P-Glycoprotein Is a Dimer in the Kidney and Brain Capillary Membranes: Effect of Cyclosporin A and SDZ-PSC 833†

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ABSTRACT: Radiation−inactivation studies were performed in order to elucidate the oligomeric nature of P-glycoprotein (P-gp) expressed in brain capillaries and renal brush border membranes (BBMs). Irradiation of renal BBMs resulted in a dose-dependent loss of P-gp, which corresponded to a target size (TS) of 255 and 211 kDa, as detected by Western blot and [^{125}I]arylazidoprazosin labeling, respectively. Similar TSs were determined for P-gp expressed in brain capillaries. These TSs correspond to approximately twice the size (120 kDa) of deglycosylated P-gp. Furthermore, the estimated TS for P-gp was not significantly different when renal BBMs were incubated with SDZ-PSC 833 (PSC) prior and during exposure to ionizing radiation. To confirm these results, the size of P-gp was evaluated from its mobility on blue-native polyacrylamide gels followed by Western blot analysis. Using this method, an apparent molecular size of 334 and 264 kDa was determined for P-gp in brain capillaries and renal BBMs, respectively. This corresponds to approximately twice the size of the glycosylated monomeric subunit of P-gp in brain capillaries (162 kDa) or renal BBMs (140 kDa). P-gp expressed in renal BBMs isolated from rats which had been treated daily with cyclosporin A (CsA) or PSC also migrated as a 264 kDa protein. These results suggest that P-gp exists mainly as a dimer in normal tissues and that resistance modulators such as CsA and PSC do not alter its oligomeric state.

Multidrug resistance (MDR)[1] is a phenomenon that is often associated with decreased intracellular drug accumulation resulting from enhanced drug efflux and is related to the overexpression of a membrane protein, P-glycoprotein (P-gp), at the surface of tumor cells (Georges et al., 1990b; Gottesman & Pastan, 1993). P-gp is thought to be responsible for the energy-dependent efflux of anticancer drugs, thereby reducing their cytotoxicity. Many compounds have been found to be potent inhibitors of P-gp activity, thus restoring the sensitivity of cancer cells to chemotherapeutic agents (Ford & Hait, 1990; Lum et al., 1993). These compounds have been termed resistance modifying agents (RMA) and include the immunosuppressive agent cyclosporin A (CsA) and its nonimmunosuppressive derivative SDZ-PSC 833 (PSC) (Boesch et al., 1991; Twentyman, 1992).

The human and mouse P-gps consist of homologous sequences of 1280 and 1276 amino acids, respectively, with potential glycosylation sites on the first extracellular loop, and a predicted molecular mass of 140 kDa (Chen et al., 1986; Gros et al., 1986). This protein has 12 predicted hydrophobic membrane segments with two cytoplasmic domains each comprising an ATP-binding sequence. The *mdr* gene products are expressed, not only in multidrug resistant tumor cells, but also in a variety of normal tissues such as the kidney and the capillaries forming the blood-brain barrier (Lieberman et al., 1989; Thiebaut et al., 1989; Cordon-Cardo et al., 1990; Jetté et al., 1995b). The expression of P-gp in these two tissues suggests that it could protect the organism against toxic xenobiotic compounds, by excreting these substances into urine, and by preventing their accumulation in critical organs such as the brain. The hypothesis of a protective role for P-gp in normal tissues has been reinforced by the generation of mice homozygous for a disruption of the class I isoform (Schinkel et al., 1994). Injection of vinblastine or ivermectin into these mice lacking cellular expression of P-gp resulted in elevated drug levels in many tissues, especially in the brain. However, little is known concerning the regulation of P-gp function in normal tissues.

Cross-linking (Naito & Tsuruo, 1992) and radiation−inactivation experiments (Boscoboinik et al., 1990) performed with membranes from multidrug resistant cells suggest that P-gp exists as a dimer in cancer cells. The isolation of detergent-solubilized P-gp by sucrose gradient velocity sedimentation has raised the possibility that P-gp may exist as mono-, di-, tri- and tetramers in MDR cells (Poruchynsky & Ling, 1994). Furthermore, the dynamics of P-gp oligomer formation and dissociation may be important in the mechanism of action of this protein and the

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[1] Abbreviations: BN-PAGE, blue-native polyacrylamide gel electrophoresis; BBM, brush border membrane; CrEL, Cremophor EL; CsA, cyclosporin A; IAAP, [^{125}I]iodoarylazidoprazosin; mAb, monoclonal antibody; MDR, multidrug resistance; P-gp, P-glycoprotein; PB, physiological buffer; pAb, polyclonal antibody; PSC, SDZ-PSC 833 ([3′-keto-bmt1]-[val2]-cyclosporin); PVDF, polyvinylidene difluoride; RIS, radiation-inactivation size; RMA, resistance modifying agents; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TS, target size; TBS, Tris-buffered saline.

