P-glycoprotein is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain

Édith BEAULIEU*, Michel DEMEULE*, Lucian GHITESCU† and Richard BÉLIVEAU*‡[1]

*Laboratoire d'Oncologie Moléculaire, Département de Chimie-Biochimie, Université du Québec à Montréal, Montréal, Québec, Canada H3C 3P8, and ‡Hôpital Ste-Justine, Montréal, Québec, Canada H1W 2C4, and †Université de Montréal, Montréal, Québec, Canada H3C 3J7

Luminal membranes of the vascular endothelium were isolated from brain, heart and lungs by modification of their density. The presence of P-glycoprotein (P-gp) was detected by Western blotting in luminal membranes from the endothelium of the three tissues. Strong enrichment in brain capillary luminal membranes, compared with brain capillaries (17-fold) and whole membranes (400–500-fold), indicates that P-gp is mainly located on the luminal side of the brain endothelium. Western blotting was also performed with antibodies directed against GLUT1, glial fibrillary acidic protein, adaptin, IP_3R-3, integrins αv and collagen IV as controls to determine whether the preparations were contaminated by other membranes. Strong enrichment of GLUT1 in brain capillary luminal membranes (9.9-fold) showed that the preparation consisted mainly of endothelial cell plasma membranes. Poor enrichment of glial fibrillary acidic protein (1.4-fold) and adaptin (2.4-fold) and a decreased level of IP_3R-3, integrins αv and collagen IV excludes the possibility of major contamination by astrocytes or internal and anti-luminal membranes. High levels of P-gp in the luminal membranes of brain capillary endothelial cells suggests that it may play an important role in limiting the access of anti-cancer drugs to the brain.

INTRODUCTION

The multidrug resistance phenotype, characterized by the overexpression of P-glycoprotein (P-gp), is a major problem encountered during chemotherapy [1,2]. P-gp, a member of the ABC transporter superfamily, is a glycosylated 140–170 kDa protein with 12 transmembrane domains and two cytoplasmically located ATP-binding sites. It has been shown to actively transport a wide variety of agents, including vinca alkaloids, colchicine, antibiotics and anthracyclines, out of cells by an ATP-dependent mechanism, thus reducing their cytosolic concentration [3–5]. P-gp is expressed in some chemotherapy-resistant tumours [6] and in many normal tissues [7,8,9] in which its physiological function still remains unclear in spite of suggested roles in detoxification by excluding toxins from normal cells and secretion of metabolites into bile, urine and the gastrointestinal tract [7,8,10]. Very high levels of P-gp were found particularly in brain capillaries [8,11], where it interacts with various drugs [12] and appears to play a critical role in host defence against certain lipophilic toxins [13].

Immunohistological studies have suggested that P-gp is localized in the luminal membrane of endothelial cells forming brain blood vessels [14,15]. It may thus contribute to multidrug resistance in brain tumours by expelling drugs from the endothelial cells into the circulation in addition to extruding them from tumoral cells [16]. P-gp may contribute greatly to the properties of the blood–brain barrier, where blood vessels are less permeable than in other tissues such as the heart and lungs. Determination of its cellular localization in blood vessels is important to understand its physiological function. It may also help to explain why combinations of chemotherapeutic agents and P-gp blockers are more toxic than expected and thereby eventually help to overcome this barrier.

In this study, luminal membranes from brain, heart and lungs were isolated by a method previously used for lung blood vessels [17]. The density of the endothelial luminal membrane is modified with cationic colloidal silica coated with a polyanion cross-linker, thus allowing its isolation by centrifugation (Figure 1). Immunoblotting analysis of P-gp, using the monoclonal antibody (mAb) C219 and the polyclonal antibody (pAb) anti-(mdr Ab-1), showed the presence of P-gp in the luminal membranes of the endothelial cells forming blood vessels in all three tissues studied, but it was most strongly enriched in the brain capillaries.

