deletion of the octapeptide repeats or by an insertional mutation within the repeats which is associated with an inherited form of human prion disease [7].

Electron microscopy studies [9, 10], as well as selective pharmacological and molecular disruption experiments [11], have shown that in neuronal cells PrP^C is endocytosed by a clathrin-mediated mechanism. PrP^C moves laterally out of detergent-insoluble lipid rafts into detergent-soluble regions of the plasma membrane prior to its endocytosis via clathrin-coated pits [10, 11]. As PrP^C is GPI anchored and lacks a cytoplasmic domain it cannot interact directly with the clathrin endocytic machinery on the cytoplasmic face of the plasma membrane. Rather, its internalisation requires it to 'piggy-back' on an integral transmembrane protein. The existence of such a transmembrane adaptor protein was first postulated over 10 years ago [9], yet its identity remains unknown. Using mutants of PrP^C that lacked either the octapeptide repeats or the polybasic KKRP tetrapeptide at the N-terminus of the mature protein, we showed that copper binding to the octapeptide repeats promotes dissociation of PrP^C from lipid rafts, while the N-terminal polybasic region mediates its interaction with a transmembrane adaptor protein that engages the clathrin endocytic machinery [11].

The low-density lipoprotein (LDL) receptor family is a group of cell-surface transmembrane proteins that bind a variety of ligands and internalise via clathrin-coated pits [12-14]. The low-density lipoprotein receptor-related protein (LRP1),

along with LRP1B and LRP2 (megalin), are the largest members of this family of endocytic receptors with multiple ligand binding sites, although only LRP1 and LRP1B are highly expressed in neuronal cells [13]. LRP1 is a 600 kDa transmembrane glycoprotein that is cleaved in the trans-Golgi network by furin to generate a 515 kDa α - and an 85 kDa β -subunit which remain non-covalently associated. Several ligands, including apolipoprotein E, amyloid precursor protein (APP) and α 2-macroglobulin, bind to the α -subunit of LRP1 [15, 16].

We hypothesised that LRP1 or LRP1B may be candidates for the transmembrane adaptor protein required by PrP^C to endocytose via clathrin-coated pits in neuronal cells. By selectively blocking the interaction of LDL family members with their ligands using soluble receptor associated protein (RAP) and by the use of siRNA, we show that LRP1, but not LRP1B, is required to mediate the Cu²⁺-stimulated endocytosis of PrP^C in human neuroblastoma SH-SY5Y cells.

Experimental

PrP constructs and cell culture

Insertion of the coding sequence of murine PrP containing a 3F4 epitope tag into pIRES_{neo} (BD Biosciences Clontech, California, U.S.A.) and the generation of SH-SY5Y cells stably expressing the protein have been reported previously [7]. SH-SY5Y cells expressing PrP^C were also stably transfected with a pIRES_{hyg} vector containing the cDNA encoding the Kunitz-type protease inhibitor (KPI) domain containing isoform of APP, APP₇₅₁. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% foetal bovine serum, 50 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were maintained in a humidified incubator at 37°C with 5% CO₂.

RNA interference studies

SH-SY5Y cells expressing PrP^C were seeded into T25 flasks at 70% confluency and incubated with 500 pmols of a 2 μ M Smartpool siRNA solution against LRP1, LRP1B or APP (Dharmacon Inc., Chicago, U.S.A.) complexed with DharmaFECT-1 transfection reagent (Dharmacon Inc.) in serum-free medium. Mock-transfectants were incubated in the presence of DharmaFECT-1 only. After 2 h, serum was added to 10 % (v/v). Cells were incubated for a further 28 h (LRP1 and LRP1B) or 46 h (APP) prior to experimentation.

Cell surface biotinylation endocytosis assay and immunoprecipitation

The cell surface biotinylation endocytosis assay was performed as described previously [7, 11]. Cells were co-incubated with 20 µg/ml recombinant RAP (Merck Biosciences, Nottingham, U.K.) during copper treatment, where indicated. Immunoprecipitation was carried out as described previously [11].

SDS PAGE and western blot analysis

Immunoprecipitated biotinylated complexes were mixed with dissociation buffer (125 mM Tris-HCl, pH6.8, 2% (w/v) sodium dodecyl sulphate, 20% (v/v) glycerol, 100 mM dithiothreitol, bromophenol blue) and boiled for 5 min. Proteins were resolved by electrophoresis through 14.5% polyacrylamide gels and then transferred to Hybond-P polyvinylidene difluoride membrane. The membrane was blocked for 1 h in phosphate-buffered saline (PBS; 1.5 mM KH₂PO₄, 2.7 mM Na₂HPO₄, 150 mM NaCl, pH 7.4) containing 5% (w/v) dried milk powder and 0.1% (v/v) Tween-20, followed by incubation with peroxidase-conjugated streptavidin (1:1000 dilution in PBS containing 0.1% (v/v) Tween-20) for 1 h. Bound peroxidase conjugates were visualised using an enhanced chemiluminescence detection system (Amersham Biosciences, Little Chalfont, U.K.). PrP^C, APP and β-actin were detected using antibodies 3F4, 22C11 and AC15, respectively, with a peroxidase-conjugated rabbit anti-mouse secondary antibody (Sigma-Aldrich, Poole, UK). For detection of LRP1 and LRP1B, cell lysate samples were prepared in non-reducing dissociation buffer. LRP1 was detected with a monoclonal antibody 5A6 (Merck Biosciences), whilst LRP1B was detected using a rabbit polyclonal antibody (a kind gift from Guojun Bu, Washington University School of Medicine, St. Louis, U.S.A.).