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modulation of its activity (Porunchynsky & Ling, 1994). It has been demonstrated that the binding of the monoclonal antibody MC 57 to its conformational epitope on the P-gp molecule is enhanced following exposure of cancer cells to PSC (Jachez et al., 1994). This suggests that RMA such as PSC could alter P-gp activity by modifying its conformation. This phenomenon could, or could not, implicate an alteration of P-gp oligomeric state. Since the quaternary structure of P-gp expressed in normal tissues has not yet been established, we have used two different methods, radiation-inactivation (Harmon et al., 1985; Beauregard et al., 1987; Jung, 1988; Béliveau & Potier, 1989) and blue-native polyacrylamide gel electrophoresis (BN-PAGE) (Schägger et al., 1994), to study the oligomeric state of P-gp expressed in renal brush border membranes (BBMs) and brain capillaries. Our results suggest that P-gp exists mainly as a dimeric complex in these two tissues and that the oligomeric state of the protein is not influenced by the presence of RMA such as CsA and PSC.

EXPERIMENTAL PROCEDURES

Chemicals. Monoclonal antibody C219 (mAb C219) was purchased from ID Labs (London, ON), and the polyclonal antibody (pAb) α-*mdr* (Ab-1) was obtained from Cedarlane Laboratories (Hornby, ON). Horseradish peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit IgGs and enhanced chemiluminescence (ECL) reagents were from Amersham (Oakville, ON). [^{125}I]Iodoarylazidoprazosin (2200 Ci/mmol) was purchased from Dupont-New England Nuclear (Markham, ON). SDZ-PSC 833 and cyclosporin A were provided by Novartis Pharmaceuticals Canada Inc. (Dorval, PQ). Dextran T-70 was purchased from Pharmacia LKB Biotechnology (Montréal, PQ) and 6-aminocaproic acid, Tricine and Bistris were from Calbiochem (La Jolla, CA). All other reagents were from Sigma (St. Louis, MO).

Isolation of Brain Capillaries and Renal Brush Border Membranes. Renal BBMs were prepared from Sprague−Dawley rats by $MgCl_2$ precipitation (Booth & Kenny, 1974). Purified membranes were resuspended in a cryoprotective medium composed of 150 mM KCl, 14% (w/v) glycerol, 1.4% (w/v) sorbitol, and 20 mM Hepes/Tris, pH 7.5, and stored at −80 °C. For capillary isolation, the rat brains were cleared of meninges, superficial large blood vessels, and choroid plexus. The cerebral cortex was homogenized in 5 vol of physiological buffer (PB) composed of 147 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose, and 15 mM Hepes/Tris, pH 7.4, with a Polytron homogenizer (Brinkman Instruments, Rexdale, ON). The homogenates were mixed with an equal volume of 31% (w/v) Dextran T-70 in PB. Brain capillaries were then purified according to the procedure of Dallaire et al. (1991). The final pellets containing isolated microvessels were resuspended in a cryoprotective medium composed of 14% (w/v) glycerol and 1.4% (w/v) sorbitol in PB, and stored at −80 °C until use. γ-Glutamyltranspeptidase activity was assayed as described previously (Jetté et al., 1995a). Protein content was determined with the method of Bradford (1976).

Cyclosporin A and SDZ-PSC 833 Treatments. Male Sprague−Dawley rats weighing 275−300 g were treated with subcutaneous injections of CsA or PSC. For all treatments, each group of animals was composed of three rats. The drugs were used in PB with 15% (w/v) Cremophor EL (CrEL) and 3% (w/v) ethanol. The control groups received the vehicle composed of PB, CrEL, and ethanol. Rats were injected daily with CsA or PSC [10 mg/(kg·day)] for 10 days. The animals were sacrificed by decapitation 24 h after the last injection followed by renal BBM and brain capillary isolation.

Radiation−Inactivation Studies. Frozen samples were exposed to γ-rays in a Model 220 Gammacell ^{60}Co irradiator (Atomic Energy of Canada) at a dose rate of approximately 0.7 Mrad/h (Beauregard et al., 1983; Béliveau et al., 1988). During this procedure, the samples were kept at −78 °C with crushed dry ice. The radiation−inactivation size (RIS) for γ-glutamyltranspeptidase was calculated from the rate of decay of the enzyme activity as a function of radiation dose using the following empirical equation (le Maire et al., 1990), which is valid for samples irradiated at −78 °C and was derived from the calibration published by Kepner and Macey (1968):

$$\text{RIS or TS} = 1.29 \times 10^6/D_{37} \qquad (1)$$

where D_{37} is the radiation dose (in megaradians) at which the measured activity has decreased to 37% of its initial value. The same equation was used to estimate the target size (TS) of P-gp, which was calculated from the rate of fragmentation of its constituting polypeptide, detected by Western blot and photolabeling analyses, as a function of irradiation dose. The D_{37} values were obtained from semilogarithmic plots of the remaining activity or protein versus irradiation dose using a least-squares fit. The fragmentation TS of proteins separated by SDS−polyacrylamide gel electrophoresis was estimated following staining of the gel with Coomassie blue and measurement of the absorbance of the band corresponding to the intact protein by laser densitometry. In these experiments, the TS was also estimated using eq 1, which was calculated from the rate of disappearance of the stained protein band as a function of irradiation dose. The values quoted in the text represent means ± SE.