MATERIALS AND METHODS

Chemicals

Dextran T-70 and fast flow Protein A–Sepharose were purchased from Pharmacia LKB Biotechnology (Montreal, Que., Canada). Electrophoresis reagents and Tween 20 were products of Bio-Rad (Mississauga, Ont., Canada). mAb C219 was from ID Labs (London, Ont., Canada), anti-[glial fibrillary acidic protein (GFAP)] and anti-α-adaptin were from Sigma (St Louis, MO, U.S.A.), anti-IP_3R-3 was from Transduction Laboratories (Mississauga, Ont., Canada), anti-(integrin αv) was from Chemicon International (Temecula, CA, U.S.A.) and anti-(collagen IV) (mAb M3F7) was from the Developmental Studies Hybridoma Bank (Iowa City, IA, U.S.A.). pAb anti-GLUT1 was from Eastacres Biologicals (Southbridge, MA, U.S.A.). Horseradish peroxidase-conjugated sheep anti-mouse IgG, donkey anti-rabbit IgG and enhanced chemiluminescence reagents were obtained from Amersham (Oakville, Ont., Canada). Endoglycosidase F/N-glycosidase F and Hepes were from Boeringher-Mannheim (Laval, Que., Canada), SDS was from Pierce

Abbreviations used: HB, homogenate buffer; GFAP, glial fibrillary acidic protein; GLUT1, Glucose transporter; IAAP, [^{125}I]iodoarylazidoprazosin; mAb, monoclonal antibody; pAb, polyclonal antibody; MBS, Mes-buffered saline; P-gp, P-glycoprotein; TBS, Tris-buffered saline; TBS-Tw: TBS/Tween 20.

[1] To whom correspondence should be sent at the address with the symbol*.

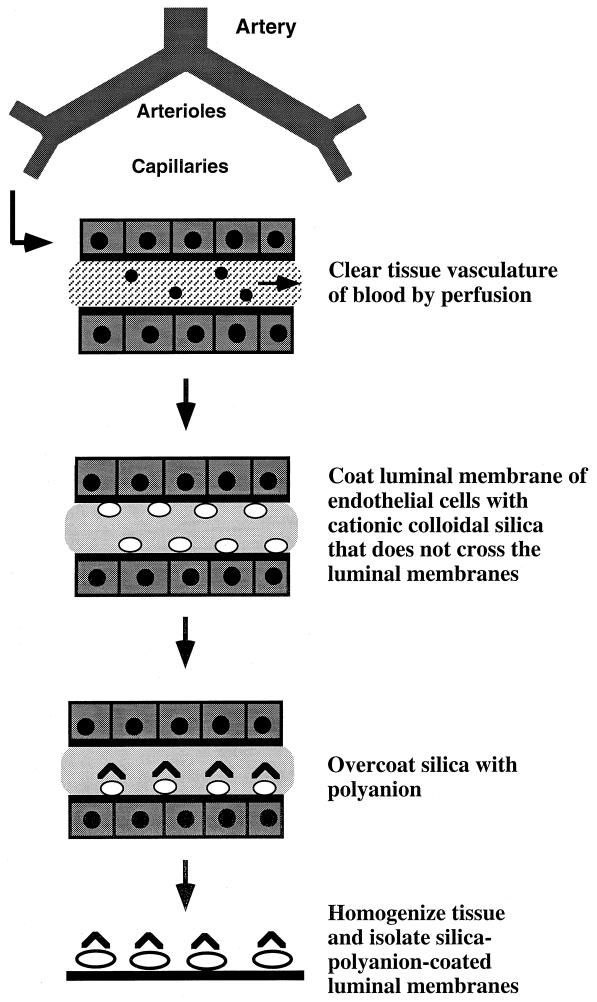

Figure 1 Outline of the procedure used for isolating luminal membranes from endothelial cells

(Rockford, IL, U.S.A.) and [^{125}I]iodoarylazidoprazosin (IAAP) was from Du Pont (Mississauga, Ont., Canada). All other reagents were purchased from Sigma.

Rats

Male Sprague–Dawley rats were obtained from Charles River (St-Constant, Que., Canada).

Luminal membranes

Luminal membranes from the endothelium of whole brain, heart and lungs were prepared as described by Jacobson et al. [17] as illustrated in Figure 1. Briefly, brain, heart or lung microvasculature was coated with colloidal silica and polyacrylate by perfusion with: (1) Dulbecco's modified Eagle's medium/nitroprusside (25–30 °C) to remove blood from the vasculature and to reduce the temperature; (2) Mes-buffered saline, pH 6.0 (MBS) to optimize the pH for coating; (3) colloidal silica; (4) MBS to eliminate unbound silica; (5) 1% sodium polyacrylate in MBS. Organs were then homogenized in Hepes-buffered sucrose, filtered through a nylon monofilament net, diluted with an equal volume of 1.02 g/ml Nycodenz and centrifuged to isolate silica-coated plasmalemmal fragments and eliminate contaminating debris.

