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Involvement of the low-density lipoprotein receptor-related protein in the transcytosis of the brain delivery vector Angiopep-2

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Abstract

The blood–brain barrier (BBB) restricts the entry of proteins as well as potential drugs to cerebral tissues. We previously reported that a family of Kunitz domain-derived peptides called Angiopeps can be used as a drug delivery system for the brain. Here, we further characterize the transcytosis ability of these peptides using an *in vitro* model of the BBB and *in situ* brain perfusion. These peptides, and in particular Angiopep-2, exhibited higher transcytosis capacity and parenchymal accumulation than do transferrin, lactoferrin, and avidin. Angiopep-2 transport and accumulation in brain endothelial cells were unaffected by the P-glycoprotein inhibitor, cyclosporin A, indicating that this peptide is not a substrate for the efflux pump P-glycoprotein. However, competition studies show that activated α_2-macroglobulin, a specific ligand for the low-density lipoprotein receptor-related protein-1 (LRP1) and Angiopep-2 can share the same receptor. In addition, LRP1 was detected in glioblastomas and brain metastases from lung and skin cancers. Fluorescent microscopy also revealed that Alexa488-Angiopep-2 co-localized with LRP1 in brain endothelial cell monolayers. Overall, these results suggest that Angiopep-2 transport across the BBB is, in part, mediated by LRP1.

Keywords: Angiopep, blood–brain barrier, low-density lipoprotein receptor-related protein, transcytosis.

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The blood–brain barrier (BBB) is mainly formed by brain capillary endothelial cells (EC) which are closely sealed by tight junctions (Abbott *et al.* 2006). In addition, brain capillaries possess few fenestrae and few endocytic vesicles, compared with the capillaries of other organs (Pardridge 1999). Because of the presence of these tight junctions and the lack of fenestrae, the BBB acts as a physical barrier and is frequently the rate-limiting factor for the penetration of proteins, pharmacological agents or peptides into the CNS (Begley 2004b; Abbott *et al.* 2006). Thus, many potential drugs, that could be effective at their site of action, have failed during development because they do not reach therapeutic concentrations within the CNS (de Boer and Gaillard 2007).

Capillary EC act as a continuous lipid blockade, preventing free diffusion through extracellular pathways. This physical barrier of the BBB forces most of the molecular traffic to take a transcellular route across the EC instead of a paracellular pathway through the intercellular junctions as is observed for most endothelia (Abbott *et al.* 2006). The transport of small hydrophobic molecules is also limited because of the presence of specific transporters on the luminal (blood side) and abluminal membranes (brain side) that regulate their transcellular traffic. In addition, there is

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Abbreviations used: α_2M*, α_2-macroglobulin; BBB, blood–brain barrier; BBCEC, bovine brain capillary endothelial cell; BSA, bovine serum albumin; CsA, cyclosporin A; EC, endothelial cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KPI, Kunitz protease inhibitor; LRP, low-density lipoprotein receptor-related protein; mAb, monoclonal antibody; MW, molecular weight; NOS, nitric oxide synthetase; pAb, polyclonal antibody; PBS, phosphate-buffered saline; P-gp, P-glycoprotein; PSC-833, an analog of cyclosporin A; RAP, receptor-associated protein; RBE4, rat brain EC; TBST, Tris-buffered saline containing 0.3% Tween 20; TfR, transferrin receptor; V_d, volume of distribution.

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 by receptor-mediated endocytosis (Fillebeen *et al.* 1999; Pardridge 1999; Tsuji and Tamai 1999). Moreover, intracellular and extracellular enzymes such as peptidases, nucleotidases, monoamine oxidase and cytochrome P450 isoforms 1A and 2B form a metabolic barrier that can metabolize peptides and ATP, and inactivate neuroactive and toxic compounds (el-Bacha and Minn 1999).

Because of the capillary EC properties, most drugs do not cross the BBB and few treatments are available against most CNS disorders such as Parkinson's disease, Alzheimer's disease, and brain tumors (Pardridge 2001, 2005). Several approaches have been described for drug delivery to the brain such as local invasive delivery by direct injection or infusion, osmotic disruption using hyperosmolar solution and induction of enhanced permeability by the bradykinin analog (RMP-7 or Cereport) (Packer *et al.* 2005). Small cationic peptides as well as various physiological targeting strategies including the Trojan horse tactic have been also under investigation in order to bring drugs to the CNS (Begley 2004b; Dietz and Bahr 2004).

We have previously identified a family of peptides, named Angiopeps, which were derived from the Kunitz domains of aprotinin and other human proteins (Demeule *et al.* 2008). Using an *in vitro* model of the BBB and *in situ* brain perfusion in mice, we showed that these Kunitz domain-derived peptides could be advantageously employed as a new brain delivery system. For example, the brain uptake of a paclitaxel-Angiopep-2 conjugate (ANG1005), presently in two parallel phase 1–2 clinical trials, was much higher than that of unconjugated paclitaxel (Regina *et al.* 2008). In the present study, we further characterized the molecular mechanism involved in the transport of Angiopep-2 across the BBB. Because Angiopep-2 is derived from Kunitz domains of aprotinin and human proteins known to be low-density lipoprotein receptor-related protein (LRP) 1 or LRP2 (megalin) ligands, we hypothesized that transcytosis of Angiopep-2 across the BBB could be receptor mediated. In the present study, we show that the passage of Angiopep-2 across the BBB is partially saturable and inhibited by LRP1 ligands. We also demonstrate that the transcytosis of activated α_2-macroglobulin (α_2M*), which is the most specific known LRP1 ligand (Hussain *et al.* 1999), is specifically inhibited by Angiopep-2. Moreover, confocal microscopy also shows that Angiopep-2 is co-localized with LRP1 in brain capillary EC. LRP1 was also detected in human brain capillaries as well as in glioblastoma biopsies and brain metastases from the lung, skin, and breast. Overall, these results suggest that the transport of Angiopep-2 peptide involved LRP1 and that it could be used as a new brain delivery system for pharmacological agents in the treatments of CNS diseases such as primary and secondary brain tumors.