Immunofluorescence microscopy

Cells were seeded onto coverslips and grown to 50% confluency. The fate of cell surface PrP^C was monitored by pre-labelling cells with antibody 3F4 for 30 min at 4°C. For endocytosis experiments, cells were incubated for 20 min at 37°C in OptiMEM in the presence or absence of 100 µM CuSO₄ presented as a histidine chelate [7]. Cells were then fixed with 4% (v/v) paraformaldehyde / 0.1% (v/v) glutaraldehyde in PBS for 15 min, permeabilised in PBS containing 0.1% Triton X-100, and then blocked for 1 h in PBS containing 3% (v/v) goat serum or 5% (v/v) fish skin gelatin (Sigma-Aldrich). Coverslips were then incubated overnight at 4°C with either a goat polyclonal antibody directed against LRP1 (N-20; Santa Cruz Biotechnology Inc., Santa Cruz, U.S.A.) or the rabbit polyclonal antibody against

LRP1B. Finally, coverslips were incubated with the appropriate fluorescent probe-conjugated secondary antibodies (Molecular Probes, Eugene, U.S.A.) for 1 h and mounted on slides using fluoromount G mounting medium (SouthernBiotech, Alabama, U.S.A.). Cells were visualised using a DeltaVision Optical Restoration Microscopy System (Applied Precision Inc., Washington, USA). Data were collected from 30-40 0.1 μm thick optical sections, and 3-D datasets were deconvolved using the softWoRx programme (Applied Precision Inc.). The presented images represent individual Z-slices taken from the middle of the cell. Co-localisation of endocytosed PrP^{C} with LDL-family members was performed using Imaris 4.0 (Bitplane AG, Zurich, Switzerland).

Results

RAP reduces the Cu^{2+} -mediated endocytosis of PrP^{C}

To initially test the hypothesis that members of the LDL family of endocytic receptors may act as transmembrane adaptors in the Cu^{2+} -mediated endocytosis of PrP^{C} , potential PrP^{C} /LDL family interactions were inhibited using RAP. RAP is a specialised chaperone that binds tightly to LDL family members in the early secretory pathway to assist correct folding and disulphide bond formation, and to prevent premature association of family members with their ligands [17]. Exogenously applied RAP has been used to universally antagonise binding of ligands to LDL family members [17]. Incubation of SH-SY5Y cells expressing murine PrP^{C} with the 3F4 epitope with 100 μM Cu^{2+} resulted in the rapid endocytosis of cell surface PrP^{C} as shown using surface biotinylation and subsequent trypsin digestion of biotinylated material remaining at the cell surface after 20 min at 37°C [7, 11]. However, in the presence of 20 $\mu\text{g}/\text{ml}$ recombinant human RAP, a concentration used in previous studies [18], the endocytosis of PrP^{C} was significantly reduced (Fig. 1A). Densitometric analysis of multiple blots from three separate experiments revealed that $44 \pm 8\%$ of biotin-labelled PrP^{C} was endocytosed in response to 100 μM Cu^{2+} . However, in cells incubated with RAP only $10 \pm 6\%$ of PrP^{C} was endocytosed in response to 100 μM Cu^{2+} (Fig. 2B), indicating a role for LDL family members in the Cu^{2+} -mediated endocytosis of PrP^{C} .

Knockdown of LRP1, but not LRP1B, by siRNA inhibits the endocytosis of PrP^{C}

Due to its ability to bind to a wide variety of ligands and its neuronal expression, LRP1 was selected as a likely candidate of the LDL family to be involved in the endocytosis of PrP^{C} , along with the more recently discovered LRP1 splice-variant,

LRP1B, which is suggested to share a number of ligands with LRP1, but internalise them at a slower rate [19]. To determine which is involved in the Cu^{2+} -mediated endocytosis of PrP^{C} , SH-SY5Y cells expressing PrP^{C} were incubated with siRNAs directed against either LRP1 or LRP1B for 30 h. The cells were then surface biotinylated, incubated with 100 μM Cu^{2+} followed by trypsin digestion. LRP1 protein expression was absent in cells incubated with the siRNA against LRP1, whilst the siRNA against LRP1B had no effect on LRP1 expression (Fig. 2A). Similarly, the siRNA against LRP1B significantly reduced its expression but had no effect on the expression of LRP1 (Fig. 2A). The amount of PrP^{C} in the cell lysates was not altered by the knockdown of either LRP1 or LRP1B (Fig. 2A). Although prior incubation with siRNA directed against LRP1B had no effect on the Cu^{2+} -mediated endocytosis of PrP^{C} , knockdown of LRP1 significantly reduced the Cu^{2+} -mediated endocytosis of PrP^{C} (Fig. 2B). Densitometric analysis of multiple blots from three separate experiments revealed that only $10 \pm 3\%$ of cell surface PrP^{C} was endocytosed in the cells incubated with the siRNA against LRP1 compared to $44 \pm 4\%$ in the untreated cells (Fig. 2C). There was no significant difference in the amount of PrP^{C} endocytosed in the cells incubated with the siRNAs directed against LRP1B as compared with the untreated cells (Fig. 2C).

