

## High transcytosis of melanotransferrin (P97) across the blood–brain barrier

Michel Demeule,\* Julie Poirier,\* Julie Jodoin,\* Yanick Bertrand,\* Richard R. Desrosiers,\* Claude Dagenais,\* Tran Nguyen,\* Julie Lanthier,\* Reinhard Gabathuler,† Malcolm Kennard,† Wilfred A. Jefferies,‡ Delara Karkan,† Sam Tsai,† Laurence Fenart,§ Roméo Cecchelli§ and Richard Béliveau\*

\*Laboratoire de Médecine Moléculaire, Département de Chimie-Biochimie, Université du Québec à Montréal-Hôpital Sainte-Justine, Montréal, Québec, Canada

†Biomarin Pharmaceutical (Canada) Inc., Vancouver, British Columbia, Canada

‡Biotechnology Laboratory and Departments of Medicals, Genetics, Microbiology and Zoology, University of British Columbia, Vancouver, British Columbia, Canada

§Laboratoire Mixte Institut Pasteur de Lille-Université d'Artois, Faculté Jean-Perrin, Lens, France

### Abstract

The blood–brain barrier (BBB) performs a neuroprotective function by tightly controlling access to the brain; consequently it also impedes access of proteins as well as pharmacological agents to cerebral tissues. We demonstrate here that recombinant human melanotransferrin (P97) is highly accumulated into the mouse brain following intravenous injection and *in situ* brain perfusion. Moreover, P97 transcytosis across bovine brain capillary endothelial cell (BBCEC) monolayers is at least 14-fold higher than that of holo-transferrin, with no apparent intra-endothelial degradation. This high transcytosis of P97 was not related to changes in the BBCEC monolayer integrity. In addition, the transendothelial transport of P97 was sensitive to temperature and was both concentration- and conformation-

dependent, suggesting that the transport of P97 is due to receptor-mediated endocytosis. In spite of the high degree of sequence identity between P97 and transferrin, a different receptor than the one for transferrin is involved in P97 transendothelial transport. A member of the low-density lipoprotein receptor protein family, likely LRP, seems to be involved in P97 transendothelial transport. The brain accumulation, high rate of P97 transcytosis and its very low level in the blood suggest that P97 could be advantageously employed as a new delivery system to target drugs directly to the brain.

**Keywords:** blood–brain barrier, low-density lipoprotein receptor-related protein, melanotransferrin, P97, transcytosis, transferrin.

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Blood–brain barrier (BBB) permeability is frequently a rate-limiting factor for the penetration of drugs or peptides into the CNS (Pardridge 1999; Bickel *et al.* 2001). The brain is shielded against potentially toxic substances by the BBB, which is formed by brain capillary endothelial cells that are closely sealed by tight junctions. In addition, brain capillaries possess few fenestrae and few endocytic vesicles, compared to the capillaries of other organs (Pardridge 1999). There is little transit across the BBB of large, hydrophilic molecules aside from some specific proteins such as transferrin, lactoferrin and low-density lipoproteins, which are taken up

Address correspondence and reprint requests to Michel Demeule, Laboratoire de Médecine Moléculaire, Université du Québec à Montréal et Hôpital Ste-Justine, C. P. 8888, Succursale Centre-ville, Montréal, Québec, Canada H3C 3P8. E-mail: oncomol@nobel.si.uqam.ca

**Abbreviations used:**  $\alpha$ 2M, activated  $\alpha$ 2-macroglobulin; A $\beta$ <sub>1–40</sub>, amyloid- $\beta$  peptide<sub>1–40</sub>; apoE, apoproteinE; BBB, blood–brain barrier; BBCEC, bovine brain capillary endothelial cell; EC, endothelial cell; EDC, *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide; LDL-R, low-density lipoprotein receptor; LR11, mosaic low-density lipoprotein-related protein; LRP, low-density lipoprotein receptor-related protein; Ltf, lactoferrin; mAb, monoclonal antibody; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RAP, receptor-associated protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPR, signal plasmon resonance; Tf, transferrin;  $V_d$ , volume of distribution; VLDL-R, very low-density lipoprotein receptor.

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by receptor-mediated endocytosis (Dehouck *et al.* 1997; Fillebeen *et al.* 1999; Partridge 1999; Tsuji and Tamai 1999; Kusahara and Sugiyama 2001).

Melanotransferrin is a glycosylated protein that was first named human melanoma antigen P97 when it was found at high levels in malignant melanoma cells (Brown *et al.* 1981, 1982). It possesses a high level of sequence homology (37–39%) with human serum transferrin, human lactoferrin and chicken transferrin (Brown *et al.* 1982; Rose *et al.* 1986). In contrast to transferrin and lactoferrin, no cellular receptor for P97 has been identified. It has also been shown that P97 reversibly binds iron and that it exists in two forms, one of which is bound to cell membranes by a glycosyl phosphatidylinositol anchor while the other form is both soluble and actively secreted (Baker *et al.* 1992; Alemany *et al.* 1993; Food *et al.* 1994). The exact physiological role of membrane-bound P97 remains to be clearly established while the function of secreted P97 is largely unexplored (Sekyere and Richardson 2000).

In the early 1980s, P97 was found to be expressed in much larger amounts in neoplastic cells and fetal tissues than in normal tissues, where it was either not present or expressed only slightly (Woodbury *et al.* 1980, 1981; Brown *et al.* 1981). More recently, it was reported that P97 mRNA is widespread in normal human tissues with the highest levels in the salivary glands (Richardson 2000). In normal human brain, P97 was shown to be present in capillary endothelium (Rothenberger *et al.* 1996) whereas in brain from patients with Alzheimer's disease it was found to be localized in microglia cells associated with senile plaques (Jefferies *et al.* 1996; Yamada *et al.* 1999). Normal serum contains very low levels of P97 (Brown *et al.* 1981), which were reported to increase by five- and sixfold in patients with Alzheimer's disease. From this observation, it was proposed that soluble P97 might be a potential biochemical marker for this disease (Kennard *et al.* 1996; Kim *et al.* 2001).

The fact that P97 levels are very low in normal serum while high P97 levels are reported in senile plaques suggests that P97 may cross the BBB to a greater extent than do other proteins present in the serum. To investigate this hypothesis we evaluated the uptake of P97 in brain following its administration in animals and compared it to those of holo-transferrin and bovine serum albumin (BSA). We further studied and characterized P97 transcytosis using a well-established model of the BBB, consisting of bovine brain endothelial cells (BBCECs) co-cultured with rat astrocytes (Dehouck *et al.* 1992; Fillebeen *et al.* 1999). We also used isolated human brain capillaries for measuring P97 uptake. The results obtained with *in vivo* and *in vitro* models provide evidence for much greater passage of P97 across the BBB than holo-transferrin and suggest that the low-density lipoprotein receptor-related protein (LRP) might be involved in its passage.