Materials and methods

Materials

Aprotinin, bovine serum albumin (BSA), lactoferrin, holo-transferrin, human antibodies (IgG) were purchased from Sigma-Aldrich (Oakville, ON, Canada) whereas the receptor-associated protein (RAP) was from Oxford Biomedical Research (Oxford, MI, USA). Peptides (Angiopep-1, -2, and -7) were synthetised by Peptidec (Pierrefonds, QC, Canada). Angiopep-2 was also synthetized by Peptisyntha (Torrance, CA, USA). Activated α_2M* by methylamine was provided by Dr Pizzo from Duke University Medical Center. LRP1 monoclonal antibody (mAb) was purchased from Research Diagnostics (Flanders, NJ, USA), LRP2 rabbit polyclonal antibody (pAb) was from Orbigen (San Diego, CA, USA) and endothelial nitric oxide synthetase (NOS)/NOS type III mAb was purchased from Transduction Laboratories (Lexington, KY, USA). Bovine brain EC, rat astrocytes and serum for the *in vitro* BBB models were from Cellial Technologies Inc. (Lens, France). [^{125}I]-Exendin-4 was from Phoenix Pharmaceutical (Belmont, CA, USA) whereas [^{125}I]-PYY was from Perkin-Elmer (Boston, MA, USA). Other biochemical reagents were purchased from Sigma-Aldrich. Fibroblast cell lines (MEF-1 and PEA-13) and all the cancer cell lines (U-87, U-118, SK-MEL-28, A-549, NCI-H460, MDA-MB231, and Caki-1) tested were from ATCC (Manassas, VA, USA) and cultured according to provider instructions.

Iodination of proteins and peptides

Proteins were radiolabeled with standard procedures using an Iodo-beads kit and D-Salt Dextran desalting columns from Pierce (Rockford, IL, USA). A ratio of two iodo-beads per iodination was used for the labeling. Briefly, beads were washed twice with 3 mL of phosphate-buffered saline (PBS) on a Whatman filter and resuspended in 60 μL of PBS, pH 6.5. Na[^{125}I] (1 mCi) from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada) was added to the bead suspension for 5 min at 20°C. 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 20°C, iodo-beads were removed and the supernatants were applied onto a desalting column prepacked with 5 mL of cross-linked dextran from Pierce. [^{125}I]-proteins were eluted with 10 mL of PBS. Fractions of 0.5 mL were collected and the radioactivity in 5 μL from each fraction was measured. Fractions corresponding to [^{125}I]-proteins were pooled and dialyzed against Ringer/HEPES, pH 7.4. Kunitz-derived peptides were radioiodinated using the same procedures as for proteins but, in the cases of radiolabeled peptides, free iodine was removed by using an AKTA column and 15RPC resin.

Preparation of astrocytes and bovine brain capillary endothelial cell culture

Primary cultures of mixed astrocytes were prepared from cerebral cortices of newborn rats (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- or 12-well microplates at a concentration of 2.5×10^5 cells/mL in 2 mL or 0.7×10^5 cells/mL in 1.5 mL (respectively) of optimal culture medium (Dulbecco's modified Eagle's medium) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and

50 μg/mL gentamycin. The medium was changed twice a week. Bovine brain capillary endothelial cells (BBCEC) were cultured in Dulbecco's modified Eagle's medium 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.

In vitro blood–brain barrier model

The *in vitro* model of BBB from Cellial Technologies Inc. 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) from Millipore (Billerica, MA, USA) or Costar 3401 0.4 μm (12-mm diameter) from Corning (Nepean, ON, Canada)] were coated on the upper surface with rat tail collagen. They were then set into six-multiwell microplates containing astrocytes prepared as described above, and BBCECs were plated on the upper sides 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 after 7 days. Experiments were performed between 5 and 7 days after confluence was reached. Integrity of the BBB model was evaluated by [^{14}C]-sucrose permeability (Pe of about 0.5/cm^3/min) as previously described (Dehouck *et al.* 1990).

Transendothelial transport experiments

Transport from the apical-to-basolateral side of the BBCEC monolayers was performed as follows. Filters were washed with Ringer HEPES solution and [^{125}I]-protein or [^{125}I]-peptide was then added to the solution bathing the upper side of the insert. At various times, the insert was sequentially transferred into a fresh well to avoid possible re-endocytosis of proteins or peptides by the abluminal side of the BBCECs. At the end of the experiment, [^{125}I]-proteins or [^{125}I]-peptides were quantitated in 500 μL of the lower chamber of each well.

Co-localization studies using confocal microscopy

First, Angiopep-2 was labeled with Alexa488 carboxylic acid, succinimidyl ester (NHS-Alexa488) as suggested by the manufacturer (Molecular Probes, Eugene, OR, USA). Briefly, peptide and NHS-Alexa488 were solubilized in dimethylsulfoxide. Angiopep-2 and Alexa488 (1 : 1.2) were incubated with excess triethylamine in the dark for 4 h at 20°C with inversion, and then at 4°C overnight. In order to remove unconjugated Alexa488 probe, the reaction mixture was then extensively dialyzed in Spectra/Por cellulose membrane [molecular weight (MW) cutoff; 1000 Da] from Spectrum Laboratories (Rancho Dominguez, CA, USA) against Ringer HEPES solution, pH 7.4 at 4°C. The labeled peptide was further purified on a Superdex peptide column from GE Healthcare (Baie d'Urfé) using an AKTAexplorer instrument. After 30 min of Alexa488-Angiopep-2 transcytosis at 37°C across the *in vitro* BBB model, BBCEC filters [Millicel-CM 0.4 μm (30 mm diameter) from Millipore] were washed three times with ice-cold PBS and fixed with 3.7% formaldehyde/PBS. BBCEC filters were then cut from the plastic insert, washed twice with PBS, permeabilized with methanol and non-specific sites were blocked with PBS/BSA (0.5%) for 30 min at 20°C. LRP1 was detected by incubation with 4 μg/mL goat anti-LRP1 (Santa Cruz, CA, USA) pAb in PBS/BSA (0.5%) for 1 h at 20°C. After three washes with ice-cold PBS/BSA (0.5%), the cells were incubated with Alexa568 conjugated donkey anti-goat secondary antibody (1 : 500 dilution) from Molecular Probes. Cells were extensively washed, mounted with prolong-gold kit (Invitrogen, Burlington, ON, Canada) and analyzed by confocal microscopy. Images of Alexa488-Angiopep-2 (in green) and LRP1 (in red) were acquired with a MRC-1024 confocal system (Bio-Rad, Hercules, CA, USA) with a Nikon TE300 microscope at 40× oil immersion. All images were treated with ImageJ software.