Whole membranes

Tissues were homogenized in 8 vol. of 250 mM sucrose/5 mM Hepes/Tris, pH 7.5. Homogenates were centrifuged at 3000 ***g*** for 10 min at 4 °C, and the supernatants were centrifuged at 33000 ***g*** for 30 min at 4 °C. Pellets were resuspended in homogenate buffer (HB) containing 50 mM mannitol and 20 mM Hepes/Tris, pH 7.5, and stored at −80 °C.

Brain capillaries

Capillaries were purified from brain cortex by the method of Dallaire et al. [18] with slight modifications as described previously [19]. γ-Glutamyltranspeptidase activity was enriched at least 15-fold.

Western-blot analysis

Proteins were solubilized in 0.5% SDS in HB for 30 min at 25 °C, centrifuged at 50000 ***g*** for 15 min, and supernatant proteins were measured using a micro-BCA (bicinchoninic acid) kit (Pierce). SDS/PAGE and P-gp immunodetection with mAb C219 were performed as described previously [19]. Immunodetection of P-gp with pAb anti-mdr (Ab-1) was performed with slight modifications. All steps were carried out in Tris-buffered saline/0.05% Tween, pH 8.0 [TBS-Tw (0.05%)]. The primary antibody was diluted 1:200, and the secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG (from donkey), was diluted 1:2000 in 1% powdered skimmed milk in TBS-Tw. Immunodetection of GLUT1 was performed as described by Pouliot and Béliveau [20]. Immunodetection of all other proteins was carried out in TBS containing 0.1% Tween 20 [TBS-Tw (0.1%)]. The primary antibodies were diluted 1:500 in TBS-Tw (0.1%) containing 1% BSA and 0.02% NaN_3, and the secondary antibodies (sheep anti-mouse IgG or donkey anti-rabbit IgG) were diluted 1:1000 in 5% milk in TBS-Tw (0.1%). For the immunodetection of IP_3R-3 and integrin αv, samples were heated at 100 °C for 5 min before electrophoresis. Molecular masses were determined using a calibration curve made with Bio-Rad standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa) and ovalbumin (45 kDa).

Deglycosylation

Capillaries were resuspended in HM at a protein concentration of 6 mg/ml, and solubilized for 10 min at 25 °C in 0.5% SDS. The samples were diluted in HM containing 0.25% 2-mercaptoethanol, 1% Triton X-100, 2 units of endoglycosidase F/N-glycosidase F/mg of protein and protease inhibitors (2 μg/ml aprotinin, 10 μg/ml pepstatin A and 100 μg/ml bacitracin). Deglycosylation was carried out overnight at 25 °C. Laemmli's sample buffer was added, and Western-blot analysis was performed as described above.

Photoaffinity labelling

Photoaffinity labelling with [^{125}I]IAAP was performed as described by Jetté et al. [21].

RESULTS

Immunodetection of P-gp was performed using two different antibodies, mAb C219 (Figure 2A) and pAb anti(mdr Ab-1) (Figure 2B), both recognizing a conserved C-terminal linear