## Materials and methods

### Brain uptake and *in situ* brain perfusion

Adult mice weighing 20–30 g were used to measure brain uptake of P97. C57BL/6 (male, female) mice were obtained from in-house breeding using mice originally from Charles River (Montreal, Quebec, Canada). The mice were anesthetized with intraperitoneal (i.p.) injection of ketamine (120 mg/kg) and xylazine (10 mg/kg). To measure the brain uptake of [<sup>125</sup>I]P97, mice were each given approximately 4 pmol of [<sup>125</sup>I]P97, [<sup>125</sup>I]BSA or human [<sup>125</sup>I]holo-transferrin in 200 µL of injection solution through the jugular vein. After 1 h, animals were killed and perfused with buffer via ascending aorta. The serum and brain samples were collected and the levels of radioactivity were measured. *In situ* brain perfusion was performed as previously described by Dagenais *et al.* (2000) using CD<sup>-1</sup> mice from Charles River. Briefly, the right hemisphere of the brain was perfused with 10 nM of [<sup>125</sup>I]P97 or [<sup>125</sup>I]holo-transferrin in Krebs–bicarbonate buffer (pH 7.4 with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at a flow rate of 2.5 mL/min for 10 min) via a catheter inserted in the right common carotid artery following ligation of the external branch. After 10 min of perfusion, the brain was further perfused for 30 s with Ringer/HEPES solution (150 mM NaCl, 5.2 mM KCl, 2.2 mM CaCl<sub>2</sub>, 0.2 mM MgCl<sub>2</sub>, 6 mM NaHCO<sub>3</sub>, 5 mM HEPES, 2.8 mM glucose, pH 7.4), to wash the excess of either [<sup>125</sup>I]-proteins. Mice were decapitated to terminate perfusion and the right hemisphere was isolated on ice before being subjected to capillary depletion (Triguero *et al.* 1990). Aliquots of homogenates, supernatants, pellets and perfusates were taken to measure their contents in [<sup>125</sup>I]-proteins by TCA precipitation and to evaluate their apparent volume of distribution ( $V_d$ ). All animal experiments were evaluated and approved by the Institutional Comity for Good Animal Practices (UQAM, Montreal, Quebec, Canada).

### Preparation of astrocytes and BBCEC culture

Primary cultures of mixed astrocytes were prepared from newborn rat cerebral cortex (Dehouck *et al.* 1992). Briefly, after removing the meninges, the brain tissue was gently forced through an 82-µm nylon sieve. Astrocytes were plated on six-well microplates at a concentration of  $1.2 \times 10^5$  cells/mL in 2 mL of optimal culture medium [Dulbecco's modified Eagle medium (DMEM)] supplemented with 10% fetal heat-inactivated calf serum. The medium was changed twice a week. BBCECs were cultured in the presence of DMEM supplemented with heat-inactivated 10% (v/v) horse and 10% calf sera, 2 mM glutamine, 50 µg/mL gentamycin, and 1 ng/mL basic fibroblast growth factor, added every other day.

### Blood–brain barrier model

The *in vitro* model of BBB was established by using a co-culture of BBCECs and newborn rat astrocytes as previously described (Dehouck *et al.* 1992). Briefly, prior to cell co-culture, plate inserts (Millicell-PC 3.0 µm; 30-mm diameter; Millipore, Bedford, MA, USA) were coated on the upper side with rat tail collagen. They were then set in the six-multiwell microplates containing astrocytes prepared as described above, and BBCECs were plated on the upper side of the filters in 2 mL of co-culture medium. BBCEC medium was changed three times a week. Under these conditions, differentiated

BBCECs formed a confluent monolayer 7 days later. Experiments were performed 5–7 days after confluence was reached. The number of cells at confluence was 400 000 cells/4.2 cm<sup>2</sup> or 90 µg of protein/4.2 cm<sup>2</sup>, as evaluated by a micro-BCA assay (Pierce, Rockford, IL, USA).

#### Sucrose permeability

The permeability coefficient of sucrose was measured to verify the integrity and tightness of the BBCEC monolayers. Brain endothelial cell monolayers grown on inserts were transferred to six-well plates containing 2 mL of Ringer/HEPES per well (basolateral compartment). In each apical chamber, the culture medium was replaced by Ringer/HEPES containing 74 nM [<sup>14</sup>C]sucrose (0.05 µCi/assay; NEN, Boston, MA, USA). At different times, the insert was transferred into other well. At the end of the experiment, the amount of radiotracer in basolateral compartments was measured in a liquid scintillation counter. The permeability coefficient (Pe) for sucrose was calculated as previously described by Dehouck *et al.* (1992), using filters either coated with endothelial cells or uncoated. Briefly, the results were plotted as the clearance of [<sup>14</sup>C]sucrose (µL) as a function of time (min). The permeability coefficient (Pe) was calculated as:  $1/Pe = (1/PS_{\text{t}} - 1/PS_{\text{f}}) / \text{filter area (4.2 cm}^2\text{)}$ , where PS<sub>t</sub> is the permeability × surface area of a filter of the co-culture; PS<sub>f</sub> is the permeability of a filter coated with collagen and astrocytes plated on the bottom side of the filter.

#### Iodination of proteins

P97 from Biomarin Pharmaceutical (Vancouver, Canada), bovine holo-transferrin and bovine lactoferrin from Sigma (Oakville, Canada) were radioiodinated with standard procedures using an iodo-beads kit and D-salt Dextran desalting columns from Pierce. A ratio of two iodo-beads was used for each protein molecule. Beads were washed twice with 3 mL of phosphate-buffered saline (PBS) on a Whatman filter and resuspended in 60 µL of PBS. Na<sup>125</sup>I (1 mCi) from Amersham-Pharmacia Biotech (Baie d'Urfé, Quebec, Canada) was added to the bead suspension for 5 min at room temperature. Iodination of each protein was initiated by the addition of 100 µg of protein (80–100 µL) diluted in 0.1 M phosphate buffer solution, pH 6.5. After incubation for 10 min at room temperature, iodo-beads were removed and the supernatants were applied onto a desalting column pre-packed with 5 mL of cross-linked dextran from Pierce. <sup>125</sup>I-proteins were eluted with 10 mL of PBS. Fractions of 0.5 mL were collected and the radioactivity in 5 µL of each fraction was measured. Fractions corresponding to <sup>125</sup>I-proteins were pooled and dialyzed against Ringer/HEPES, pH 7.4.