In situ mouse brain perfusion

The uptake of [^{125}I]-proteins or [^{125}I]-peptides from the luminal side of mouse brain capillaries was measured using the *in situ* brain perfusion method adapted in our laboratory for the study of drug uptake in the mouse brain (Dagenais *et al.* 2000; Cisternino *et al.* 2001). Briefly, the right common carotid artery of mice anesthetized with ketamine/xylazine (140/8 mg/kg i.p.) was exposed and ligated at the level of the bifurcation of the common carotid, rostral to the occipital artery. The common carotid was then catheterized rostrally with polyethylene tubing filled with heparin (25 U/mL) and mounted on a 26-gauge needle. The syringe containing the perfusion fluid ([^{125}I]-proteins or [^{125}I]-peptides in Krebs/bicarbonate buffer at pH 7.4, gassed with 95% O_2 and 5% CO_2) was placed in an infusion pump (Harvard pump PHD 2000; Harvard Apparatus, South Natick, MA, USA) and connected to the catheter. Prior to the perfusion, the contralateral blood flow contribution was eliminated by severing the heart ventricles. The brain was perfused for 5 min at a flow rate of 1.15 mL/min at 37°C. After perfusion of radiolabeled molecules, the brain was further perfused for 60 s with Krebs buffer, to wash away excess [^{125}I]-proteins or [^{125}I]-peptides. Mice were then decapitated to terminate perfusion and the right hemisphere was isolated on ice and capillary depletion immediately performed with ice-cold solutions on Dextran-70 cushion as previously described (Banks *et al.* 2002). Aliquots of homogenates, supernatants, pellets, and perfusates were taken to measure their contents and to evaluate the apparent volume of distribution (V_d).

RBE4 cell line

Rat brain endothelial cells (RBE4) (Roux *et al.*, 1994) were plated on collagen I-coated plates and maintained in α-minimal essential medium/Ham's F-10 (1 : 1 vol/vol) medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 300 μg/mL geneticin (G418), and 1 ng/mL basic fibroblast growth factor in humidified 5% CO_2/95% air at 37°C.

Drug accumulation studies in RBE4 cells

Cellular uptake of [^{14}C]-doxorubicin (GE Healthcare, Piscataway, NJ, USA) and [^{125}I]-Angiopep-2 were measured in RBE4 confluent cells, grown in 24-well plates. Cells were washed three times with PBS and pre-incubated for 30 min at 37°C in culture medium without serum with or without the following inhibitors: cyclosporin A (CsA) (10 μM), PSC833 (10 μM). Radiolabeled molecules (50 nM) were then added for 60 min. The cells were rapidly washed three times with ice-cold PBS and then lysed in 500 μL of 0.1 M NaOH. The amount of radiolabeled molecules retained in the cells was counted by β-scintillation counting

(Packard model 1900 TR; Packard Instrument Company, Meriden, CT, USA) for [^{14}C]-doxorubicin and in a gamma counter for [^{125}I]-Angiopep-2. An aliquot of cell lysate was used in parallel to determine cellular protein concentration.

Brain tumor

Primary and metastasic human brain tumors were obtained from the Brain Tumor Tissue Bank (London, ON, Canada) or from Notre-Dame Hospital (Montreal, QC, Canada). Two of normal human brain were also obtained from autopsies at Notre-Dame Hospital. Tissues were weighed and homogenized in five volumes of buffer containing 250 mM sucrose and 10 mM HEPES/Tris pH 7.5 with a Polytron (Brinkmann Instruments, Roxdale, ON, Canada). Brain capillaries were purified from human brain cortex as described previously (Dallaire *et al.* 1991; Beaulieu *et al.* 1995). Protein content was determined with the Coomassie Plus Protein Assay Reagent (Pierce) using BSA as a standard. After preparation, samples were aliquoted and frozen at −80°C until use.

RT–PCR

The PCR primers were designed using MACVECTOR software, based on published mRNA sequences from *Homo sapiens*. The specific primers (BioCorp., Montreal, QC, Canada) are as follows: LRP1-S: 5′-AGAAGTAGCAGGACCAGAGGG-3′; LRP1-AS: 5′-ACAGT-ACCCAGGCAGTTATGA-3′ 300 bp: LRP1b-S: 5′-AACCCAAC-CCCTTACACTACACTG-3′ 284 bp; LRP1b-AS: 5′-CCAGAG-AAACCTGAATACCAGAGC-3′; LRP2-S: 5′-CGGAGCAGTGT-GGCATATTTTC-3′; LRP2-AS: 5′-CAGGTGTATTGGGTGTC-AAGGC-3′ 280 bp: GAPDH-S 5′-CCATCACCATCTTCCAGGA-G-3′; GAPDH-AS: 5′-CCTGCTTCACCACCTTCTTG-3′.

Immunoblotting procedures

Proteins from human brain homogenates or capillaries and treated cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, proteins were electro-transferred to polyvinylidene difluoride membranes, which were then blocked for 1 h at 20°C with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween 20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (2.5 μg/mL) in TBST containing 3% BSA and 0.02% NaN_3, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1 : 2500 dilution) in TBST containing 5% non-fat dry milk. Three washes with TBST were performed after the incubations with the first and secondary antibodies. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences).

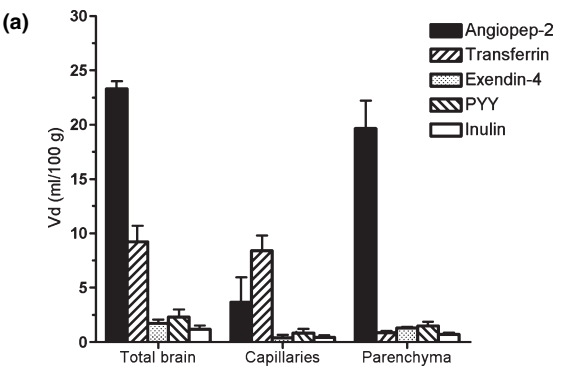
Data analysis

Data were expressed as mean ± SD. Statistical analyses were performed using Student's *t*-test when one group was compared with the control group. To compare two or more groups with the control group, one-way ANOVA with Dunnett's *post hoc* test was used. In addition, curve slopes were used to determine whether two curves were statistically different. All statistical analyses were performed using GraphPad Prism version 4.0C for Macintosh (GraphPad Software, Sand Diego, CA, USA). Significance was assumed for $p < 0.05$.

Results

The alignment of aprotinin and human proteins previously allowed us to design a family of peptides that we called Angiopeps as a potential peptide-based vector to deliver drug to the CNS (Demeule *et al.* 2008). A set of 96 peptides was tested and among them, Angiopep-2 was selected based on its initial transport rate measured by *in situ* brain perfusion and using an *in vitro* BBB model. In the present study, *in situ* brain perfusion allowed us to compare the V_d of Angiopep-2 with that obtained for transferrin, a protein reported to cross the BBB (Zhang and Pardridge 2005) and two unrelated peptides with similar MW (Fig. 1a and b). After a 5 min perfusion, capillary depletion was performed and the V_d (mL/100 g) was calculated for the Angiopep-2 (MW 2301 Da), transferrin and two other peptides, exendin-4 (4186 Da) and PYY (4279 Da). Results indicate that the V_d for the Angiopep-2 was the highest in the total brain and in the brain parenchyma.