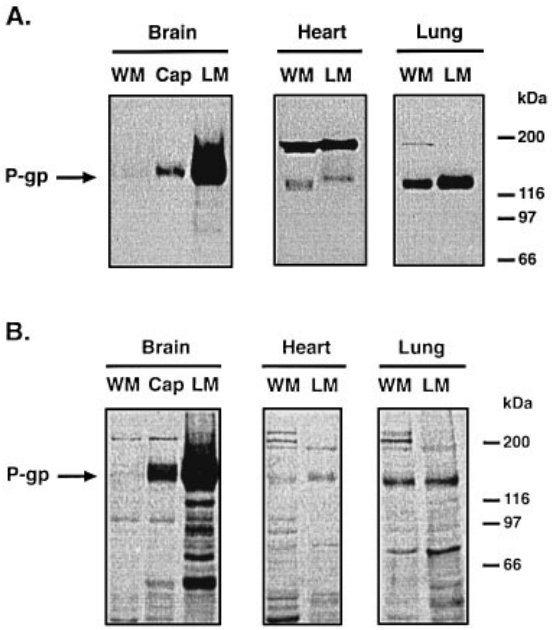

Figure 2 Immunodetection of P-gp in luminal membranes

After solubilization, proteins from whole membranes (WM) (30 μg), endothelial luminal membranes (LM) from heart (30 μg), lungs (30 μg) and brain (3 μg) and brain capillaries (Cap) (3 μg) were resolved on a single gel by SDS/PAGE. Immunodetection with mAb C219 (**A**) and pAb Ab-1 (**B**) was performed as described in the Materials and methods section. Appropriate exposure times were chosen for the different tissues.

Table 1 Enrichment of proteins in capillary luminal membranes compared with corresponding total membranes and brain capillaries

Autoradiograms were scanned with a laser densitometer and the relative area under each peak was calculated. Values are the ratio of the amount of protein detected in capillary luminal membranes to the amount found in isolated brain capillaries or in whole membranes of the corresponding tissue

		Whole membranes		
	Brain capillaries	Brain	Heart	Lung
P-gp	17	400–500	1.2	2.2
GLUT1	9.9	240–280	—	—
GFAP	1.4	2.6	—	—
Adaptin	2.4	3.5	0.24	2.7
IP_3R-3	0.2	9.2	0	0.3
Integrin αv	0.4	0.3	0.2	0.2
Collagen IV	0.3	7.5	0.2	0.9

sequence. P-gp was detected in whole tissue membranes and luminal membranes isolated from the capillary endothelium of brain, heart and lungs, and in brain capillaries. In brain tissues, it was enriched 17-fold in luminal membranes compared with capillaries and 400-500-fold over whole membranes (Table 1). These results confirmed that P-gp is localized on the luminal side of the endothelial cells of the blood–brain barrier. P-gp was not enriched in the luminal membranes of heart capillaries and was only enriched 2-fold in those of the lung (Table 1).

Since two proteins (p155 and p190 kDa) were detected by the mAb C219, deglycosylation and photoaffinity labelling with IAAP were performed to identify which protein was P-gp. First,

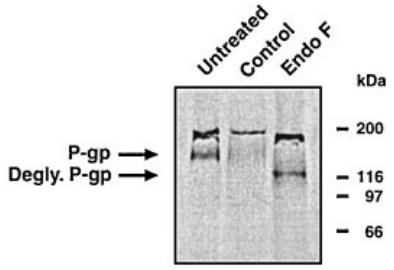

Figure 3 Deglycosylation of P-gp from brain capillaries

Solubilized brain capillary proteins were incubated for 16 h at 4 °C in the presence (Endo F) or absence (Control) of endoglycosidase F/N-glycosidase F, as described in the Materials and methods section. The samples and untreated brain capillary proteins (Untreated) were resolved by SDS/PAGE. Immunodetection with mAb C219 was performed as described in the Materials and methods section.

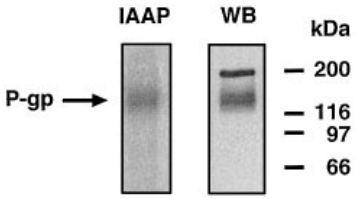

Figure 4 Photoaffinity labelling of P-gp in brain capillaries

Brain capillary proteins (50 μg) were incubated with IAAP, irradiated at 254 nm and immunoprecipitated with mAb C219 as described previously [21]. Proteins were then resolved by SDS/PAGE. Gels were dried and exposed to Kodak films (IAAP). Untreated brain capillary proteins (10 μg) were resolved by SDS/PAGE, and Western-blot analysis was performed with mAb C219 as described in the Materials and methods section (WB).

deglycosylation of brain capillary proteins (Figure 3) shifted the apparent molecular mass of p155 to 130 kDa, whereas that of p190 was only reduced by 5 kDa. The decrease of 25 kDa is in agreement with previous results [22], indicating that p155 is P-gp. Second, photoaffinity labelling with IAAP (Figure 4) confirmed that p155 is P-gp, since it is photolabelled by IAAP, whereas p190 is not.