The subcellular localisation of LRP1 and LRP1B relative to that of PrP^{C} was also examined. In order to visualise the fate of cell surface PrP^{C} only, cells were pre-labelled with antibody 3F4 prior to incubation in the presence or absence of Cu^{2+} . The cells were then fixed and permeabilised before incubation with antibodies against either LRP1 or LRP1B. Both LRP1 and LRP1B were localised at the cell surface and in intracellular compartments in both Cu^{2+} and non- Cu^{2+} -treated cells (Fig. 3A and B). In cells incubated in the absence of Cu^{2+} , 3F4-labelled PrP^{C} was located exclusively at the cell surface, with little co-localisation with either LRP1 or LRP1B. After Cu^{2+} treatment, a portion of the 3F4-labelled PrP^{C} was endocytosed into intracellular compartments. In these Cu^{2+} -treated cells, co-localisation of PrP^{C} with both LRP1 and LRP1B was seen to increase at the cell surface. However, only LRP1, but not LRP1B, co-localised with intracellular PrP^{C} in Cu^{2+} -treated cells (Fig. 3C), consistent with a role for LRP1 in the Cu^{2+} -mediated endocytosis of PrP^{C} .

Overexpression or knockdown of APP has no effect on the endocytosis of PrP^{C}

As the neuronal protein APP is also endocytosed by LRP1 [20], we examined whether APP might modulate the endocytosis of PrP^{C} . SH-SY5Y cells expressing PrP^{C} were also stably transfected with a pIRES hyg vector containing the cDNA

encoding APP₇₅₁. Immunoblotting of cell lysates revealed that there was an approximately 2-fold increase in APP₇₅₁ expression in these cells above endogenous levels (Fig. 4A). When lysate samples from mock and APP₇₅₁ transfected cells were immunoblotted for PrP^C the levels of PrP^C expression were identical (Fig. 4A). The Cu²⁺-mediated endocytosis of PrP^C in the APP₇₅₁ transfected cells was then examined. There was no difference in the amount of cell surface PrP^C endocytosed in the APP₇₅₁ transfected cells as compared to cells transfected with the empty pIRES_{hyg} vector (Fig. 4B and E). Similarly, there was no change in the amount of PrP^C endocytosed when the non-KPI containing isoform of APP, APP₆₉₅, was overexpressed in the SH-SY5Y cells (data not shown).

To confirm that APP does not interfere with the endocytosis of PrP^C, the expression of APP in the SH-SY5Y cells was knocked down using siRNA. A significant reduction in APP expression ($87 \pm 6\%$) was observed in the cells incubated with the siRNA against APP, with no effect on total PrP^C levels (Fig. 4C). Cells were then surface biotinylated and incubated in the presence or absence of 100 μ M Cu²⁺ for 20 min followed by trypsin digestion. There was no significant difference in the amount of cell surface PrP^C endocytosed in the absence of APP siRNA as compared to the presence of the APP siRNA ($43 \pm 4\%$ and $41 \pm 5\%$ of cell surface PrP^C was endocytosed, respectively) (Fig. 4D and E). Together these data indicate that APP does not modulate the endocytosis of PrP^C by LRP1.

Discussion

To date PrP^C has been reported to bind to several proteins present at the cell surface, including stress-inducible protein 1 [21], neural cell adhesion molecules [22, 23] and the 37 kDa/67 kDa laminin receptor [24]. Of these proteins, only the 37 kDa/67 kDa laminin receptor has been directly implicated in the internalisation of PrP^C [25]. However, the 37 kDa/67 kDa laminin receptor was responsible for the internalisation of only 25 to 50% of membrane-bound recombinant PrP^C [25] and the binding of PrP^C to all these proteins involves regions C-terminal to residue 90, making them unlikely candidates to act as transmembrane adaptors in the Cu²⁺-mediated endocytosis of PrP^C which is dependent on the polybasic region at the extreme N-terminus of the protein [11]. Consistent with the existence of other transmembrane receptors for PrP^C, we show here for the first time that LRP1 is required for the Cu²⁺-mediated endocytosis of PrP^C. By exploiting the fact that ligand binding to LDL-family members can be universally antagonised by soluble RAP [17],

we have shown that soluble RAP significantly reduces the Cu^{2+} -mediated endocytosis of PrP^{C} in SH-SY5Y cells. More selective targeting of individual LDL-family members with siRNAs revealed that LRP1, but not LRP1B, was responsible for facilitating the Cu^{2+} -mediated endocytosis of PrP^{C} in SH-SY5Y cells. We cannot rule out the involvement of other LDL-family members or other as yet unidentified proteins in the endocytosis of PrP^{C} in other cell types.