In order to determine whether Angiopep-2 could be a substrate of the efflux pump P-glycoprotein (P-gp), we



(b)

Peptides	Amino acid sequence	Molecular weight (Dalton)
Angiopep-2	TFFYGGCRGKRNNFKTEEY-COOH	2301
Exendin-4	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH_2	4186
PYY	YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2	4279

Fig. 1 Volume of distribution of Angiopep-2, transferrin, and two unrelated peptides. (a) After iodination, *in situ* brain perfusion was performed for radiolabeled Angiopep-2, transferrin, extendin-4, and PYY as described in the Materials and methods section. Brain mice were perfused with 250 nM of [^{125}I]-Angiopep or 25 nM of either [^{125}I]-exendin-4 and [^{125}I]-PYY for 5 min. Mice were killed and capillary depletion was performed as described in the Materials and methods section. Radioactivity associated with the brain homogenate (total brain), the pellet (capillaries), and supernatant (parenchyma) were quantified using a gamma scintillator counter. Results were expressed in terms of distribution volume (mL/100g). Data represent the mean ± SD obtained from at least three mice. (b) Amino acid sequences as well as the molecular weights of Angiopep-2, extendin-4, and PYY are presented.

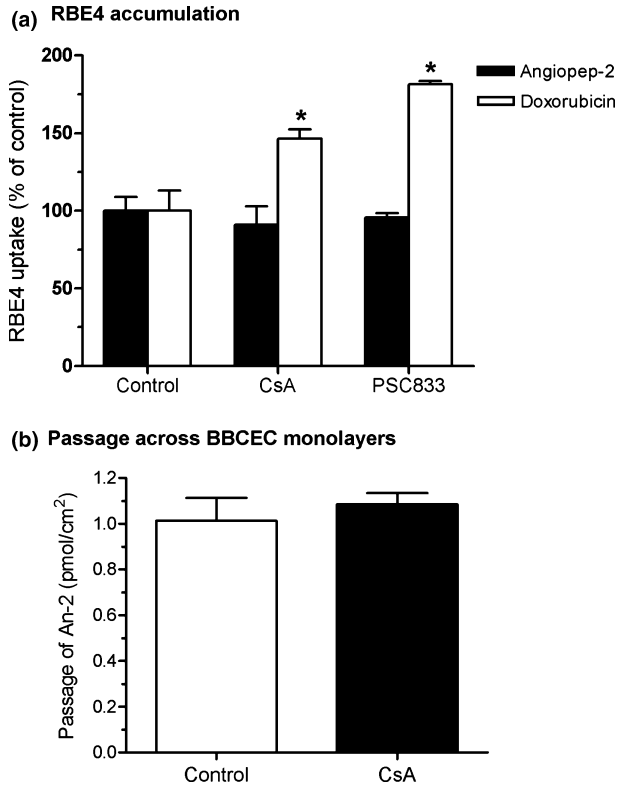


Fig. 2 Effect of P-glycoprotein (P-gp) inhibitors on Angiopep-2 transport. (a) RBE4 cells were incubated with [^{125}I]-Angiopep-2 or [^{14}C]-doxorubicin for 60 min at 37°C in the presence or absence of cyclosporine A (CsA) or PSC-833 (10 μM). After the incubation, endothelial cells were washed three times with cold phosphate-buffered saline (PBS) and incorporated radioactivity in RBE4 cells measured. Results were expressed as the percentage of radioactivity measured for [^{125}I]-Angiopep-2 or [^{14}C]-doxorubicin in RBE4 cells in absence of inhibitors (n = 6). (b) The impact of CsA (10 μM) on [^{125}I]-Angiopep-2 transcytosis from the apical-to-basolateral side of BBCEC monolayers was also evaluated. Results were expressed as transcytosis of [^{125}I]-Angiopep-2 from the apical-to-basolateral side of the BBCEC monolayers (n = 3 different filters). *p < 0.05.

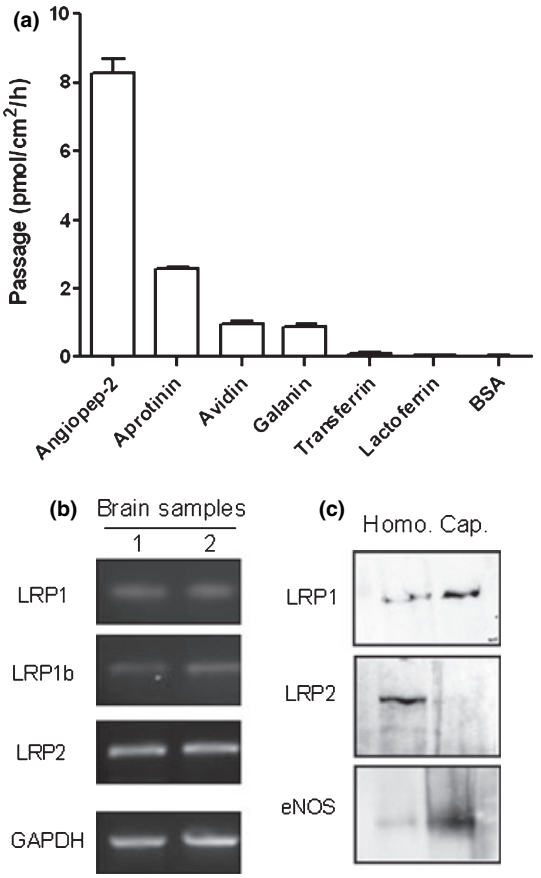


Fig. 3 Passage of Angiopep-2 and aprotinin across the *in vitro* blood–brain barrier (BBB) model and LRP1 detection in human brain. (a) After iodination, the passage of Angiopep-2, aprotinin, avidin, galanin, lactoferrin, transferrin, and bovine serum albumin (BSA) at 250 nM were measured from the apical-to-basolateral sides of endothelial cells (EC) monolayers as described in the Materials and methods section. Data represent the mean ± SD. (b) RT-PCR detection of LRP1, LRP1b, and LRP2 with GAPDH as control in two different samples of human brain. (c) Capillary depletion was performed on one biopsy of normal human brain. LRP1 and LRP2 in brain tissue and capillary fraction were detected by western blot analysis with endothelial nitric oxide synthetase (eNOS) as control for endothelial cells enrichment.

evaluated the effect of two P-gp inhibitors, CsA and PSC-833, on accumulation in immortalized RBE4 and in transcytosis across the *in vitro* BBB model (Fig. 2). As shown in Fig. 2a, the accumulation of [^{125}I]-Angiopep-2 in the immortalized RBE4 was unaffected by the addition of both P-gp inhibitors CsA or PSC-833. As positive controls, both inhibitors significantly increased the accumulation of [^{14}C]-doxorubicin in RBE4. Transcytosis of [^{125}I]-Angiopep-2 across the *in vitro* BBB model was unaffected by 10 μM CsA (Fig. 2b), a concentration known to inhibit P-gp (Jodoin *et al.* 2003). These results confirm that the anticancer agent doxorubicin is a P-gp substrate and indicate that Angiopep-2 is not.