#### Transcytosis and binding experiments

Transcytosis experiments were performed as follows. One insert covered with BBCECs was set into a six-well microplate with 2 mL of Ringer–HEPES and was preincubated for 2 h at 37°C. [<sup>125</sup>I]P97 (0.5–1.5 µCi/assay) was then added to the upper side of the insert. At various times, the insert was sequentially transferred into a fresh well to avoid possible reendocytosis of P97 by the abluminal side of the BBCECs. At the end of the experiment, [<sup>125</sup>I]P97 was quantitated in 500 µL of the lower chamber of each well by TCA precipitation. We also measured P97 in 50 µL of the lower chamber

of each well by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970). Proteins were separated on 7.5% acrylamide gels, stained with Coomassie blue, dried and analyzed by densitometry. For the binding experiments, cells were treated with or without saponin and leupeptin to permeabilize cellular membranes and gain access to all P97 receptors with minimal degradation, as previously described by Descamps *et al.* (1996). Briefly, BBCECs were pre-incubated for 1 h at 25°C in Ringer–HEPES solution supplemented with NaHCO<sub>3</sub> (2 g/L), 0.5% saponin (wt/vol), 0.1% BSA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 µg/mL leupeptin. The cells were washed in DMEM (2 × 10 min) containing 25 mM NaOAc (pH 5.4), 1 mM PMSF and 1 µg/mL leupeptin (medium A). Binding experiments were carried out for 2 h at 4°C in Ringer–HEPES in the presence of [<sup>125</sup>I]P97 (25 nM) and increasing concentrations of unlabeled P97. At the end of the incubation, filters were gently washed four times with 2 mL of PBS. Then the radioactivity associated with endothelial cells was determined by removing the coated-filter from the culture insert and measuring radioactivity in a gamma counter.

#### P97 accumulation in human brain capillaries

Human brain capillaries were isolated by a procedure previously described (Dallaire *et al.* 1991; Demeule *et al.* 2001). A rapid filtration technique was used to measure the accumulation of [<sup>125</sup>I]P97 in human brain capillaries. Accumulation of [<sup>125</sup>I]P97 was measured at 37°C for 1 h in isolated human brain capillaries (100 µg/assay). The incubation medium contained [<sup>125</sup>I]P97 and a final concentration of 100 nM P97 in Ringer–HEPES solution. The accumulation of [<sup>125</sup>I]P97 was performed in the presence or absence of 5 µM of unlabeled P97, holo-transferrin or lactoferrin. After incubation, the accumulation was stopped by addition of 1 mL of ice-cold stop solution (150 mM KCl, 0.1% BSA and 5 mM HEPES, pH 7.5). The suspension was filtered under vacuum through a 0.45-µm pore size Millipore filter. The filter was rinsed with 8 mL of stop solution, and the radioactivity was counted. Non-specific binding of radioactivity to the capillaries was determined by addition of the ice-cold stop solution to the capillaries before adding the incubation medium. This value was subtracted from the values obtained following a 1-h incubation.

#### BIAcore analysis

The mAb L235 (ATCC, Richmond, VA, USA) was covalently coupled to a CM5 sensor chip via primary amine groups using the *N*-hydroxysuccinimide (NHS)/*N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide (EDC) coupling agent as previously described (Johnsson *et al.* 1991). Briefly, the carboxymethylated dextran was first activated with 50 µL of NHS/EDC (50 mM/200 mM) at a flow rate of 5 µL/min. The mAb L235 (5 µg) in 10 mM acetate buffer, pH 4.0 was then injected and the unreacted NHS-esters were deactivated with 35 µL of 1 M ethanolamine hydrochloride, pH 8.5. Approximately 8000–10 000 relative units of mAb 235 were immobilized on the sensor chip surface. Ringer–HEPES buffer was used as the eluent buffer to monitor the signal plasmon resonance (SPR). P97 diluted in the same eluent buffer was boiled for various lengths of time, cooled to room temperature and injected onto the sensor chip surface. The SPR obtained was compared to that of unboiled P97.

## Results

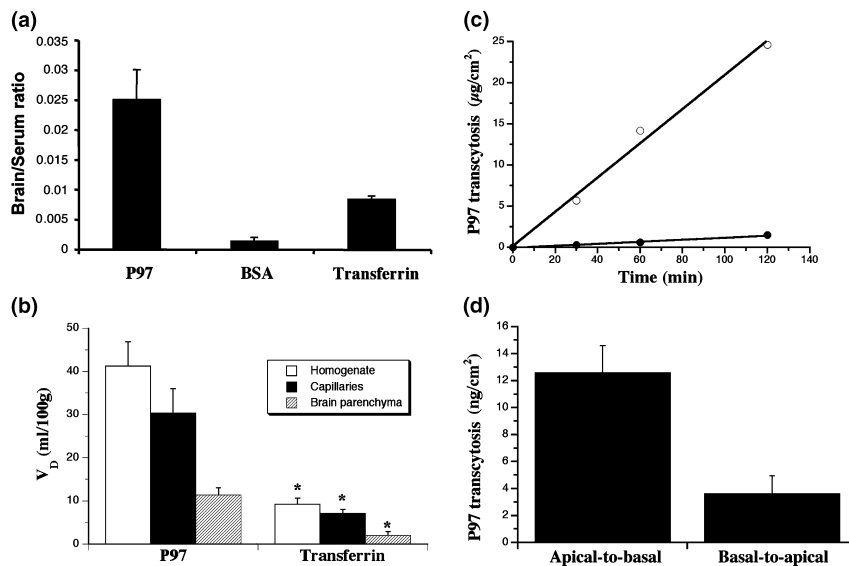
### Uptake of P97 in brain and transcytosis of P97 using BBCEC monolayers