The involvement of LRP1 in PrP^{C} endocytosis may not be entirely surprising, as over 40 ligands have been reported to bind to this scavenger receptor [14]. For example, LRP1 is an endocytic receptor for another GPI-anchored protein, the urokinase-type plasminogen activator receptor (uPAR). LRP1 internalises uPAR after uPAR has bound to a complex of uPA and plasminogen activator inhibitor-1 (PAI-1) [26, 27]. Like PrP^{C} , uPAR has been localised to lipid rafts [28] and its endocytosis is inhibited by both RAP and LRP1 specific antibodies [13]. Although Cu^{2+} causes PrP^{C} to dissociate from detergent-insoluble lipid rafts [11], it is not known where PrP^{C} initially interacts with LRP1. A recent study demonstrated that LRP1 is capable of transiently associating with lipid rafts [29], thus the initial interaction between PrP^{C} and LRP1 may occur within lipid rafts after the octapeptide repeats become fully loaded with Cu^{2+} and a conformational change in the N-terminus of PrP^{C} [30] brings the polybasic region, critical for the clathrin-dependent endocytosis of PrP^{C} [10, 11] into an orientation that favours interaction either directly or indirectly with the ligand binding domains of LRP1. A recent study suggested that clusters of basic residues are also critical in mediating the interaction between the uPA/PAI-1 complex and LRP1 [31]. It has been postulated that endogenous heparan sulphate proteoglycans (HSPGs) may be involved in the clathrin-dependent endocytosis of PrP^{C} [10]. Interestingly, HSPGs have been implicated in facilitating the binding of many ligands to LRP1 [13], and thus it is possible that cell-surface HSPGs may be involved in the LRP1-dependent endocytosis of PrP^{C} , perhaps through the formation of large multimeric protein complexes.

LRP1 mediates the endocytosis of another protein involved in neurodegenerative diseases, the Alzheimer's APP from which the neurotoxic amyloid- β peptide is proteolytically cleaved [32]. LRP1 has been shown to associate directly with the ectodomain of APP isoforms containing the KPI-domain [33]. When cells were cultured in the presence of RAP the level of cell surface APP was shown to increase and blocking the interaction of APP with LRP1 reduced the production of the amyloid- β peptide [18]. The interaction of both the KPI-containing and non-KPI-containing

isoforms of APP with LRP1 can also occur via the cytoplasmic adaptor protein Fe65, which bridges the cytoplasmic domains of APP and LRP1 [34, 35]. Neither over-expression of KPI-containing or non-KPI-containing APP isoforms nor knockdown of APP by siRNA affected the Cu^{2+} -mediated endocytosis of PrP^{C} . This may be due to PrP^{C} and APP binding to different regions in the ectodomain of LRP1 and/or, that as a scavenger receptor, LRP1 is not limiting in the cell.

In conclusion, we have shown for the first time that LRP1 mediates the endocytosis of PrP^{C} in neuronal cells. Further studies will be required to elucidate whether interaction with LRP1 has a role to play in the conversion of PrP^{C} to PrP^{Sc} .

Acknowledgements

This work was supported by grants from the Medical Research Council (MRC) of Great Britain (G9824728) and the Wellcome Trust (080229 and the Bioimaging Facility, University of Leeds). DRT was in receipt of a studentship from the MRC.

References

- 1 Aguzzi, A. and Polymenidou, M. (2004) Mammalian prion biology: one century of evolving concepts. *Cell* **116**, 313-327
- 2 Campana, V., Sarnataro, D. and Zurzolo, C. (2005) The highways and byways of prion protein trafficking. *Trends Cell Biol.* **15**, 102-111
- 3 Taylor, D. R. and Hooper, N. M. (2006) The prion protein and lipid rafts. *Mol. Membr. Biol.* **23**, 89-99
- 4 Moir, R. D., Atwood, C. S., Huang, X., Tanzi, R. E. and Bush, A. I. (1999) Mounting evidence for the involvement of zinc and copper in Alzheimer's disease. *Eur. J. Clin. Invest.* **29**, 569-570.
- 5 Stockel, J., Safar, J., Wallacen, A. C., Cohen, F. E. and Prusiner, S. B. (1998) Prion protein selectively binds copper(II) ions. *Biochemistry* **37**, 7185-7193
- 6 Pauly, P. C. and Harris, D. A. (1998) Copper stimulates endocytosis of the prion protein. *J. Biol. Chem.* **273**, 33107-33110
- 7 Perera, W. S. S. and Hooper, N. M. (2001) Ablation of the metal ion-induced endocytosis of the prion protein by disease-associated mutation of the octarepeat region. *Curr. Biol.* **11**, 519-523
- 8 Lee, K. S., Magalhaes, A. C., Zanata, S. M., Brentani, R. R., Martins, V. R. and Prado, M. A. (2001) Internalization of mammalian fluorescent cellular prion protein and N-terminal deletion mutants in living cells. *J. Neurochem.* **79**, 79-87.