Using the *in vitro* BBB model, we also found that the apical-to-basolateral transport of Angiopep-2 was about threefold-higher than that of aprotinin (Fig. 3). We next compared the passage of Angiopep-2 to that of proteins (avidin, lactoferrin, and transferrin) which have been reported to be transported across the BBB (Fishman *et al.* 1987; Pardridge and Boado 1991; Fillebeen *et al.* 1999). Transport of Angiopep-2 across BBCEC monolayers was higher than that of avidin and much higher (> 50-fold) than that of transferrin and lactoferrin. Also the neuropeptide galanin (MW of 3210 Da) exhibited a passage which was eightfold lower than for Angiopep-2 whereas the transport of BSA was negligible (Fig. 3a). Intact [^{125}I]-Angiopep-2 was detected in the lower compartment of the *in vitro* BBB model after transport measurement (data not shown). Previous

studies have shown that aprotinin is a LRP1 and LRP2 ligand (Hussain *et al.* 1999). Both receptors were then detected by RT-PCR (Fig. 3b) and western blot analysis (Fig. 3c) in human brain homogenate and capillaries. RT-PCR analysis indicates that both LRP1 and LRP2 mRNA are expressed in brain homogenate as well as in brain capillaries. However, western blot analysis showed that only LRP1 could be detected in human brain capillaries whereas LRP2 was undetectable. As a control for capillary depletion, endothelial NOS was detected as a marker of EC. These results suggest that LRP1 is expressed at the BBB and in other brain cell types whereas LRP2 is mostly expressed in cell types other than EC.

To determine whether Angiopep-2 could be recognized by LRP1, the accumulation of [^{125}I]-RAP, a chaperone molecule that inhibits the interactions of all ligands to LRP1 (Hussain *et al.* 1999) was measured in fibroblasts expressing LRP1 (MEF-1) and in fibroblasts that do not express LRP1 (PEA-13). As shown in Fig. 4, both cell lines were incubated with [^{125}I]-RAP and increasing concentration of aprotinin. The addition of aprotinin inhibited, in a dose-dependent manner, the accumulation of [^{125}I]-RAP in LRP1 expressing cells (Fig. 4a). LRP-dependent accumulation of [^{125}I]-RAP was calculated by subtracting the results obtained with PEA-13 cells which do not express LRP1 (Fig. 4b). Furthermore, the LRP1-dependent accumulation of [^{125}I]-RAP was also measured in the presence of saturating (25 μM) aprotinin and Angiopep-2 concentrations (Fig. 4c). Both aprotinin and Angiopep-2 significantly inhibited the LRP1-dependent accumulation of [^{125}I]-RAP.

Using the *in vitro* BBB model, the effects of aprotinin and Angiopep-2 were examined on the passage of [^{125}I]-α_2M*, a specific LRP1 ligand. The transport of [^{125}I]-α_2M* (2.5 nM) was measured from the apical-to-basolateral side of the BBCEC in the presence of a saturating concentration of unlabeled α_2M* and RAP (Fig. 5a). The presence of either unlabeled α_2M* and RAP inhibited the passage of [^{125}I]-α_2M* by 70%. Furthermore, the presence of Angiopep-2 (25 μM) inhibited, by 50%, the transport of [^{125}I]-α_2M* across EC monolayers (Fig. 5b). Similar inhibition was also measured for aprotinin (data not shown). In contrast, transport of [^{125}I]-α_2M* was unaffected by the presence of the same concentration of holo-transferrin and Angiopep-7 (Fig. 5b). In addition, the reverse experiment (Fig. 6) shows that a 30 min pre-incubation of BBCEC with a fivefold molar excess (500 nM) of α_2M* inhibited the passage of [^{125}I]-Angiopep-2 (100 nM) by 26%. The slopes were significantly different, 0.035 pmol/cm^2/min for the control and 0.026 pmol/cm^2/min for the transcytosis of [^{125}I]-Angiopep-2 measured in the presence of α_2M*.

To further investigate the involvement of LRP1 in Angiopep-2 transport across BBCEC, confocal fluorescent microscopy studies were performed. Angiopep-2 was first labeled with the fluorescent dye Alexa488. BBCEC monolayers were incubated with Alexa488-Angiopep-2 or the neutralized Alexa488 dye (5 μM) for 30 min, washed three times on both monolayer sides and fixed. As shown in Fig. 7a, incorporated Alexa488-Angiopep-2 (in green) is detected in vesicular structures of the BBCEC. In contrast, the neutralized Alexa488 dye was undetected indicating that this dye

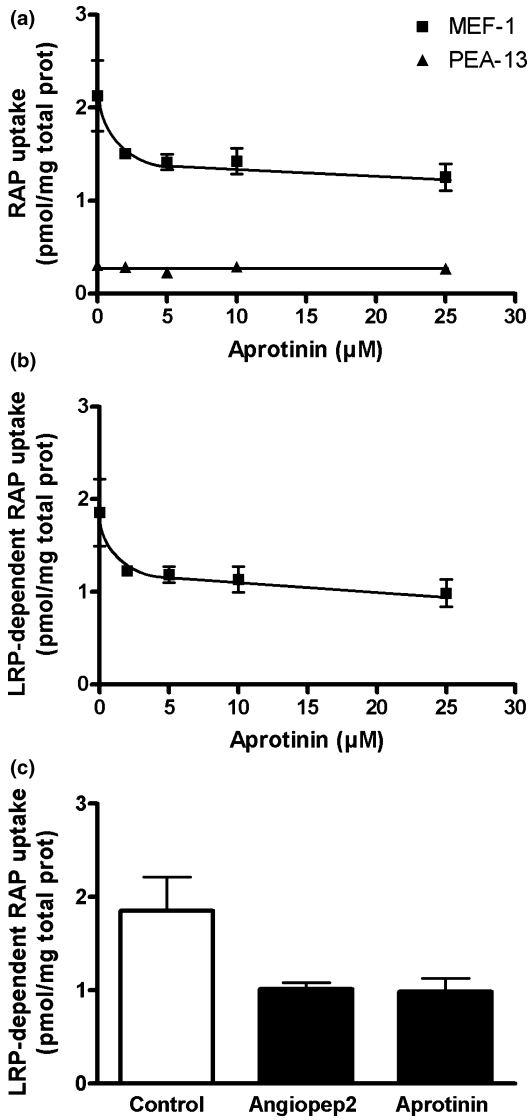


Fig. 4 Transport of the receptor-associated protein (RAP). (a) Uptake of [^{125}I]-RAP at 250 nM in fibroblast cells (MEF-1) which expressed LRP1 (■) and in fibroblast cells that do not expressed LRP1 (PEA-13) (▲) was measured in the presence of different concentrations of aprotinin as described in the Materials and methods section. (b) Difference between uptakes measured in MEF-1 and PEA-13 cells was calculated and presented as the LRP-dependent uptake of [^{125}I]-RAP. (c) The uptake of [^{125}I]-RAP was also measured in the presence of 25 μM aprotinin and Angiopep-2 in both LRP positive and negative cells. The effect of aprotinin and Angiopep-2 on LRP-dependent RAP uptake is presented. Data represent the mean ± SD. One representative experiment is shown (n = 3).