We first evaluated the brain uptake of human [ $^{125}$ I]P97 in mice, 1 h after intravenous (i.v.) injection and compared it to that obtained for [ $^{125}$ I]BSA or human [ $^{125}$ I]transferrin (Fig. 1a). The brain/serum ratios for holo-P97, BSA and holo-transferrin are, respectively, 0.025, 0.002 and 0.008 indicating a higher brain accumulation for P97. To determine whether this observation is related to a greater rate of brain penetration, we measured the apparent  $V_d$  of P97 and transferrin by *in situ* brain perfusion in mice (Fig. 1b). After a 10-min perfusion, the apparent  $V_d$  for both proteins was calculated for the whole brain homogenates as well as for brain capillaries and brain parenchyma. Under these conditions the apparent  $V_d$  of transferrin in the brain parenchyma is 2.0 mL/100 g which is slightly higher than the brain  $V_d$  for the vascular marker [ $^{14}$ C]inulin at 1.7 mL/100 g (data not shown). Importantly, the apparent  $V_d$  of P97 in the brain parenchyma is 11.4 mL/100 g, 5.7-fold higher than for transferrin, indicating a greater passage through brain capillaries. To further investigate the transport of P97 across the BBB, the passage of [ $^{125}$ I]P97 across an *in vitro* model

of the BBB was measured at 37°C and at 4°C (Fig. 1c). A dramatic reduction in the transport from the apical to the basolateral surface of BBCEC monolayers of [ $^{125}$ I]P97 is observed at 4°C, suggesting that the transcytosis of P97 requires an active mechanism. Transcytosis of [ $^{125}$ I]P97 at 37°C was measured both in the apical-to-basolateral direction and in the basolateral-to-apical direction across BBCEC monolayers to ascertain any vectorial transport of P97 (Fig. 1d). After 2 h, [ $^{125}$ I]P97 transport is about 3.5-fold higher when measured in the apical-to-basolateral direction, suggesting preferential transport of P97 towards the brain.

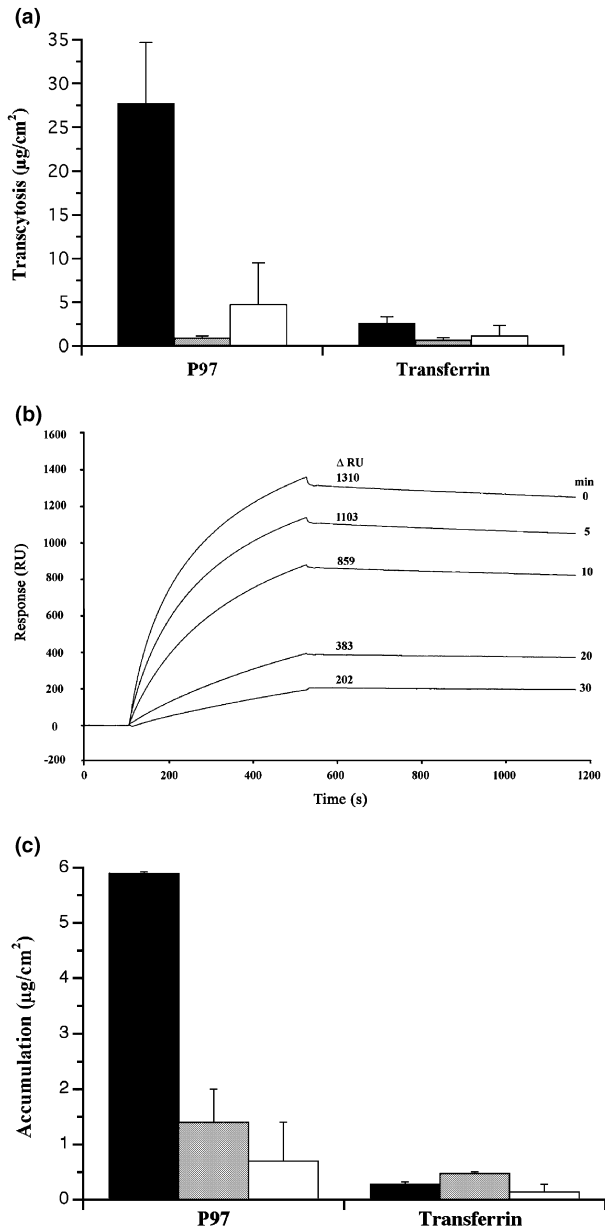
### Efficiency of P97 transcytosis

We examined the efficiency of P97 transcytosis by comparing the passage of both holo-P97 and bovine holo-transferrin under identical conditions (Fig. 2a). Transport of P97 from the apical to the basolateral surface of ECs is much higher than for transferrin at 37°C (Fig. 2a). Heat-denaturation reduced the passage of both P97 and holo-transferrin through the BBCEC monolayers, indicating that their transcytosis is conformation-dependent. As P97 is resistant to heat denaturation in Ringer-HEPES solution, it was necessary to determine the denaturing conditions (Fig. 2b). As no enzymatic activity has yet been defined for this protein, the



**Fig. 1** Brain uptake and transcytosis of P97 in brain. (a) Uptake of [ $^{125}$ I]P97, [ $^{125}$ I]BSA and [ $^{125}$ I]holo-transferrin was evaluated 1 h after i.v. injection. The brain/serum ratio of radioactivity is compared across the three compounds. Results represent means  $\pm$  SD ( $n = 3$ ). (b) *In situ* brain perfusion was performed with human [ $^{125}$ I]P97 or [ $^{125}$ I]holo-transferrin at 10 nM in Krebs–bicarbonate buffer (pH 7.4) for 10 min. The volume of distribution ( $V_d$ ) of [ $^{125}$ I]-proteins was calculated in whole brain homogenate (white bars), in brain capillaries (solid bars) and in brain parenchyma (hatched bars) after isolation of the right hemisphere and capillary depletion. Results represent means  $\pm$  SE ( $n = 8$  mice for P97;  $n = 6$  mice for transferrin). Statistically significant differences between P97 and transferrin corresponding  $V_d$  are

indicated by \* $p < 0.01$  (Student's *t*-test). (c) Transcytosis of [ $^{125}$ I]P97 across BBCEC monolayers was performed at 37°C ( $\circ$ ) and 4°C ( $\bullet$ ). [ $^{125}$ I]P97 (0.5–1.5  $\mu\text{Ci}/\text{assay}$ ) at a final concentration of 1 mg/mL was added to the upper side of the cell-covered filter. At the end of the experiment, [ $^{125}$ I]P97 was assessed in the lower or upper chambers of each well by TCA precipitation. The results of a representative experiment are shown ( $n = 4$ ). (d) Preferential transport of P97 across the BBCEC monolayers. Apical-to-basal and basal-to-apical transport of [ $^{125}$ I]P97 (0.5–1.5  $\mu\text{Ci}/\text{assay}$ ) at a final concentration of 25 nM was measured for 2 h at 37°C. At the end of the experiment, [ $^{125}$ I]P97 was assessed in the lower or upper chambers of each well by TCA precipitation. Results represent means  $\pm$  SD ( $n = 4$ ).