- 9 Shyng, S. L., Heuser, J. E. and Harris, D. A. (1994) A glycolipid-anchored prion protein is endocytosed via clathrin-coated pits. *J. Cell Biol.* **125**, 1239-1250
- 10 Sunyach, C., Jen, A., Deng, J., Fitzgerald, K. T., Frobert, Y., Grassi, J., McCaffrey, M. W. and Morris, R. (2003) The mechanism of internalization of glycosylphosphatidylinositol-anchored prion protein. *EMBO J.* **22**, 3591-3601
- 11 Taylor, D. R., Watt, N. T., Perera, W. S. and Hooper, N. M. (2005) Assigning functions to distinct regions of the N-terminus of the prion protein that are involved in its copper-stimulated, clathrin-dependent endocytosis. *J. Cell Sci.* **118**, 5141-5153
- 12 Willnow, T. E. (1999) The low-density lipoprotein receptor gene family: multiple roles in lipid metabolism. *J. Mol. Med.* **77**, 306-315
- 13 Nykjaer, A. and Willnow, T. E. (2002) The low-density lipoprotein receptor gene family: a cellular Swiss army knife? *Trends Cell Biol.* **12**, 273-280
- 14 Strickland, D. K., Gonias, S. L. and Argraves, W. S. (2002) Diverse roles for the LDL receptor family. *Trends Endocrinol. Metab.* **13**, 66-74
- 15 Hyman, B. T., Strickland, D. and Rebeck, G. W. (2000) Role of the low-density lipoprotein receptor-related protein in beta-amyloid metabolism and Alzheimer disease. *Arch. Neurol.* **57**, 646-650
- 16 Pietrzik, C. U., Busse, T., Merriam, D. E., Weggen, S. and Koo, E. H. (2002) The cytoplasmic domain of the LDL receptor-related protein regulates multiple steps in APP processing. *EMBO J.* **21**, 5691-5700
- 17 Bu, G. (1998) Receptor-associated protein: a specialized chaperone and antagonist for members of the LDL receptor gene family. *Curr. Opin. Lipidol.* **9**, 149-155
- 18 Ulery, P. G., Beers, J., Mikhailenko, I., Tanzi, R. E., Rebeck, G. W., Hyman, B. T. and Strickland, D. K. (2000) Modulation of beta-amyloid precursor protein processing by the low density lipoprotein receptor-related protein (LRP). Evidence that LRP contributes to the pathogenesis of Alzheimer's disease. *J. Biol. Chem.* **275**, 7410-7415
- 19 Li, Y., Knisely, J. M., Lu, W., McCormick, L. M., Wang, J., Henkin, J., Schwartz, A. L. and Bu, G. (2002) Low density lipoprotein (LDL) receptor-related protein 1B impairs urokinase receptor regeneration on the cell surface and inhibits cell migration. *J. Biol. Chem.* **277**, 42366-42371
- 20 Knauer, M. F., Orlando, R. A. and Glabe, C. G. (1996) Cell surface APP751 forms complexes with protease nexin 2 ligands and is internalized via the low density lipoprotein receptor-related protein (LRP). *Brain Res.* **740**, 6-14

- 21 Zanata, S. M., Lopes, M. H., Mercadante, A. F., Hajj, G. N., Chiarini, L. B., Nomizo, R., Freitas, A. R., Cabral, A. L., Lee, K. S., Juliano, M. A., de Oliveira, E., Jachieri, S. G., Burlingame, A., Huang, L., Linden, R., Brentani, R. R. and Martins, V. R. (2002) Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection. *EMBO J.* **21**, 3307-3316
- 22 Schmitt-Ulms, G., Legname, G., Baldwin, M. A., Ball, H. L., Bradon, N., Bosque, P. J., Crossin, K. L., Edelman, G. M., DeArmond, S. J., Cohen, F. E. and Prusiner, S. B. (2001) Binding of neural cell adhesion molecules (N-CAMs) to the cellular prion protein. *J. Mol. Biol.* **314**, 1209-1225.
- 23 Santuccione, A., Sytnyk, V., Leshchyns'ka, I. and Schachner, M. (2005) Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth. *J. Cell Biol.* **169**, 341-354
- 24 Hundt, C., Peyrin, J. M., Haik, S., Gauczynski, S., Leucht, C., Rieger, R., Riley, M. L., Deslys, J. P., Dormont, D., Lasmezas, C. I. and Weiss, S. (2001) Identification of interaction domains of the prion protein with its 37- kDa/67-kDa laminin receptor. *EMBO J.* **20**, 5876-5886.
- 25 Gauczynski, S., Peyrin, J. M., Haik, S., Leucht, C., Hundt, C., Rieger, R., Krasemann, S., Deslys, J. P., Dormont, D., Lasmezas, C. I. and Weiss, S. (2001) The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *EMBO J.* **20**, 5863-5875.
- 26 Cubellis, M. V., Wun, T. C. and Blasi, F. (1990) Receptor-mediated internalization and degradation of urokinase is caused by its specific inhibitor PAI-1. *EMBO J.* **9**, 1079-1085
- 27 Nykjaer, A., Petersen, C. M., Moller, B., Jensen, P. H., Moestrup, S. K., Holtet, T. L., Etzerodt, M., Thogersen, H. C., Munch, M., Andreasen, P. A. et al. (1992) Purified alpha 2-macroglobulin receptor/LDL receptor-related protein binds urokinase.plasminogen activator inhibitor type-1 complex. Evidence that the alpha 2-macroglobulin receptor mediates cellular degradation of urokinase receptor-bound complexes. *J. Biol. Chem.* **267**, 14543-14546
- 28 Stahl, A. and Mueller, B. M. (1995) The urokinase-type plasminogen activator receptor, a GPI-linked protein, is localized in caveolae. *J. Cell Biol.* **129**, 335-344
- 29 Wu, L. and Gonias, S. L. (2005) The low-density lipoprotein receptor-related protein-1 associates transiently with lipid rafts. *J. Cell. Biochem.* **96**, 1021-1033
- 30 Morante, S., Gonzalez-Iglesias, R., Potrich, C., Meneghini, C., Meyer-Klaucke, W., Menestrina, G. and Gasset, M. (2004) Inter- and intra-octarepeat Cu(II) site geometries in the prion protein: implications in Cu(II) binding cooperativity and Cu(II)-mediated assemblies. *J. Biol. Chem.* **279**, 11753-11759