Detection of P-Glycoprotein. P-glycoprotein was detected by Western blot analysis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the method of Laemmli (1970). Membranes were resuspended in sample buffer to a final protein concentration of 1 mg/ml and loaded onto 6.0 or 7.5% acrylamide/bis(acrylamide) (29.1:0.9) gels without prior heating. The proteins were transferred electrophoretically onto a 0.45 μm pore size polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). P-gp was detected with mAb C219, as described previously (Jetté et al., 1996), or pAb α-*mdr* (Ab-1). Both antibodies recognize linear epitopes corresponding to amino acid sequences conserved among the three P-gp isoforms. pAb α-*mdr* (Ab-1) was diluted in Tris-buffered saline (TBS, pH 7.4) containing 0.05% Tween-20 and 1% (w/v) bovine serum albumin. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as secondary antibody. Detection was carried out using ECL reagents according to the manufacturer's instructions. The blots were exposed to preflashed Fuji films.

Photoaffinity Labeling with [^{125}I]Iodoarylazidoprazosin. BBM or brain capillary proteins (50 μg) were incubated with 20 nM of [^{125}I]iodoarylazidoprazosin (IAAP) in a reaction buffer containing 20 mM Tris/HCl, pH 8.0, and proteinase

inhibitors (2 μg/mL aprotinin, 10 μg/mL pepstatin A, and 100 μg/mL bacitracin). The incubation was carried out in the dark for 1 h at 25 °C and followed by cross-linking under a UV Stratalinker 2400 lamp (Stratagene, La Jolla, CA) at 254 nm for 5 min at 4 °C. The labeled P-gp was recovered by immunoprecipitation with mAb C219 (1 μg/assay), as described previously (Jetté et al., 1996). The immunoprecipitated proteins were electrophoresed on a 6.0% SDS−polyacrylamide gel. The photolabeled P-gp was detected following exposure of the gels to preflashed Kodak films with an intensifying screen (Picker, Montréal, PQ) at −80 °C for 2−4 weeks.

Blue-Native Polyacrylamide Gel Electrophoresis. BN-PAGE was performed according to Schägger et al. (1994). Brain capillary proteins (100 μg) were solubilized with 1% (w/v) Triton X-100 in a buffer containing 750 mM 6-aminocaproic acid and 50 mM Bistris, pH 7.0, and centrifuged at 40000*g* for 20 min. Shortly before starting BN-PAGE, Coomassie blue G-250 (Baker, Toronto, ON) was added from a 5% (w/v) stock solution in 500 mM 6-aminocaproic acid to obtain a 1/1 (g/g) detergent/Coomassie blue ratio. Samples (10 μg of protein per lane) were loaded onto 7.5% Bistris-polyacrylamide/(bisacrylamide) (48:1.5) gels overlayed by a 4% sample gel. The anode buffer contained 50 mM Bistris/HCl, pH 7.0 (4 °C). The cathode buffer was composed of 50 mM Tricine, 15 mM Bistris/HCl, pH 7.0 (4 °C), and 0.02% (w/v) Coomassie blue. Electrophoresis was performed at 4 °C using a Miniprotean II apparatus (Bio-Rad). It was started at 100 V until the sample was within the stacking gel and continued with current limited to 7.5 mA/gel. For better visualization of protein bands, the cathode buffer was replaced after one-third of the run by a cathode buffer containing 0.002% (w/v) Coomassie blue. Proteins were then transferred electrophoretically onto PVDF membrane for Western blot analysis.

Densitometric and Statistical Analyses. The intensity of the bands obtained from Western blot analyses and from photolabeling studies was estimated with a Personal densitometer SI (Molecular Dynamics, Sunnyvale, CA). Relative molecular mass determination was performed using the following standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa). For BN-PAGE, relative molecular mass determination was based on a calibration curve prepared with the following standards: thyroglobulin (669 kDa), mono- and dimeric forms of catalase (232 and 464 kDa), ferritin (440 kDa), and aldolase (158 kDa). Statistical analyses were performed using paired Student's *t* test with the StatView program and a Macintosh computer.

RESULTS

Gel Electrophoresis of Brain Capillary Proteins. The radiation−inactivation technique has been used to study the size and structure of both soluble and membrane-bound proteins (Kepner & Macey, 1968; Beauregard et al., 1983, 1987; Harmon et al., 1985; Béliveau et al., 1988; Jung, 1988; Béliveau & Potier, 1989; le Maire et al., 1990). Experiments have shown that this method can be applied to renal BBM vesicles to obtain molecular characteristics of transport systems (Béliveau et al., 1988; Béliveau & Potier, 1989). However, this method has never been used to study proteins from isolated brain capillaries, which is a metabolically active tissue. Experiments were performed to verify if the irradiation procedure and the composition of the cryoprotective medium used to study renal BBM proteins are adequate to study the oligomeric state of proteins found in isolated brain capillaries. The fragmentation of proteins separated by SDS−PAGE was first investigated. Exposure of brain capillaries to ionizing radiation (0−12 Mrad) resulted in a progessive destruction of proteins with molecular masses higher than 60 kDa, while those with lower molecular masses were less affected (Figure 1A). The TS of two arbitrarily chosen proteins, α and β, was estimated. The electrophoresis gel was scanned and the intensity of the bands corresponding to proteins α and β was evaluated and plotted as a function of the radiation dose (Figure 1B). On the basis of eq 1, the estimated target sizes of protein α and protein β were 143 ± 12 and 90 ± 9 kDa, respectively. The relative molecular mass of these proteins, according to a calibration plot of Rf obtained with the migration of protein standards, was 161 kDa for protein α and 89 kDa for protein β. The results obtained by the radiation−inactivation method are thus in good agreement with those obtained by SDS−PAGE.