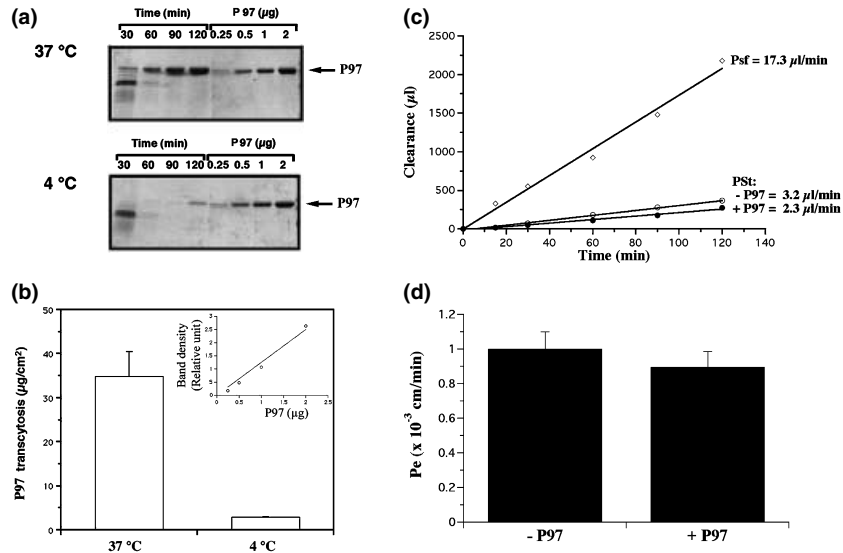
conformation of the protein was assessed using the biological interaction analysis in real-time between P97 and the monoclonal antibody (mAb) L235, which recognizes a conformational epitope on P97. For this analytical approach, mAb L235 was immobilized on the surface of a sensor chip and exposed to native P97 as well as to P97 which had been boiled for 5, 10, 20 or 30 min. The surface plasmon resonance signal generated by the interaction between P97 and immobilized mAb L235 decreased from 1310 relative unit (RU) to 202 RU, indicating that the protein should be boiled for at least 30 min to lose 85% of its ability to interact with mAb L235. Thus, the difference between the passage measured with native and denatured proteins (Fig. 2a), which corresponds to their conformation-dependent transcytosis is

**Fig. 2** Transcytosis and accumulation of P97 and transferrin in BBCEC monolayers. (a) Transcytosis experiments were performed at 37°C (solid bars) or 4°C (white bars). [<sup>125</sup>I]P97 or bovine [<sup>125</sup>I]holo-transferrin (0.5–1.5 µCi/assay) at a final concentration of 1 mg/mL was added to the upper side of the cell-covered filter. At the end of the experiment, radiolabeled proteins were measured in the lower chamber of each well by TCA precipitation. Results represent means ± SE (*n* = 4). Control experiments were also performed at 37°C with denatured [<sup>125</sup>I]P97 or bovine [<sup>125</sup>I]holo-transferrin boiled for 30 min (grey bars; *n* = 2; means ± SD). (b) Biospecific interaction analysis was performed with native or boiled P97 for the indicated times. mAb L235 (5 µg) was immobilized on a sensor chip (CM5) using standard procedures incorporating NHS, EDC and ethanolamine. Native and boiled P97 (5–30 min) diluted at 1 mg/mL in Ringer-HEPES was cooled and injected into the BIAcore. The surface plasmon resonance response obtained for native P97 and boiled P97 was plotted (in relative units (RU)) as a function of time. (c) The accumulation of both proteins into BBCECs were also measured. Briefly, after incubation at 37°C (solid bars) or 4°C (white bars) with either [<sup>125</sup>I]-protein, cells were washed four times with cold PBS. Accumulation of both denatured proteins (grey bars) was also measured at 37°C. Filters were then removed, and the radioactivity associated with the cells was quantified (*n* = 3).

14-fold higher for P97 (26.8 µg/cm<sup>2</sup>) than for bovine holo-transferrin at 37°C (1.9 µg/cm<sup>2</sup>). In addition to transcytosis, the intracellular accumulation at 37°C and the membrane binding at 4°C of P97 are also higher than the corresponding values for bovine holo-transferrin (Fig. 2c). When the accumulation values obtained with denatured proteins are subtracted from those of native proteins, the accumulation of P97 in BBCECs is 4.5 µg/cm<sup>2</sup>, whereas no significant accumulation is observed for bovine transferrin. These results on transcytosis and accumulation show that the P97 transport system has much greater capacity than has the transferrin transport system.

#### P97 stability and BBCEC monolayer integrity following transendothelial transport

To examine P97 integrity after transcytosis at 37°C and 4°C, 50 µL of the lower compartment of the wells were recovered after 30, 60, 90 and 120 min. Proteins were then separated by SDS-PAGE and visualized by gel staining (Fig. 3a). Time-dependent transcytosis of recombinant P97 is observed, with no apparent degradation. Transcytosis of this protein is much higher when the experiment is performed at 37°C than at 4°C. The low molecular weight proteins observed at 30 min are only serum proteins remaining in the assay. Furthermore, the gels were scanned and the amount of P97 that passed through the BBCEC monolayers was evaluated using known quantities of P97 (Fig. 3b). The total amount of intact P97 after transendothelial transcytosis is 35 µg/cm<sup>2</sup>, which is very similar to the amount shown in Fig. 2(a) after TCA precipitation, indicating that the iodination of P97 does not interfere with its transcytosis.



**Fig. 3** Stability of P97 and integrity of the BBCEC monolayers following P97 transcytosis. (a) Transcytosis experiments were performed at 37°C and 4°C by adding P97 (1 mg/mL) to the upper compartment. At the end of the experiment, 50 µL from each lower chamber was used for SDS-PAGE. After electrophoresis, the gels were stained with Coomassie blue. A standard curve was also made with known amounts of recombinant P97 (0–2 µg). (b) The gels were dried and scanned to quantify the amount of intact P97 that crossed the BBCEC monolayers at 37°C and 4°C. Represent means  $\pm$  SD ( $n = 3$ ). (c) Effect of P97 on sucrose permeability of BBCE cell monolayers

As P97 is transported much faster than is transferrin, the permeability to [<sup>14</sup>C]sucrose was measured in the presence of a high concentration of P97 (Fig. 3c). No significant increase in the clearance of sucrose is detectable in the presence of P97. The permeability coefficient for sucrose in the presence of P97 is  $1.0 \pm 0.1 \times 10^{-3}$  cm/min, not significantly different from the value of  $0.9 \pm 0.1 \times 10^{-3}$  cm/min measured in the absence of P97 (Fig. 3d). These data indicate that the rapid passage of P97 is unrelated to changes in the integrity of BBCEC monolayers.