- 31 Skeldal, S., Larsen, J. V., Pedersen, K. E., Petersen, H. H., Egelund, R., Christensen, A., Jensen, J. K., Gliemann, J. and Andreasen, P. A. (2006) Binding areas of urokinase-type plasminogen activator-plasminogen activator inhibitor-1 complex for endocytosis receptors of the low-density lipoprotein receptor family, determined by site-directed mutagenesis. *FEBS J.* **273**, 5143-5159
- 32 Vardy, E. R. L. C., Catto, A. J. and Hooper, N. M. (2005) Proteolytic mechanisms in amyloid-beta metabolism: therapeutic implications for Alzheimer's disease. *Trends Mol. Med.* **11**, 464-472
- 33 Kounnas, M. Z., Moir, R. D., Rebeck, G. W., Bush, A. I., Argraves, W. S., Tanzi, R. E., Hyman, B. T. and Strickland, D. K. (1995) LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell* **82**, 331-340
- 34 Kinoshita, A., Whelan, C. M., Smith, C. J., Mikhailenko, I., Rebeck, G. W., Strickland, D. K. and Hyman, B. T. (2001) Demonstration by fluorescence resonance energy transfer of two sites of interaction between the low-density lipoprotein receptor-related protein and the amyloid precursor protein: role of the intracellular adapter protein Fe65. *J. Neurosci.* **21**, 8354-8361
- 35 Pietrzik, C. U., Yoon, I. S., Jaeger, S., Busse, T., Weggen, S. and Koo, E. H. (2004) FE65 constitutes the functional link between the low-density lipoprotein receptor-related protein and the amyloid precursor protein. *J. Neurosci.* **24**, 4259-4265

Figure legends

Figure 1. The Cu²⁺-stimulated endocytosis of PrP^C is inhibited by RAP

(A) SH-SY5Y cells expressing PrP^C were surface biotinylated and then either untreated or treated with 20 µg/ml recombinant RAP in the presence or absence of 100 µM Cu²⁺ for 20 min at 37°C. Prior to lysis the cells were incubated with trypsin where indicated to digest cell surface PrP^C. Cells were then lysed and total PrP^C immunoprecipitated from the sample using antibody 3F4 and then subjected to western blot analysis. The biotin-labelled PrP^C fraction was detected with peroxidase-conjugated streptavidin. (B) Densitometric analysis (mean ± S.E.M) of multiple blots (lanes 4 and 5 in panel A) from three separate experiments.

Figure 2. The endocytosis of PrP^C is blocked by siRNA directed against LRP-1 but not LRP-1B

SH-SY5Y cells expressing PrP^C were incubated with a 2 µM solution of the indicated LRP siRNA prepared with DharmaFECT-1 transfection reagent in OptiMEM for 30 h. (A) Cell lysates were immunoblotted for LRP1 expression using Ab 5A6 and LRP1B expression using a goat polyclonal antibody, along with PrP^C and β-actin. (B) Cells were surface biotinylated and treated with or without 100 µM Cu²⁺ for 20 min at 37°C. Prior to lysis the cells were incubated with trypsin where indicated to digest cell surface PrP^C. Cells were then lysed and total PrP^C immunoprecipitated from the sample using antibody 3F4. Samples were then subjected to western blot analysis. The biotin-labelled PrP^C fraction was detected with peroxidase-conjugated streptavidin. (C) Densitometric analysis (mean ± S.E.M) of multiple blots (lanes 5 and 6 in panel B) from three separate experiments. * Students t-test with values of $P < 0.05$ were taken as statistically significant.