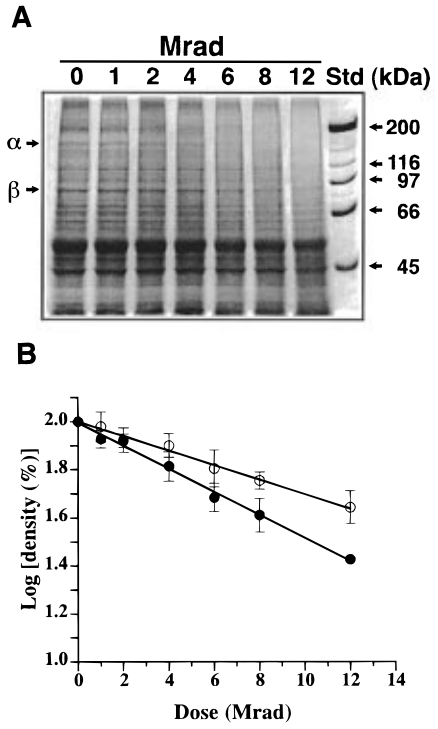

FIGURE 1: Polyacrylamide gel electrophoresis of irradiated brain capillary proteins. Isolated brain capillaries were irradiated and electrophoresis was performed in the presence of SDS, under reducing conditions. (A) Thirty micrograms of protein was used per lane. Staining was done with Coomassie blue. Samples were irradiated at doses ranging from 0 to 12 Mrad. Molecular mass standards (Std) are shown in the last lane. The position of protein α and protein β is indicated. (B) Disappearance of the band of proteins α (●) and β (○) was measured by laser densitometry. The results are expressed as the log of the percent band density relative to the unirradiated control ($n = 3$).

Molecular Size Determination of γ-Glutamyltranspeptidase. The inactivation of an intrinsic enzyme marker, γ-glutamyltranspeptidase, of brain capillaries was also evaluated. The activity of the enzyme decreased as a function of the radiation dose and the inactivation curve gave a molecular size of 66 ± 4 kDa (Figure 2). This value is similar to the size reported for the rat renal γ-glutamyltranspeptidase established from its nucleotide sequence (61 kDa) and to

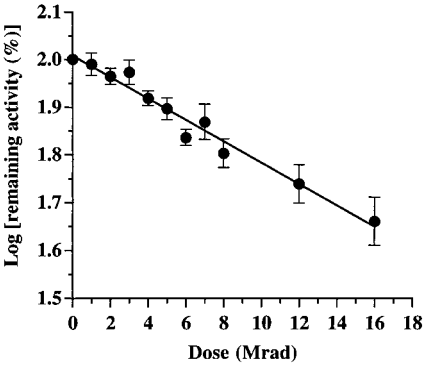

FIGURE 2: Radiation-inactivation curve for brain capillary γ-glutamyltranspeptidase. Brain capillaries were irradiated at doses ranging from 0 to 16 Mrad, and γ-glutamyltranspeptidase activity was measured as described in the Experimental Procedures. Results are expressed as the log of the percentage of activity remaining relative to that of the unirradiated control ($n = 4$).

the size of this heterodimeric enzyme (61−66 kDa) evaluated from its electrophoretic migration on SDS−polyacrylamide gel after deglycosylation (Frey, 1993). γ-Glutamyltranspeptidase is synthesized as a propeptide of 61 kDa and is subsequently cleaved to yield a heterodimer (20 and 41 kDa). The two subunits do not form homodimers, are not covalently linked, but contain hydrophobic domains which may serve as contact sites between them (Frey, 1993). Furthermore, both subunits are required for full functional activity of γ-glutamyltranspeptidase, and the catalytic properties of the enzyme are retained following its detergent solubilization. In the present study, the inactivation size estimated for γ-glutamyltranspeptidase is similar to the one measured for the detergent-solubilized enzyme. This confirmed that the radiation−inactivation procedure used to study renal BBM proteins is adequate for measuring the molecular size of brain capillary proteins.

Molecular Size Determination of P-Glycoprotein. Two different antibodies, one monoclonal (mAb C219) and one polyclonal (pAb α-mdr Ab-1), were used to detect P-gp by Western blot analysis in samples irradiated with doses up to 8 Mrad. Both antibodies recognize a linear cytoplasmic amino acid sequence within the P-gp (Georges et al., 1990a). Exposure to ionizing radiation resulted in a dose-dependent decrease in the labeling intensity of the brain capillary (Figures 3A and 4A) and kidney (Figures 3B and 4B) P-gps. A single immunoreactive band was detected with pAb α-mdr (Ab-1). mAb C219 detected, in addition to P-gp, a 190 kDa protein from brain capillaries which has been shown previously to be unrelated to P-gp (Beaulieu et al., 1995; Jetté et al., 1995b). The intensity of this cross-reactive protein band was not taken into account in the quantification of P-gp. In both tissues, the radiation-induced degradation of the P-gp molecule was a function of the radiation dose (Figure 3C and 4C). A semilogarithmic plot was used to fit the data to a single exponential dependance on radiation dose since there was a good correlation (Figure 3C, $r = 0.981$ and 0.988; Figure 4C, $r = 0.994$ and 0.980 for brain capillaries and renal BBMs, respectively) between the log of protein density and the irradiation dose. The target sizes calculated from experiments performed with the mAb C219 were 244 ± 14 kDa for the brain capillary P-gp and 255 ± 27 kDa for that of the renal BBMs. The target sizes calculated from experiments performed with the pAb α-mdr (Ab-1) were 268 ± 15 and 267 ± 21 kDa for the brain capillary and renal