#### P97 binding on BBCEC monolayers

Previous studies have shown that the majority of transferrin receptors are intracellular and that saponin treatment is needed to permeabilize the cellular membranes in order to gain access to all receptors (Descamps *et al.* 1996). Thus, to increase the accessibility of intracellular P97 binding sites, BBCECs were treated with saponin (Fig. 4a). This permeabilization of ECs increased the amount of [<sup>125</sup>I]P97 bound to BBCECs by fourfold. Moreover, the binding of [<sup>125</sup>I]P97 after saponin treatment was decreased in the presence of unlabeled P97 (Fig. 4b). A 200-fold molar excess of unlabeled P97 inhibited radiolabel binding by approximately 50%, suggesting that the interaction of P97 with ECs is saturable. Values for specific P97 binding were calculated by subtracting the non-specific binding of P97 measured in the

co-cultured with astrocytes. The passage of [<sup>14</sup>C]sucrose was measured with filters (◇) or with filters coated with BBCE cells in the absence (○) or in the presence of P97 1 mg/mL (●). One representative experiment is shown. The results were plotted as the sucrose clearance (µL) as a function of time (min). (d) The sucrose permeability coefficient (Pe) was determined in the presence (+ P97) or in the absence (– P97) of P97, and was calculated as described in the Materials and Methods section. Results represent means  $\pm$  SD ( $n = 3$ ).

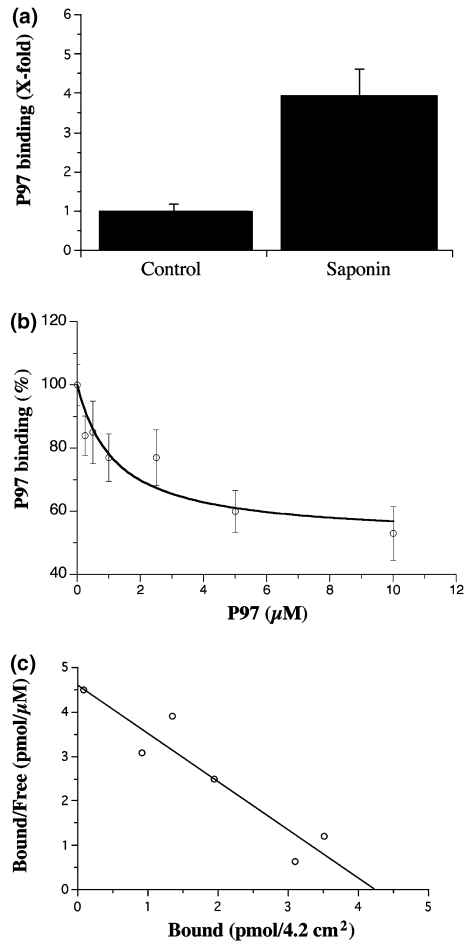
presence of a high concentration of unlabeled P97 and are expressed in a Scatchard plot (Fig. 4c). Analysis of this plot is consistent with a single-binding site for P97 with a  $K_d$  of about 1 µM and  $4 \times 10^6$  sites/cell.

#### Effect of P97 and transferrin on [<sup>125</sup>I]P97 transcytosis

To establish whether this P97 transport was saturable, and whether it involved the transferrin receptor, apical-to-basal transport of [<sup>125</sup>I]P97 across BBCEC monolayers was measured in the presence of a 200-fold molar excess of P97, bovine holo-transferrin or human holo-transferrin (Fig. 5). An excess of unlabeled P97 reduced the transport of [<sup>125</sup>I]P97 by 69% (Fig. 5a), whereas the presence of either bovine or human holo-transferrin had no impact (Fig. 5b). This indicates that P97 transcytosis is a saturable process that does not employ the transferrin receptor. Furthermore, this assumption is supported by the fact that mAb OX-26, which binds to the transferrin receptor, does not significantly reduce P97 transcytosis as compared to transcytosis measured in the presence of non-specific IgGs (Fig. 5c).

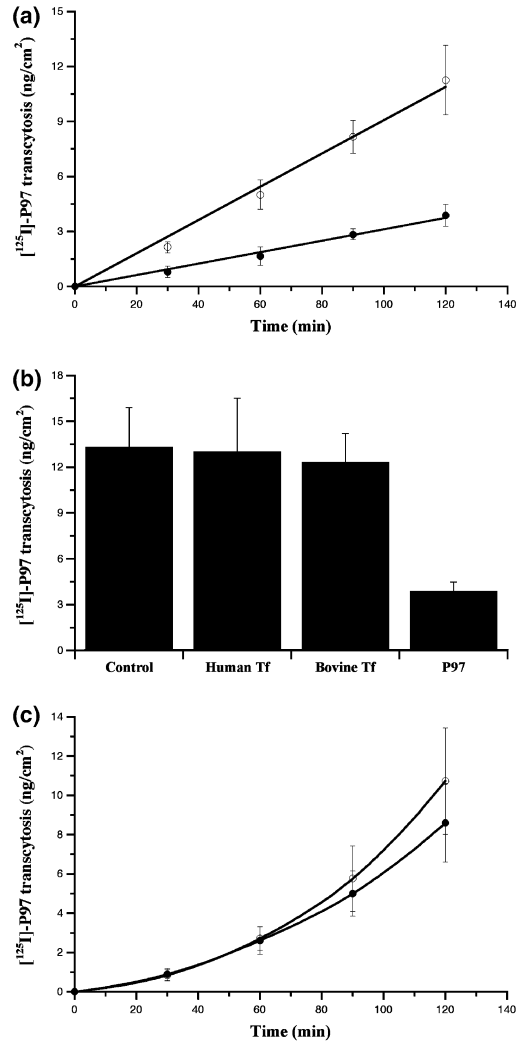
#### Identification of LRP as a potential receptor for P97

We also assessed the uptake of [<sup>125</sup>I]P97 into isolated human brain capillaries incubated for 1 h at 37°C (Fig. 6a). A 50-fold molar excess of unlabeled P97 inhibited the uptake of [<sup>125</sup>I]P97 by 60%. Human lactoferrin caused a