A brain capillary is seen in cross-section in electron micrography (Figure 5A). The capillary lumen (CL) is surrounded by the luminal membrane of endothelial cells (E). The luminal membrane is coated with electron-dense silica/polyanion which is seen as block dots. Electron microscopy also shows that the final preparation is almost exclusively composed of luminal membranes from capillaries coated with electron-dense silica/polyanion (Figure 5B). Thus the method that was previously used to isolate lung luminal membranes can be used successfully with tissues other than lung.

Luminal membrane preparations were further characterized by immunodetecting various membrane markers. Immunodetection of the glucose transporter GLUT1 and GFAP, an astrocyte-related protein, was performed as a first control (Figure 6A) to determine the cellular membrane type. Two different isoforms of GLUT1 were detected. An isoform of 52 kDa was enriched 10-fold in brain capillary luminal membranes (Table 1). This isoform is a good marker for endothelial cells of the brain since it is expressed selectively in the brain microvascular endothelium in both luminal and abluminal membranes [23]. Thus enrichment of this isoform indicates that this preparation isolated from brain is vascular endothelium. Another GLUT1 isoform of 45 kDa was

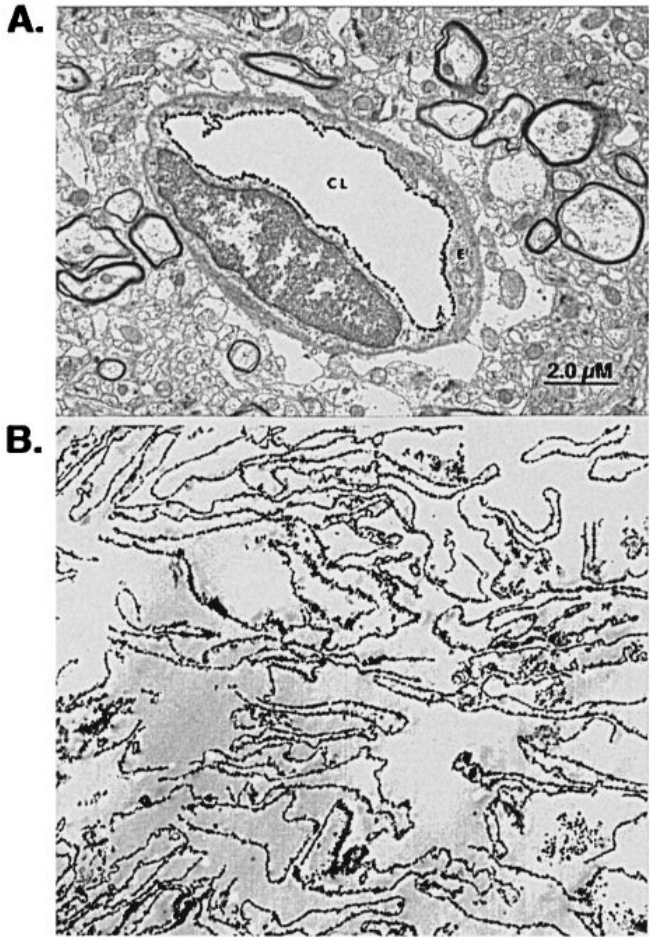

Figure 5 Electron micrographs of luminal membranes from brain

(**A**) Cross-sectional view of brain capillary endothelial cells (E) after coating with cationized silica particles and polyanion cross-linker which surround the capillary lumen (CL). (**B**) The final membrane preparation coated with electron-dense silica/polyanion which is seen as black dots.

detected in whole membranes and capillaries isolated from brain but not in luminal membranes from brain blood vessels. GLUT1 was not detected in the luminal membranes of the endothelia from lungs and heart. This absence is not surprising since the vascular bed of these tissues is not a site of expression of this protein, according to immunohistochemical studies [24]. Immunodetection of GFAP (Figure 6B) revealed two proteins of about 50 kDa in whole membranes, capillaries and capillary luminal membranes from brain. These proteins were weakly enriched in capillary luminal membranes from brain compared with brain capillaries (1.4-fold) and whole membranes (2.6-fold) (Table 1). This indicates a minor contamination of the brain luminal membrane preparation by astrocytes. No GFAP was detected in capillary luminal membranes from heart or lung as expected. These results suggest that mainly plasma membranes of endothelial cells were present in the luminal membrane preparations.