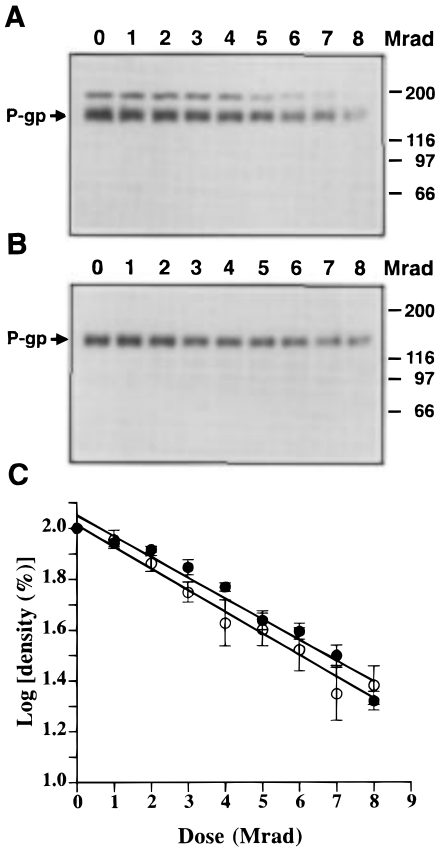

FIGURE 3: Determination of the target size of P-gp immunodetected with mAb C219. (A) Isolated brain capillaries and (B) renal BBMs were irradiated, and proteins (10 μg) were resolved by SDS−PAGE and transferred onto PVDF membranes. Immunodetection of P-gp was performed using mAb C219. (C) The intensity of the specifically labeled protein bands corresponding to proteins of $M_r = 162\,000$ from the brain capillaries (●) and $M_r = 140\,000$ from the renal BBMs (○) was measured by densitometric scanning of the autoradiograms. The results are expressed as the log of the percent band density relative to the unirradiated control ($n = 5$).

P-gps, respectively (Table 1). These results suggest that P-gp is expressed mainly as a dimer in both tissues. However, it remains possible that, in brain capillaries, some of the P-gp could be present as monomers since a resistance to low doses of radiation was observed (Figure 3C and 4C). Under our experimental conditions, we cannot eliminate completely this possibility, although the majority of P-gp is clearly inactivated as a dimer.

The fragmentation TS was also estimated using IAAP, a photoaffinity probe which is specific for P-gp (Greenberger et al., 1990). Brain capillary and renal BBM proteins were cross-linked with IAAP before exposure to ionizing radiation, and the photolabeled P-gp was then recovered by immunoprecipitation with mAb C219 and detected by autoradiography. A dose-dependent decrease in intensity of the brain capillary (Figure 5A) and the renal BBM (Figure 5B) photolabeled P-gp was observed. In both cases, the radiation-induced degradation of the labeled protein was a simple exponential function of the radiation dose (Figure 5C). The fragmentation target sizes estimated from these results were 204 ± 22 and 211 ± 40 kDa for P-gp expressed in brain capillaries and renal BBMs, respectively.

The effect of ionizing radiation on the IAAP binding activity of P-gp was studied to determine the P-gp oligomeric state required for IAAP binding. In these experiments, the

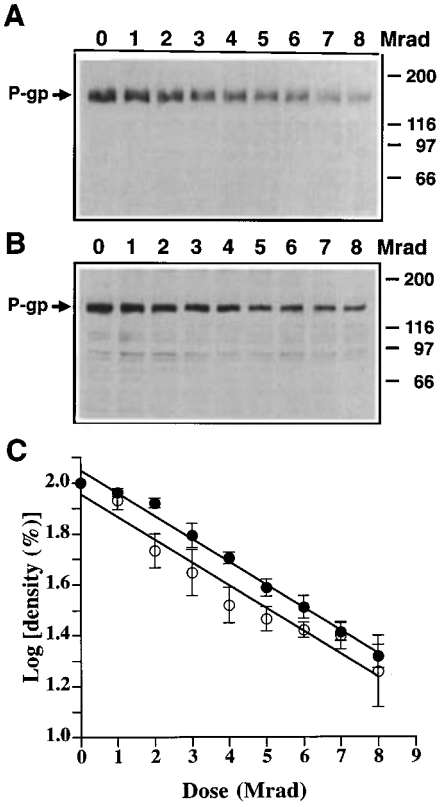

FIGURE 4: Determination of the target size of P-gp immunodetected with pAb α-mdr (Ab-1). (A) Isolated brain capillaries and (B) renal BBMs were irradiated, and proteins (10 μg) were resolved by SDS−PAGE and transferred onto PVDF membranes. Immunodetection of P-gp was performed using pAb α-mdr (Ab-1). (C) The intensity of the specifically labeled protein bands from brain capillaries (●) and renal BBMs (○) was measured by densitometric scanning of the autoradiograms. The results are expressed as the log of the percent band density relative to the unirradiated control ($n = 4$).

samples were exposed to ionizing radiation before cross-linking with IAAP, and the photolabeled P-gp was recovered by immunoprecipitation with mAb C219 and detected by autoradiography. A dose-dependent decrease in intensity of the brain capillary (Figure 6A) and the renal BBM (Figure 6B) photolabeled P-gp was observed. In both tissues, the radiation-induced degradation of the labeled protein was a simple exponential function of the radiation dose (Figure 6C). The RISs estimated for P-gp, as measured from its IAAP binding activity, were 215 ± 17 and 222 ± 27 kDa for brain capillaries and renal BBMs, respectively.