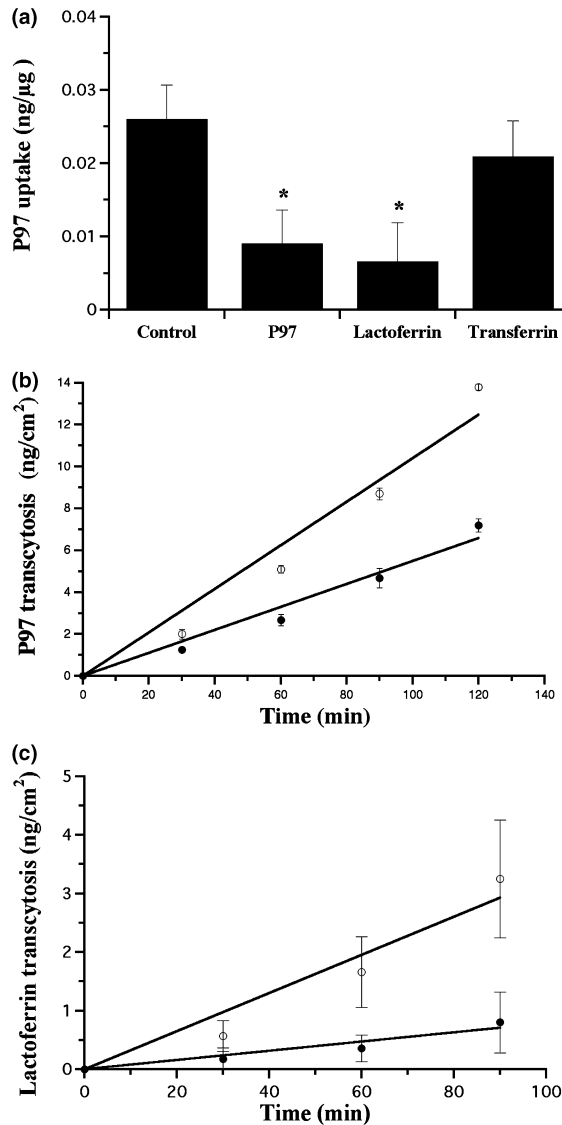
**Fig. 4** Binding of P97 to BBCE cells. (a) P97 binding experiments were performed with BBCECs that were either pre-incubated in Ringer-HEPES solution or pretreated with saponin. BBCECs were then incubated for 2 h at 4°C with [ $^{125}$ I]P97 (0.5–1.5  $\mu$ Ci/assay) at a final concentration of 25 nM. At the end of the incubation, the filters were gently washed with cold PBS and then the radioactivity associated with the ECs was quantified. Results represent means  $\pm$  SD ( $n = 3$ ). (b) The binding of [ $^{125}$ I]P97 was also performed with increasing concentrations of unlabeled P97 following saponin treatment. The results were expressed as the percentage of the [ $^{125}$ I]P97 binding measured in the absence of unlabeled P97. Results represent means  $\pm$  SEM ( $n = 5$ ). (c) The results were also transformed with a Scatchard plot and expressed as the ratio of bound P97/free P97 as a function of the bound P97.

similar inhibition of [ $^{125}$ I]P97 uptake, whereas human holo-transferrin had no effect. These results suggest that LRP, which binds and transports lactoferrin across BBCEC monolayers (Fillebeen *et al.* 1999), is also involved in the uptake of [ $^{125}$ I]P97 into brain capillaries and in the transcytosis of P97. To further investigate the role of LRP in the transport of P97, transcytosis experiments across BBCEC monolayers were performed in the presence of the receptor-associated protein (RAP), a protein chaperone that regulates LRP (Fig. 6b). Recombinant RAP (25  $\mu$ g/mL)



**Fig. 5** [ $^{125}$ I]P97 transcytosis across BBCEC monolayers in the presence of unlabeled P97, transferrin or the mAb OX-26 directed against the transferrin receptor. (a) Transport from the apical to the basolateral side of ECs of [ $^{125}$ I]P97 (0.5–1.5  $\mu$ Ci/assay) at a final concentration of 25 nM was measured in the absence ( $\circ$ ) or in the presence ( $\bullet$ ) of a 200-fold molar excess of unlabeled P97 (5  $\mu$ M). Results represent means  $\pm$  SD ( $n = 6$ ). (b) The effects of a 200-fold molar excess of either human or bovine transferrin (Tf) and P97 were also evaluated on the transcytosis of [ $^{125}$ I]P97 (0.5–1.5  $\mu$ Ci/assay) at a final concentration of 25 nM after 120 min. Results represent means  $\pm$  SD ( $n = 5$  for human transferrin;  $n = 3$  for bovine transferrin and  $n = 6$  for unlabeled P97). (c) Transcytosis of [ $^{125}$ I]P97 (0.5  $\mu$ Ci/assay) at a final concentration of 25 nM was also measured in the presence of mouse IgGs ( $\circ$ ) or mAb OX-26 ( $\bullet$ ) at a concentration of 5  $\mu$ g/mL. Results represent means  $\pm$  SD ( $n = 3$ ).

reduced the initial rate of [ $^{125}$ I]P97 transport across EC monolayers by more than 50%. In addition, the transcytosis of bovine [ $^{125}$ I]lactoferrin is inhibited by more than 75% by a 200-fold molar excess of unlabeled P97 (Fig. 6c).



**Fig. 6** Identification of P97 receptor. (a) Uptake of [ $^{125}$ I]P97 (0.5–1  $\mu$ Ci/assay) at a final concentration of 100 nM (control) into isolated human brain capillaries was measured for 1 h at 37°C in the presence of a 50-fold molar excess of unlabeled P97, human holo-transferrin or human lactoferrin. Results represent means  $\pm$  SEM ( $n = 5$ ). Statistically significant differences are indicated by \* $p < 0.05$  (Student's  $t$ -test). (b) Apical-to-basal transport of [ $^{125}$ I]P97 (0.5–1.5  $\mu$ Ci/assay) at a final concentration of 25 nM was measured in the presence (●) or absence (○) of RAP (25  $\mu$ g/mL). Results represent means  $\pm$  SD ( $n = 5$ ). (c) Inhibition of bovine [ $^{125}$ I]lactoferrin transport by P97. Transcytosis of bovine [ $^{125}$ I]lactoferrin (0.5–1  $\mu$ Ci/assay) at a final concentration of 50 nM was measured in the presence (●) or absence (○) of unlabeled P97 (5  $\mu$ M) at 37°C. Results represent means  $\pm$  SD ( $n = 6$ ).