Figure 3. LRP1 but not LRP1B co-localises with PrP^C after its Cu²⁺-mediated endocytosis

SH-SY5Y cells expressing PrP^C were seeded onto glass coverslips and grown to 50% confluency. Cells were then pre-incubated with antibody 3F4 at a dilution of 1:1000 in PBS for 30 min at 4°C, washed 3 times in PBS and then incubated for 20 min at 37°C in OptiMEM in either the absence or the presence of 100 µM Cu²⁺. Cells were fixed, permeabilised and then incubated with either (A) a goat polyclonal antibody against LRP1 or (B) a rabbit polyclonal antibody against LRP1B overnight at 4°C. Cells were then incubated with Alexa Fluor 488 conjugated rabbit anti-mouse antibody and then with either Alexa Fluor 594 conjugated rabbit anti-goat (for LRP1)

or Alexa Fluor 594 conjugated goat anti-rabbit (for LRP1B). Cell nuclei were stained using DAPI. Cells were viewed using a Deltavision Optical Restoration Microscopy System. Images are representative of 3 individual experiments. Bar = 10 μm . (C) The co-localisation of intracellular PrP^C, internalised in response to Cu²⁺, with LRP1 or LRP1B was quantified using Imaris 4. The data represents the percentage of intracellular PrP^C-containing vesicles that also stained positive for LRP1 or LRP1B (\pm S.E.M), $n \geq 10$ cells.

Fig. 4. The Cu²⁺-stimulated endocytosis of PrP^C is unaltered either by overexpression or knockdown of APP

(A) SH-SY5Y cells expressing PrP^C were stably transfected with a pIRES_{hyg} vector containing the cDNA encoding APP₇₅₁. Mock transfectants were created by transfection of an empty pIRES_{hyg} vector. Lysates were immunoblotted for APP, PrP^C and β -actin. (B) Cells stably expressing APP₇₅₁ were surface biotinylated and incubated with or without 100 μM Cu²⁺ for 20 min at 37°C. Prior to lysis, the cells were incubated with trypsin to digest cell surface PrP^C. Cells were then lysed and total PrP^C immunoprecipitated from the sample using antibody 3F4 and then subjected to western blot analysis. The biotin-labelled PrP^C fraction was detected with peroxidase-conjugated streptavidin. (C) SH-SY5Y cells expressing PrP^C were incubated for 48 h with a 2 μM solution of the siRNA against APP, prepared with DharmaFECT-1 transfection reagent in OptiMEM. Mock-transfectants were incubated in the presence of DharmaFECT-1 only. Lysates were immunoblotted for APP, PrP^C and β -actin. (D) Endocytosis experiment as described in (B) using the cells incubated in the presence or absence of APP siRNA. (E) Densitometric analysis (mean \pm S.E.M) of multiple blots from three separate experiments performed in (B) and (D).