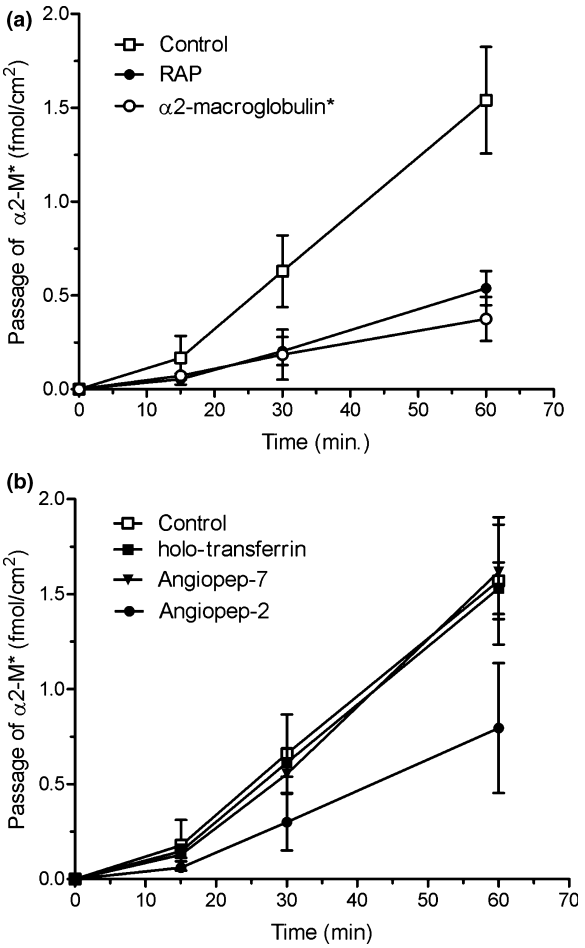


Fig. 5 Effect of Angiopep on the transport of activated α_2-macroglobulin (α_2M*). (a) Transport of [^{125}I]-α_2M* (2.5 nM) from the apical-to-basolateral side of bovine brain capillary endothelial cell (BBCEC) monolayers was measured in the presence and absence (□) a 100-fold excess of RAP (●) or unlabeled α_2M* (○). (b) Transcytosis of [^{125}I]-α_2M* was measured in the presence and absence (□) of 25 μM of Angiopep-2 (●), Angiopep-7 (▼) or holo-transferrin (■). Data represent the mean ± SD of three different experiments performed in triplicate.

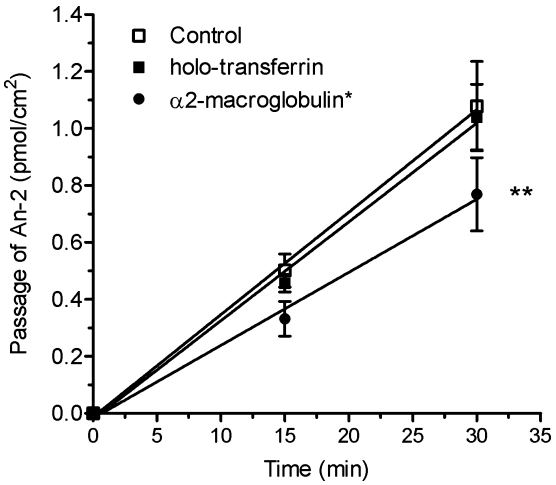


Fig. 6 Effect of α_2-macroglobulin (α_2M*) on the passage of Angiopep-2. (a) Bovine brain capillary endothelial cell (BBCEC) monolayers were pre-incubated with (●) or without (□) 500 nM of α_2M* or 5 μM of holo-transferrin (■) for 30 min at 37°C. Transcytosis of [^{125}I]-Angiopep-2 (100 nM) was then measured for 15 and 30 min at 37°C as described in the Materials and methods section. Data represent the mean ± SD of three different experiments performed in triplicate (**$p < 0.01$, significant difference in the curve slope when compared with curve slope for control conditions).

alone does not accumulate in BBCEC monolayers (Fig. 7b). LRP1 was then labeled in red (Fig. 7c) using a rabbit pAb which recognizes bovine LRP1 and a secondary antibody linked to the fluorescent dye Alexa568. Detection made with only the anti-goat secondary antibody was negative (Fig. 7d). Pictures of Alexa488-Angiopep-2 (in green) and LRP1 (in red) were then merged. Co-localization of Alexa488-Angiopep-2 and LRP1 is clearly observed in yellow (Fig. 7e). Image in the white square was then enlarged and yellow spots indicated by arrows strongly suggest a co-localization of the fluorescent labeled Angiopep-2 with LRP1 mostly at the cell surface of BBCEC (Fig. 7f).

As Angiopep-2, after its passage across the BBB, will reach the parenchyma and target cells, we further investigated whether LRP1 is also expressed in brain tumors (Fig. 8a). Western blot analysis was performed using different biopsies from glioblastomas and brain metastases of lung, skin, and breast cancers. In all tumors, LRP1 was detected at similar levels as in the U-87 glioblastoma cell line (Fig. 8b). Moreover, expression of LRP1 and LRP2 was evaluated by RT-PCR in various cancer cell lines (Fig. 8c). LRP1 is expressed in all the tested cancer lines. Interestingly, LRP2 was undetectable in the two glioblastoma cell lines (U-87 and U-118). The mRNA of GAPDH was detected as a control in each tested cancer cell lines. Overall, our results indicate that LRP1 is expressed at the BBB as well as in primary and secondary brain tumors.

Discussion

The BBB is a major obstacle against the use of drugs to treat disorders of the CNS (Begley 2004a). In a previous study, we reported a strategy for identifying and designing peptides derived from the Kunitz domain of aprotinin and other human proteins which have a higher brain penetration capability than do other potential brain drug delivery systems, such as transferrin (Demeule *et al.* 2008). From these results, a family of peptides were selected and were called Angiopeps. In the present study, we further characterized the mechanism involved in the transport of these peptides across the BBB using an *in vitro* model, *in situ* brain perfusion and fluorescent microscopy.