Molecular Size of P-gp Detected on Native Gels. BN-PAGE was used to separate native forms of P-gp. The addition of Coomassie blue G-250, which binds to hydrophobic domains on the surface of proteins, induces a charge shift and prevents their aggregation (Schägger et al., 1994). Proteins were solubilized with Triton X-100 and resolved on 7.5% Bistris-polyacrylamide gels. From migration distances and known molecular masses of the standard proteins, a calibration curve was deduced, which showed a linear relationship between log M_r and migration distance (Figure 7A). Brain capillary and renal BBM proteins were electrophoretically transferred onto PVDF membranes for P-gp immunodetection. Following this procedure, a diffuse protein band corresponding to P-gp was detected with pAb α-mdr (Figure 7B). This pattern of migration is probably due to differences in the level of glycosylation of the protein and to the percentage of polyacrylamide used to prepare the

Table 1: Molecular Sizes of P-Glycoprotein as Determined by Various Methods[a]

	brain capillaries		renal BBMs	
method	molecular size (kDa)	multimeric state	molecular size (kDa)	multimeric state
TS, Western blot, mAb C219	244 ± 14	1.7	255 ± 27	1.8
TS, Western blot, pAb α-*mdr*	268 ± 15	1.9	267 ± 21	1.9
TS, IAAP photolabeling	204 ± 22	1.5	211 ± 40	1.5
TS, Western blot, PSC-treated samples			285 ± 17	2.0
RIS, IAAP binding	215 ± 17	1.5	222 ± 27	1.6
BN-PAGE, mAb C219	334 ± 9	2.1	264 ± 13	1.9
BN-PAGE, pAb α-*mdr*	331 ± 3	2.0	265 ± 3	1.9
SDS−PAGE				
glycosylated form	162	1.0	140	1.0
deglycosylated form	120	1.0	120	1.0
molecular weight deduced from sequence	140		140	

[a] For the radiation-inactivation (TS and RIS) experiments, the multimeric state was calculated by dividing the measured molecular size by the molecular mass of P-gp, which was deduced from its amino acid sequence (Chen et al., 1986; Gros et al., 1986). For the BN-PAGE experiments, the multimeric state was calculated by dividing the measured molecular size by that of the glycosylated forms of P-gp. The apparent molecular sizes of the glycosylated and deglycosylated forms of P-gp were determined from their migration on SDS−polyacrylamide gel followed by Western blot analysis and from Lieberman et al. (1989) and Jetté et al. (1995b).

gels. Although P-gp migrated as a sharper protein band on a 10% polyacrylamide gel (not shown), the experiments were performed using 7.5% polyacrylamide gels since the standard proteins (M_r 158000−669000) were better resolved in these latter conditions. The average molecular size of P-gp was evaluated by measuring the migration distance from the middle of the immunoreactive protein bands. Using this procedure, a molecular size of 331 and 265 kDa was determined for P-gp detected in brain capillary and renal BBM samples, respectively. Furthermore, P-gp from renal BBMs migrated as a 259 kDa protein when a 10% polyacrylamide gel was used to separate proteins (not shown). Similar values were obtained when the detection was made using mAb C219 (Table 1) or when the samples were solubilized with *n*-dodecyl β-D-maltoside (results not shown). No immunoreactive band corresponding to the molecular size of the monomeric subunit of P-gp (140−170 kDa) was detected. When the samples were solubilized with Triton X-100 and resolved by SDS-PAGE, P-gp from brain capillaries and renal BBMs migrated as 162 and 140 kDa proteins, respectively (Figure 7B).

Influence of Resistance Modifying Agents on the Oligomeric State of P-gp. The influence of RMA on the oligomeric state of P-gp was studied since it has been suggested that PSC, a nonimmunosuppressive analog of CsA, could alter P-gp activity by modifying its conformation, a phenomenon which could implicate alteration of the oligomeric state of the protein (Jachez et al., 1994). To see if PSC could alter the size of P-gp measured in radiation−inactivation studies, samples were incubated with this drug (2 μg/mL) prior and during exposure of the membranes to ionizing radiation. Proteins were then resolved by SDS−PAGE and P-gp detected by Western blot analysis using the pAb α-mdr (Ab-1). A progressive decrease in the amount of P-gp was detected (Figure 8A). A semilogarithmic plot of these results indicated a simple exponential dependence