## Discussion

In the present study, we report that the *in vivo* brain uptake of P97 is much higher than that of other proteins such as BSA

and transferrin. We also further investigated the transport of P97 with an *in vitro* model of the BBB, which previously has been used to characterize the transcytosis of various proteins such as transferrin, lactoferrin, low-density lipoproteins and insulin (Descamps *et al.* 1996; Dehouck *et al.* 1997; Fillebeen *et al.* 1999). As was seen with these proteins, transendothelial transport of P97 requires energy and is concentration-dependent, suggesting receptor-mediated endocytosis. In addition, preferential transport of P97 from the apical to the basolateral surface of BBCECs is observed with no detectable degradation of P97. The conformation of P97 also seems to be very important for its transcytosis because heat-denaturation considerably reduced the transendothelial transport of this protein. Thus, the *in vitro* results strongly confirm and support the *in vivo* observations on high P97 uptake in the brain.

These results are the first regarding P97 transendothelial transport, accumulation and binding by ECs of brain capillaries. Our findings are consistent with the presence of a low-affinity receptor for P97 with a high capacity. As all the experiments comparing bovine transferrin with human P97 are performed in a heterologous system, we can expect that the binding constant for the P97 receptor would be even greater in a human homologous system. It has been postulated that P97 is an alternate ligand for the transferrin receptor (Rothenberger *et al.* 1996) because P97 shares many properties with human transferrin and because the transferrin receptor has been detected in the same tissues as P97. However, our results strongly support that the transferrin receptor is not responsible for P97 transendothelial transport. First, the transcytosis, binding and accumulation of P97 are much higher than those for transferrin indicating that the P97 receptor has a much higher capacity and lower affinity than those previously reported for the transferrin receptor (Descamps *et al.* 1996). Second, the transcytosis of P97 is unaffected by either bovine or human transferrin, indicating that P97 does not compete with transferrin for its receptor. Third, the mAb OX-26 directed against the transferrin receptor, which was previously shown to inhibit the uptake of transferrin (Descamps *et al.* 1996), has no effect on P97 transport.

In addition to the transcytosis experiments using BBCEC monolayers, the competition of [ $^{125}$ I]P97 uptake by unlabeled P97 in isolated human brain capillaries confirmed the presence of a receptor for P97. Moreover, lactoferrin competed [ $^{125}$ I]P97 uptake efficiently, better than transferrin or any other tested proteins, suggesting that lactoferrin and P97 share the same receptor. The receptor for lactoferrin transcytosis in brain ECs is LRP (Fillebeen *et al.* 1999), a member of the large LDL-receptor family (Bu and Rennke 1996). To further investigate whether LRP could be involved in P97 transcytosis, experiments were performed with BBCEC monolayers in the presence of RAP, a protein which inhibits the binding of ligands to members of the

LDL-receptor family (Willnow *et al.* 1992; Bu and Rennke 1996; Bu and Schwartz 1998). Known members of this family also include LDL-R, LRP1B, megalin, VLDL-R, apoE-receptor 2 and the mosaic LDLR-related protein (LR11; Hussain 2001; Liu *et al.* 2001). Among these receptors, megalin, is also known to bind lactoferrin (Willnow 1998; Hussain 2001). However, megalin is mainly expressed in the kidney, whereas the major site of LRP expression is in brain (Hussain 2001). Thus, the diminution of P97 transcytosis by RAP and the inhibition of lactoferrin transcytosis by P97 also indicate that LRP is implicated in the transport of P97 across BBCECs.

Indirect lines of evidence also suggest that LRP may be involved in P97 transport in brain. P97 and other LRP ligands [amyloid- $\beta$  peptide (A $\beta$ )<sub>1-40</sub>, apolipoprotein E (apoE) and activated  $\alpha$ 2-macroglobulin ( $\alpha$ 2M)] have been reported to accumulate during Alzheimer's disease (Jefferies *et al.* 1996; Shibata *et al.* 2000; Qiu *et al.* 2001). It was also reported that LRP levels increased in the brains of Alzheimer's patients (Qiu *et al.* 2001). These previous studies and our results suggest that P97 and other LRP substrates such as A $\beta$ <sub>1-40</sub>, apoE and  $\alpha$ 2M might compete for the same receptor leading to an increase in their intracerebral levels. Additional experiments are required to investigate whether other LRP substrates can affect the transendothelial transport of P97. Because the members of the LDL-R family share similar substrates, we cannot exclude the possibility that other receptors of this family could also be involved in P97 transcytosis.

The concept of using receptor-mediated endocytosis to deliver peptides into the brain was initially described with the findings on the transendothelial transport of insulin across the BBB (Frank *et al.* 1986). Subsequent studies demonstrated that a neuropeptide could be delivered into the CNS using receptor-mediated endocytosis by targeting the transferrin receptor with the mAb OX-26 (Pardridge *et al.* 1991; Bickel *et al.* 2001). The development of chimeric proteins containing this mAb, specific linkers and a neurotropic peptide has permitted delivery into the brain of significant levels of this peptide (Pardridge *et al.* 1998; Bickel *et al.* 2001; Zhang and Pardridge 2001). In addition, the transendothelial transport of mAb OX-26 was also reported in these studies to be similar to the transport of human transferrin across the BBB. Our results therefore suggest that P97 crosses the BBB at least as well as OX-26. Another advantage of using P97 is its very low concentration in the serum (100 000-fold lower than transferrin; Jefferies *et al.* 1996; Kim *et al.* 2001), which suggests that it could deliver P97-conjugate(s) directly into the CNS. However, as P97 was found to be associated with senile plaques in Alzheimer's disease, a better understanding of the physiological roles of P97 in normal and pathological conditions in the future will be helpful to estimate the safety for the utilisation of P97 as a drug vector.

In conclusion, these are the first *in vivo* and *in vitro* results indicating that intact P97 can cross brain ECs without

affecting the integrity of the BBB and with a much higher rate than is seen with transferrin. The inhibition of P97 transcytosis by RAP in BBCEC monolayers, the competition of P97 uptake in brain capillaries by human lactoferrin and the reduction of lactoferrin transcytosis by P97, suggest that LRP, a member of the LDL-R family, may be involved in the transendothelial transport of P97. Further studies are now undergoing to elucidate the molecular events underlying the trafficking of P97 across the BBB and to determine whether P97-conjugates can be used to deliver drugs, peptides or enzymes to the brain.

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