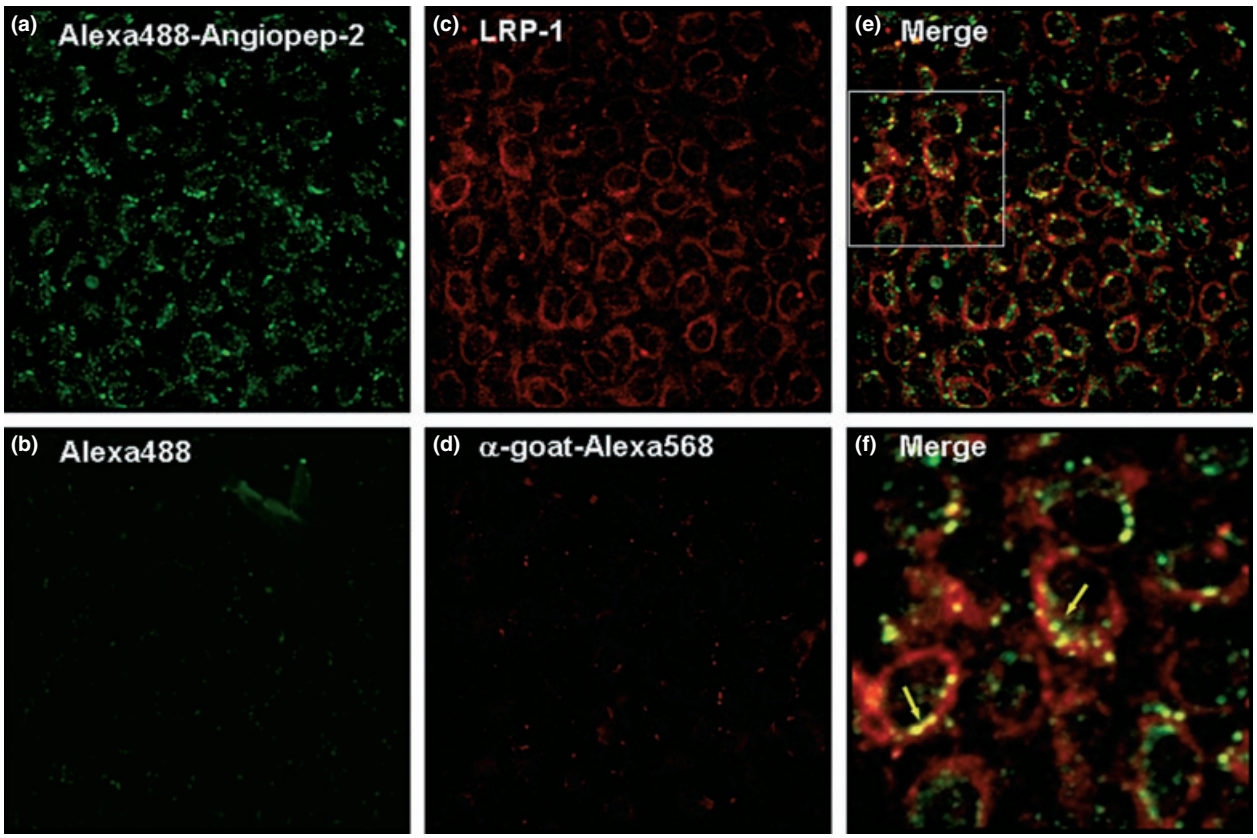


Fig. 7 Co-localization of internalized Alexa488-Angiopep-2 and LRP1. After Angiopep-2 labeling with Alexa488, 30 min transcytosis across the bovine brain capillary endothelial cell (BBCEC) monolayers was performed for Alexa488-Angiopep-2 conjugate (a) or the neutralized Alexa488 dye (b). Incorporated Alexa488-Angiopep-2 (in green) was visualized by confocal microscopy as described in the Materials and methods section. (c) LRP1 in BBCEC was labeled in red using a goat polyclonal anti-LRP1 antibody and Alexa568 conjugated donkey anti-goat secondary antibody (in red). (d) As a negative control, detection of LRP1 was made with only the secondary antibody. (e) Co-localization of Alexa488-Angiopep-2 and LRP1 is represented in yellow in the merged image. (f) Image in the white square was enlarged and co-localization of Alexa488-Angiopep-2 with LRP1 (in yellow) is indicated by arrow. One representative field is shown of experiments performed on three different filters.

Results obtained with the *in vitro* BBB model and *in situ* brain perfusion showed that both transport across BBCEC monolayers and the parenchymal V_d are higher for Angiopep-2 than for BSA (which is considered as a vascular marker), transferrin, avidin, or galanin. The transferrin receptor (TfR) is one of the best characterized receptor-mediated transcytosis systems for the targeting of drugs to the brain. Drug targeting to the TfR can be achieved by either using the endogenous ligand transferrin or a murine mAb (OX-26) directed against the rat TfR (Pardridge *et al.* 1991). It is important to note that chemical conjugation of avidin to OX-26 led to reduce plasma area under the curve and a marked reduction of brain targeting compared with OX-26 (Penichet *et al.* 1999). In addition, the exact molecular mechanism involved in transcytosis of transferrin and OX-26 across the BBCEC remains unclear. Although the mechanism of transcytosis of transferrin and OX-26 is not yet fully elucidated, it should be emphasized that drug delivery to the brain via the TfR has been shown to be possible. Avidin has been previously proposed as a potential brain delivery vector (Pardridge and Boado 1991; Bickel *et al.* 2001). However, it was shown that the half-life of avidin in serum was very short because biotinidase is present in serum and because the cationic charge of avidin leads to its degradation and/or rapid clearance by tissues (Jeong Lee and Pardridge 2000). In addition, transport of Angiopep-2 was much higher than that of avidin as well as for the human 30 amino acid neuropeptide galanin (Lang *et al.* 2007). Furthermore, the V_d of Angiopep-2 was also higher than the peptides exendin-4 which induces satiety as well as weight loss in both lean and obese mice and rats (Young *et al.* 1999) and PYY which is known to play a role in regulating appetite (Ballantyne 2006). Interestingly, both exendin-4 and $PYY_{3–36}$ have been reported to be able to cross the BBB (Kastin and Akerstrom 2003; Nonaka *et al.* 2003). Reported influx rates for both exendin-4 and $PYY_{3–36}$ were lower than