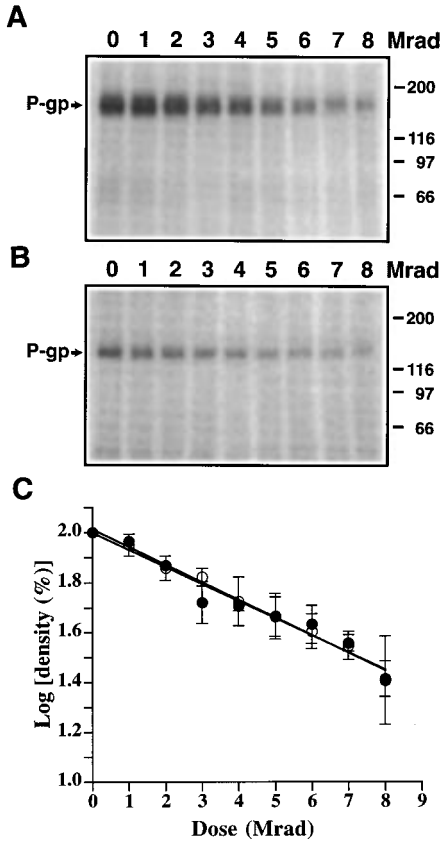

FIGURE 5: Determination of the target size of P-gp photolabeled with IAAP. (A) Proteins (50 μg) from isolated brain capillaries and (B) renal BBMs were incubated with IAAP, cross-linked under UV light and submitted to ionizing radiation. P-gp was then immunoprecipitated with mAb C219 (1 μg/assay), and the gels were exposed to preflashed Kodak films at −80 °C for 2−4 weeks. (C) The intensity of the specifically photolabeled protein bands from brain capillaries (●) and renal BBMs (○) was measured by densitometric scanning of the autoradiograms. The results are expressed as the log of the percent band density relative to the unirradiated control ($n = 3$).

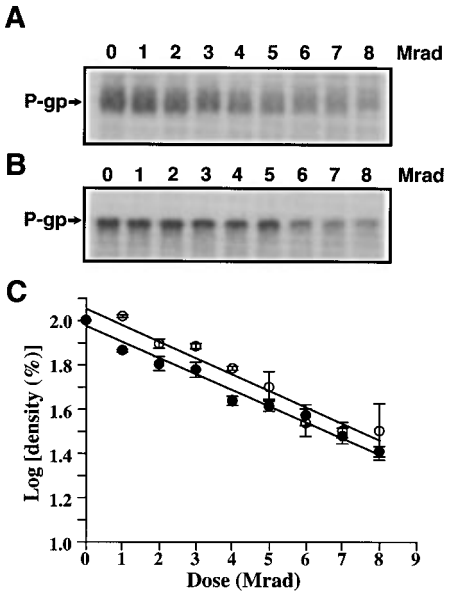

FIGURE 6: Determination of the radiation-inactivation size of P-gp from its IAAP binding activity. (A) Brain capillary and (B) renal BBM samples were submitted to ionizing radiation, and proteins (50 μg) were incubated with IAAP and cross-linked under UV light. P-gp was then immunoprecipitated with mAb C219 (1 μg/assay) and the gels were exposed to preflashed Kodak films at −80 °C for 2−4 weeks. (C) The intensity of the specifically photolabeled protein bands from brain capillaries (●) and renal BBMs (○) was measured by densitometric scanning of the autoradiograms. The results are expressed as the log of the percent band density relative to the unirradiated control ($n = 2$).

on the radiation dose (Figure 8B). The molecular size determined for P-gp was 285 ± 17 kDa, a value which is not significantly different from the size estimated for non-treated samples (Table 1) using pAb α-mdr (Ab-1) ($p = 0.679$) or mAb C219 ($p = 0.549$).

The influence of RMA on P-gp oligomerization was also evaluated by treating rats with daily injections of CsA or PSC for 10 days. At the end of the treatments, the animals were sacrificed and renal BBMs were purified. Proteins from these samples were resolved by BN-PAGE and processed for Western blot. P-gp was detected with the pAb α-mdr (Ab-1) in samples from both control and treated animals (Figure 9). This antibody detected a more than 2-fold increase in P-gp expression in membranes isolated from CsA- and PSC-treated rats, as we have reported previously (Jetté et al., 1996; Jetté et al., personal communication). This increase in P-gp expression was visualized as darker and larger immunoreactive bands, both in samples from CsA- and PSC-treated animals. This widening of the immunodetected protein bands could reflect an alteration of the postranslational modifications of the overexpressed P-gp, such as phosphorylation or glycosylation. It has been demonstrated that the level of glycosylation alters the electrophoretic mobility of P-gp on polyacrylamide gels (Greenberger et al., 1997; Ichikawa et al., 1991). However, the molecular size of the immunodetected P-gp was similar in all samples (265 kDa). Similar results were obtained when the detection was made using the mAb C219. This demonstrates that PSC and CsA treatments did not alter the oligomeric state of P-gp expressed in renal BBMs, even if these drugs increased the expression level of the protein.

DISCUSSION

The sizes of two arbitrarily chosen brain capillary proteins estimated by a standard SDS−PAGE procedure and by the radiation-inactivation method combined with densitometric evaluation of stained proteins were found to be similar. In addition, the size of γ-glutamyltranspeptidase obtained by the radiation−inactivation method was similar to the functional size reported for the detergent-solubilized enzyme (Frey, 1993). These control experiments demonstrated that the irradiation procedure and the composition of the cryoprotective medium used to study renal BBM proteins are adequate to study the oligomeric state of proteins from isolated brain capillaries.