Figure 1

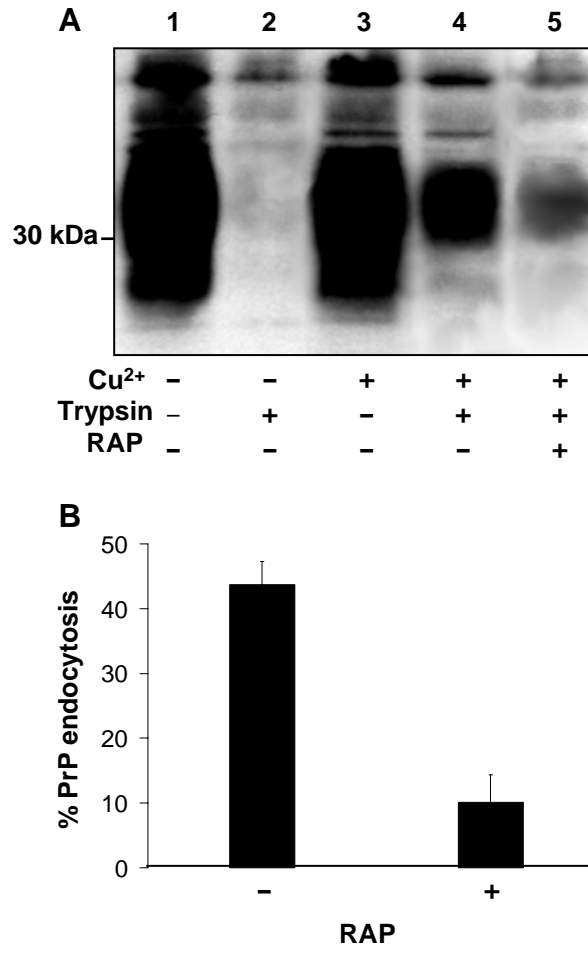


Figure 2

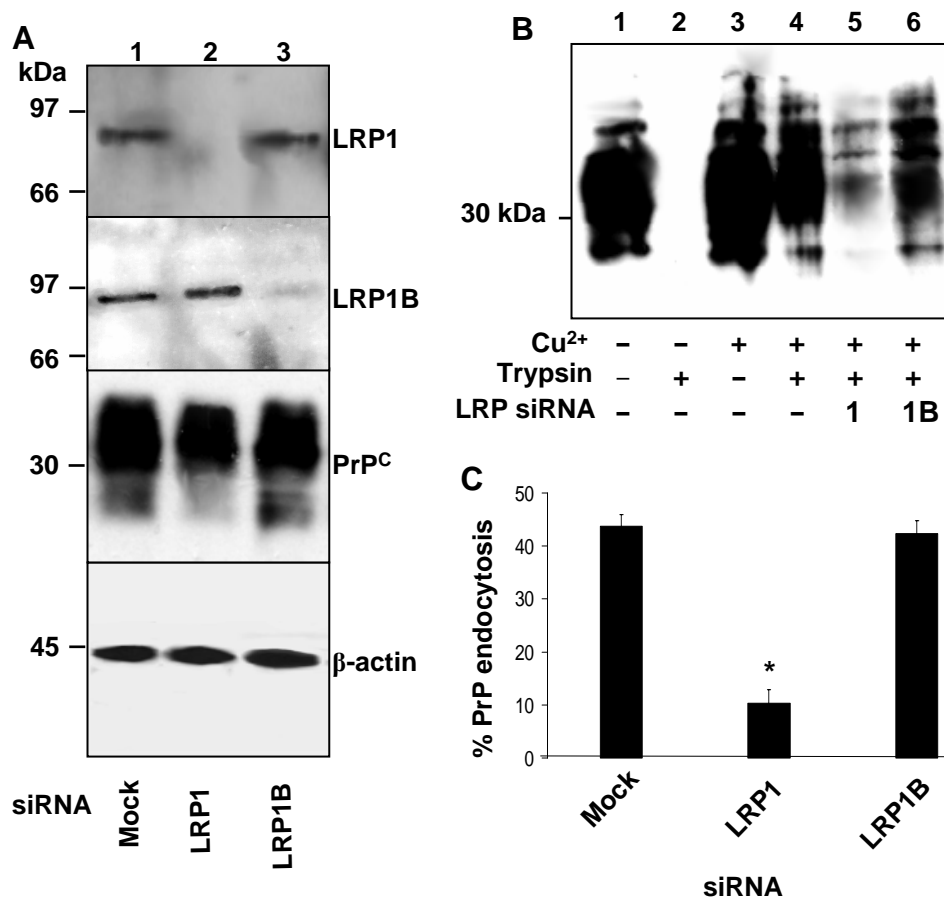


Figure 3

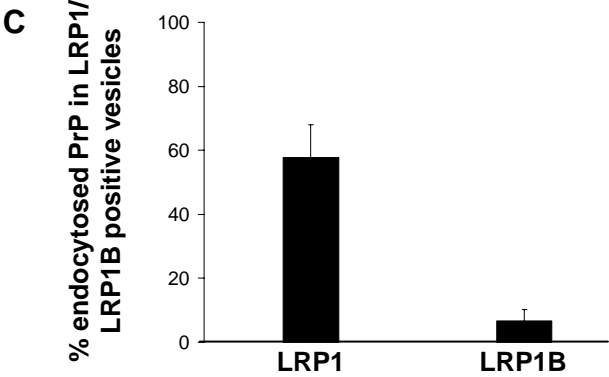
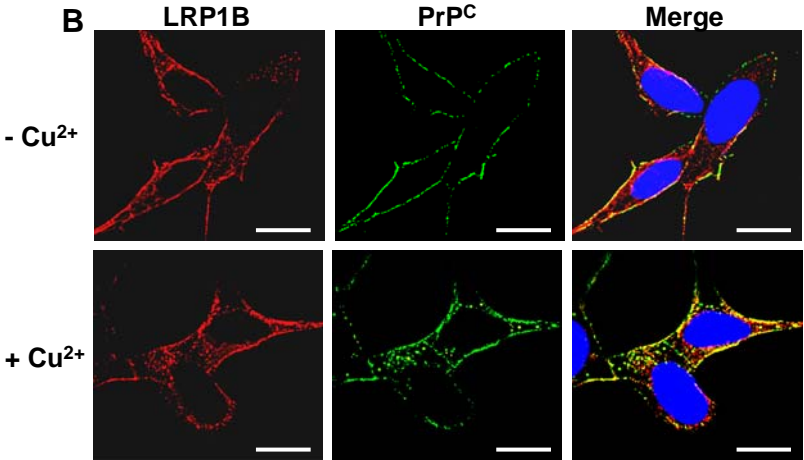
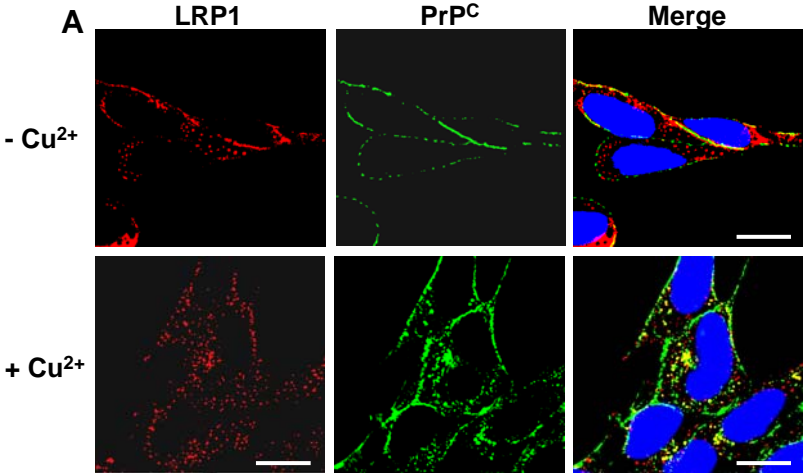


Figure 4