Contamination by internal membranes was evaluated by immunodetection of adaptin (Figure 7A), a protein located in the Golgi apparatus membrane, and IP_3R-3 (Figure 7B), a protein located in the endoplasmic-reticulum membrane. With anti-adaptin, two proteins that correspond to adaptin (105 and 110 kDa) were detected in the capillary luminal membranes,

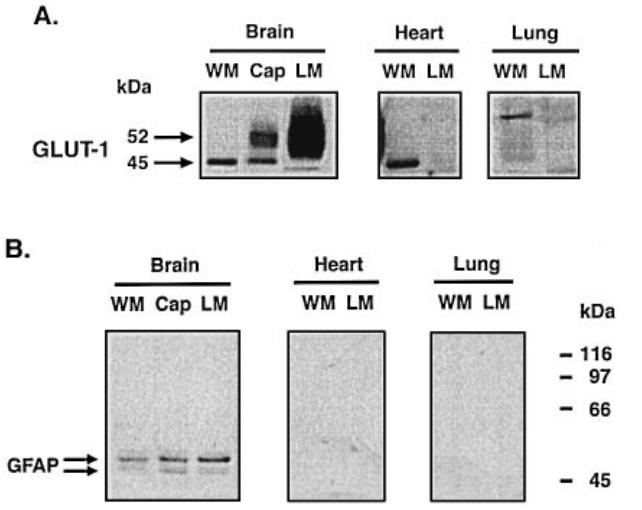

Figure 6 Determination of cellular membrane type

(**A**) After solubilization, proteins from whole membranes of each tissue (WM) (30 μg), endothelial luminal membranes (LM) from heart (30 μg), lungs (30 μg) and brain (3 μg) and brain capillaries (Cap) (3 μg) were resolved on a single gel by SDS/PAGE. Immunodetection with pAb anti-GLUT1 was performed as described in the Materials and methods section. (**B**) After solubilization, proteins from brain capillaries (Cap) (15 μg), whole membranes (WM) and luminal membranes (LM) from brain (15 μg), heart (30 μg) and lung (30 μg) capillaries were resolved on a single gel by SDS/PAGE. Immunodetection with mAb anti-GFAP was performed as described in the Materials and methods section. Appropriate exposure times were chosen for the different tissues.

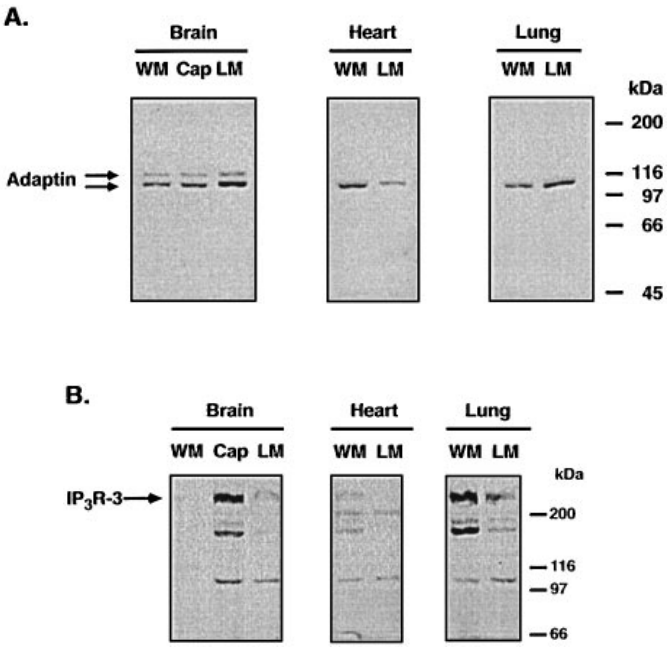

Figure 7 Immunodetection of intracellular membrane markers

(**A**) After solubilization, proteins from brain capillaries (Cap) (15 μg), whole membranes (WM) and luminal membranes (LM) from brain (15 μg), heart (30 μg) and lung (30 μg) capillaries were resolved on a single gel by SDS/PAGE. Immunodetection with mAb anti-adaptin was performed as described in the Materials and methods section. (**B**) After solubilization, proteins from brain capillaries (Cap) (20 μg), whole membranes (WM) (20 μg) and luminal membranes (LM) from brain (20 μg), heart (15 μg) and lung (20 μg) capillaries were resolved on a single gel by SDS/PAGE. Immunodetection with mAb directed against anti-IP_3R-3 was performed as described in the Materials and methods section. Appropriate exposure times were chosen for the different tissues.