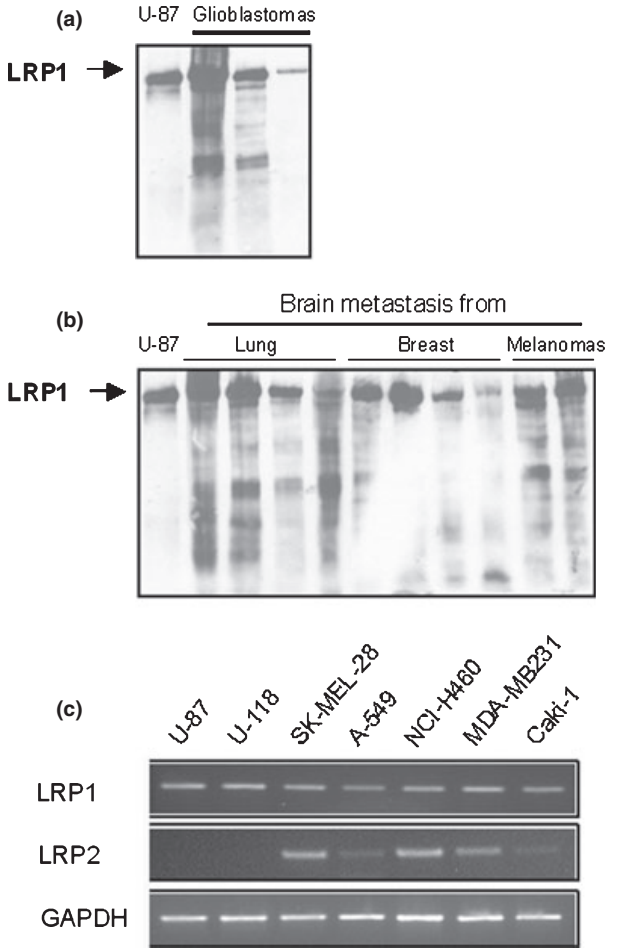


Fig. 8 Detection of low-density lipoprotein related-receptor protein (LRP) 1 in brain and cell tumors. Immunodetection of LRP1 in three different biopsies from primary human brain tumors (a) and in 10 different biopsies of human brain metastases by western blot analysis (b). (c) RT-PCR analyses of LRP1, LRP2, and GAPDH in various human cancer cell lines are shown. A representative experiment is shown.

that of Angiopep-2. We are also demonstrating that Angiopep-2 is not recognized by the efflux pump P-gp as the transport of this peptide was unaffected by both P-gp inhibitors CsA and PSC-833.

Among the transporters and receptors that have been described as playing a role at the BBB, LRP1 has been reported to possess the ability to mediate transport of ligands (RAP, lactoferrin, and melanotransferrin) across EC of the BBB (Fillebeen *et al.* 1999; Demeule *et al.* 2002). Both LRP1 and megalin (LRP2) have also been shown to be responsible for endocytosis of the secreted β-amyloid precursor protein (Kounnas *et al.* 1995; Cam and Bu 2006). It has also been demonstrated that the Kunitz protease inhibitor (KPI) domain is important for the recognition, internalization, and clearance of amyloid precursor protein by LRP1 (Kounnas *et al.* 1995). Angiopep peptides were designed by the alignment of different human proteins that possess a KPI-domain and which are known to be LRP1 ligands (Moestrup *et al.* 1995; Hussain *et al.* 1999; Demeule *et al.* 2008). As the Angiopep peptide family, including Angiopep-2, were derived from the KPI domain, the present data show the possible involvement of LRP1 in Angiopep-2 transport. In our previous study, we also showed that lysine residues at positions 10 and 15 provide specificity for the transport of Angiopep-2 across the BBB. These two lysines have been previously reported to be in the minimal amino acid sequence required for the binding of aprotinin to LRP2 (Moestrup *et al.* 1995). As LRP1 shares many LRP2 ligands and the level of sequence identity between these two receptors is high, our results indicate that both lysine residues at position 10 and 15 provide specificity for the transport of Angiopep-2 across the BBB.

Here, we then investigated whether Angiopep peptides could be recognized by LRP1. Previous studies have reported that LRP1 is one of the major receptors for the clearance and internalization of α_2M* (Gonias *et al.* 1994; Hussain *et al.* 1999). Our data obtained with the *in vitro* BBB model are in agreement with these studies and indicate that transcytosis of α_2M* across the BBB is also LRP1 dependent and affected by both Angiopep-2 and aprotinin. Angiopep-2, and not Angiopep-7, could inhibit α_2M* transcytosis to the same extent as an excess of unlabeled α_2M*. These results demonstrate that Angiopep-2 maintains the capacity to interact with LRP1. Taken together, our data suggest that Angiopep-2, by interacting with LRP1, led to the inhibition of [^{125}I]-α_2M* transcytosis across the BBCE monolayers. Confocal fluorescent microscopy shows that only Alexa488-Angiopep-2 is present at detectable levels in the BBCEC and not the dye alone. Moreover, we observed the incorporation of Alexa488-Angiopep-2 in vesicular structures that were also positively stained for LRP1 indicating that Alexa488-Angiopep-2 conjugate co-localized with LRP1 in BBCEC. Confocal microscopy has been previously used to demonstrate co-localization of LRP1 with the urokinase/plasminogen activator inhibitor type-2 complex in prostate cancer cell (Croucher *et al.* 2006). In the present study, these are the first results showing that Angiopep-2 co-localized with LRP1 in vesicular structures of BBCEC.

Interestingly, RT-PCR analysis showed that LRP1 and LRP2 are expressed in brain homogenate and isolated brain capillaries. However, only LRP1 could be detected by western blot in brain capillaries. Moreover, western blots as well as RT-PCR analysis showed that LRP1 is expressed in glioblastomas. This is in agreement with a previous study in which LRP1 was reported to be present in the cellular cytoplasm and on the cell surface of glioblastomas (Yamamoto *et al.* 1997). In addition, LRP1 mRNA was shown to be more frequently expressed in glioblastomas and anaplastic astrocytomas, compared with low-grade astrocytomas, by RT-PCR analysis. Interestingly, higher

LRP1 levels were detected by immunochemistry in EC of brain tumors when compared with EC from normal brain (Yamamoto *et al.* 1998). In the present study, we also detected LRP1 in brain metastastic biopsies from breast, skin, and lung cancers. Overall, these results suggest that Angiopep-2, by interacting with LRP1, could target primary and secondary brain tumors.

In conclusion, the present data were compatible with the hypothesis that Angiopep-2 transport across the BBB is, in part, receptor mediated. Competitive transport experiments with known LRP1 ligands indicate the involvement of LRP1. However, as Angiopep-2 transcytosis is only partially inhibited by LRP1 ligands, other transport mechanism(s) could also be responsible for its internalization and transport across the BBB. Further co-localization experiments are presently ongoing in order to identify and characterize the molecular events associated with Angiopep-2 transcytosis across EC of the BBB. Taken together, our data suggest that the transport of the Kunitz-derived Angiopep-2 peptide involves LRP1 and that it could be advantageously employed as a brain delivery system for pharmacological agents for targeting LRP1 in primary and secondary brain tumors.

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