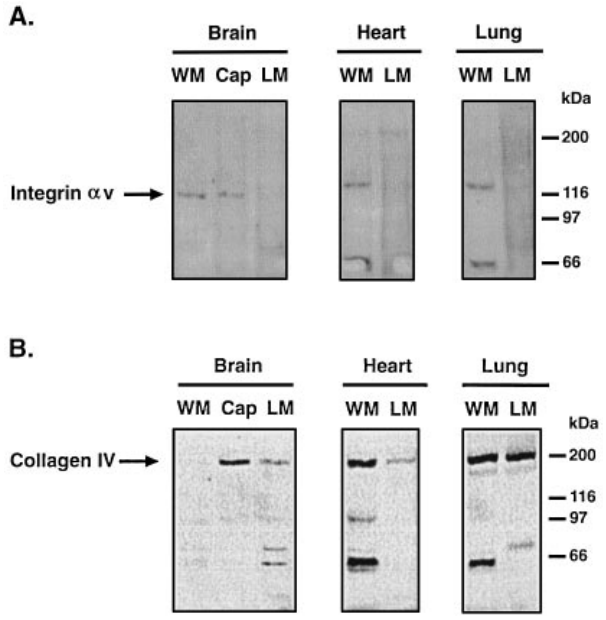

Figure 8 Immunodetection of anti-luminal and basal membrane markers

After solubilization, proteins from brain capillaries (Cap) (20 μg), whole membranes (WM) (20 μg) and luminal membranes (LM) from brain (20 μg), heart (15 μg) and lung (20 μg) capillaries were resolved on a single gel by SDS/PAGE. Immunodetection with mAb 1960 directed against integrin αv (**A**) and with mAb M3F7 directed against collagen IV (**B**) was performed as described in the Materials and methods section. Appropriate exposure times were chosen for the different tissues.

whole membranes and capillaries isolated from the brain. This protein was only weakly enriched in brain luminal membranes compared with brain capillaries (2.4-fold) and whole membranes from brain (3.5-fold, Table 1). This weak enrichment could be due to the fact that adaptin has also been reported in plasma membrane. Only one band was detected in the luminal membrane of capillaries and in whole membranes from heart and lungs. In heart, expression of adaptin was weaker in capillary luminal membranes (0.2-fold) than in whole membranes in contrast with lung where it was enriched, but only 2.7-fold (Table 1). IP_3R-3 was detected as a protein of about 300 kDa in the three fractions isolated from brain. The enrichment of this protein in the luminal membranes, compared with whole membranes, was 9.2-fold (Table 1). In contrast, this protein was expressed at lower levels in capillary luminal membranes than in brain capillaries (0.2-fold). In heart, no IP_3R-3 was detected in capillary luminal membranes, whereas in lung, IP_3R-3 levels in capillary luminal membranes were very low compared with whole membranes (0.3-fold). These very low levels of adaptin and IP_3R-3 in capillary luminal membrane preparations from the three tissues indicate weak contamination by Golgi apparatus and endoplasmic reticulum membranes.

Contamination of capillary luminal membrane preparations by anti-luminal membranes and basal laminae was evaluated by immunodetection of integrin αv (Figure 8A) and collagen IV (Figure 8B). The integrin αv (125 kDa) was detected at very low levels in capillary luminal membranes and at higher levels in brain capillaries and whole membranes. In brain tissues, the expression in capillary luminal membranes was 0.4-fold that in brain capillaries and 0.3-fold that in whole membranes (Table 1). In heart and lungs, the ratio of expression levels between capillary luminal membranes and whole membranes was only 0.2-fold. Thus very little anti-luminal membrane contaminated the capillary luminal membrane preparations. Collagen IV (185 kDa) was found in capillary luminal membranes from the three tissues studied. In brain tissues, it was enriched 7.5-fold in capillary luminal membranes compared with whole membranes (Table 1) but only 0.3-fold when compared with brain capillaries. In heart, the expression level was lower in capillary luminal membranes than in whole membranes (0.2-fold) and in lungs the expression level was similar in both capillary luminal membranes and whole membranes. These lower detection levels, compared with brain capillaries and whole membranes from heart and lungs, exclude the possibility of a major contamination by basal laminae in all capillary luminal membrane preparations.

DISCUSSION

Isolation of the luminal membranes from endothelial cells of blood vessels of the lungs, devoid of blood cells, with high yield and purity was previously accomplished by Jacobson et al. [17]. This method takes advantage of the exposed nature of luminal membranes of the endothelial cells to coat them, by perfusion, with a layer of cationic colloidal silica which is then overcoated with a polyanion cross-linker. The density of the luminal membranes is thus changed, allowing their isolation by centrifugation. In this paper, the method was applied to lung, heart and brain.

The high level of P-gp expression and its strong enrichment in brain capillary luminal membranes confirmed its localization on the luminal side of endothelial cells forming the blood vessels of the brain. This also suggests a fundamental role for P-gp at this site. Possible involvement of P-gp in the blood–brain barrier was previously demonstrated using various methods. Transport of vincristine by primary cultured bovine brain capillary endothelial cells, which is vectorial, suggests that P-gp functions as a drug-efflux pump at the luminal side of the endothelial cells of brain capillaries [25]. This hypothesis is reinforced by the vectorial transport of cyclosporin A, suggesting a detoxifying role for P-gp [26]. Mice with genetic disruption of class I P-gp accumulate higher levels of drugs in the brain, supporting the notion that P-gp is involved in blood–brain barrier functions [27]. These results suggest that P-gp may contribute to multidrug resistance in brain tumours by blocking drug entry into the brain or tumour cells.

Detection of P-gp in luminal membranes from lungs suggests that it is also localized on the luminal side of the lung endothelium. However, the relatively weak enrichment suggests that it may also be located in other lung cells. In fact, the presence of P-gp in bronchial cells has previously been reported [28]. In the heart, P-gp was detected in endothelial luminal membranes but it does not appear to be restricted to this region since it was not enriched in luminal membranes. The detection of P-gp in the myocardium [28] may explain the absence of enrichment in the heart capillary luminal membranes.

Proteins other than P-gp were studied as controls to evaluate the purity of the luminal membrane preparations. The strong enrichment of GLUT1 (55 kDa isoform) in luminal membranes from brain endothelium indicates that the material is vascular endothelium. The absence of the 45 kDa isoform indicates that the luminal membrane preparation is not contaminated with non-endothelial cells since this isoform was shown to be associated with such cells [29]. However, the presence of the 45 kDa isoform in whole heart membranes and its absence from heart capillary luminal membranes was expected since it was previously demonstrated in cardiac myocytes [30,31]. This indicates that there was no major contamination of the heart capillary luminal membrane preparations by myocytes.

Contamination of luminal membrane preparations by astrocytes, Golgi apparatus, endoplasmic reticulum, anti-luminal membranes and basal laminae was evaluated by immunodetection of GFAP, adaptin, IP_3R-3, integrin αv and collagen IV respectively. Absence or very low enrichment of all these markers in the luminal membrane fractions from each tissue showed that the preparations were not contaminated by these membranes to any great extent. Thus the strong co-enrichment of P-gp (400–500-fold) and GLUT1 (240–280-fold) in brain capillary luminal membranes compared with whole brain membranes versus low enrichment of other proteins such as IP_3R-3 (9.2-fold) and collagen IV (7.5-fold) and still lower enrichment of other markers show clearly that P-gp is mainly a luminal protein in the brain vascular endothelium.

In conclusion, in the lungs and heart, P-gp is present in endothelial luminal membranes but is not restricted to this site. In the brain endothelium, the luminal membrane appears to be the major site of P-gp expression. This localization of P-gp adds a new and important dimension to the tissue distribution of anticancer drugs, since it indicates that part of the multidrug resistance in the clinical treatment of cancer could originate from the vascular endothelium, in addition to the induced expression of P-gp in the drug-resistant cancer cells.

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