Western blot analysis was used to detect P-gp in irradiated samples since it was shown that the amount of polypeptide detected with mAb C219 is a linear function of the amount of protein loaded onto the gel for rat brain capillaries (Jetté et al., 1993) and cancer cells (Boscoboinik et al., 1990). The radiation fragmentation sizes (TS) reported here for P-gp expressed in renal BBMs and brain capillaries vary between 244 and 268 kDa, as detected by Western blot analysis using two different antibodies. Following endoglycosidase/N-glycanase F treatment, the apparent molecular mass of the protein component of P-gp has been estimated to 120 000 in these two tissues (Lieberman et al., 1989; Jetté et al., 1995b). This size is similar to the size evaluated for P-gp

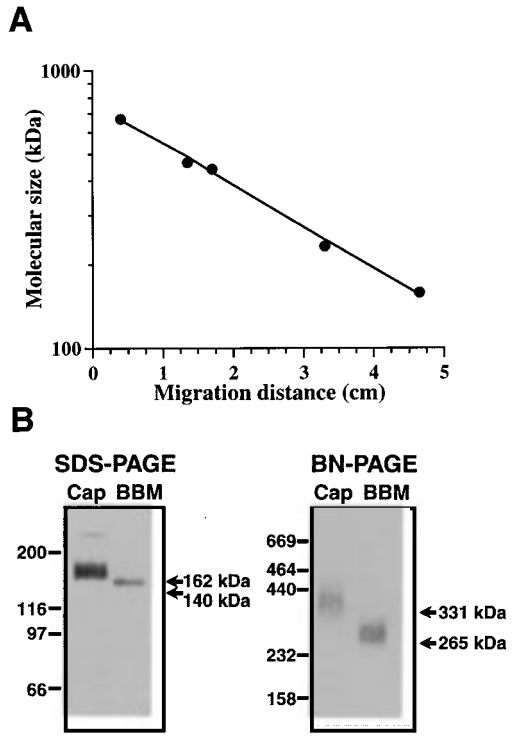

FIGURE 7: Blue-native gel electrophoresis of brain capillary and renal BBM proteins. Renal BBM and brain capillary proteins were solubilized with Triton X-100 and Coomassie blue G-250 was added to the samples. Proteins (10 μg) were then loaded onto a 7.5% Bistris−polyacrylamide gel. The electrophoresis was performed at 4 °C in the presence of Coomassie blue G-250 in the cathode buffer. Proteins with known molecular mass (see Experimental Procedures) were used as molecular standards and were prepared as described for the renal BBM and brain capillary samples. (A) The calibration curve of a representative experiment is shown and was deduced from migration distances of the molecular standards. (B) Proteins separated by SDS− and BN-PAGE were transferred onto PVDF membranes and P-gp was detected using the pAb α-mdr (Ab-1) (n = 4).

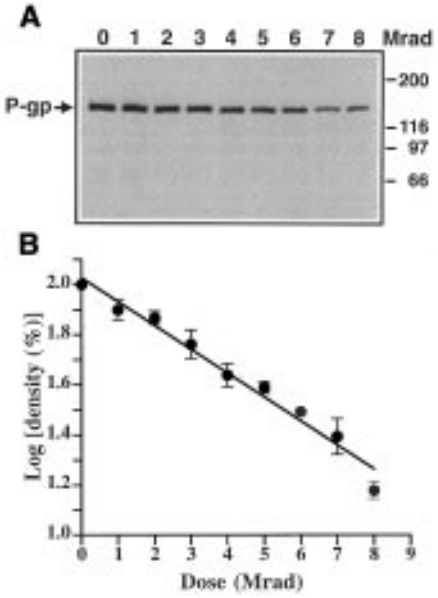

FIGURE 8: Determination of P-gp target size following incubation of renal BBMs with PSC. Renal BBM samples were incubated with PSC (2 μg/mL) prior to exposure of the membranes to ionizing radiation. The drug remained present during the course of the irradiation procedure. (A) Proteins (10 μg) were resolved by SDS−PAGE and transferred onto PVDF membranes. Immunodetection of P-gp was performed using pAb α-mdr (Ab-1). (B) The intensity of the specifically immunolabeled protein bands was measured by densitometric scanning of the autoradiograms. The results are expressed as the log of the percent band density relative to the unirradiated control (n = 3).

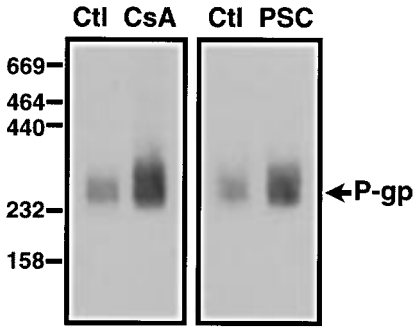

FIGURE 9: Detection of P-gp in renal BBMs isolated from CsA- and PSC-treated rats. Rats were treated with daily injections of Cremophor EL (Ctl) or 10 mg/(kg d) of CsA or PSC. Rats were sacrificed 24 h after the last injection and renal BBMs were isolated from the different animal groups. Following solubilization with Triton X-100, protein samples (10 μg) were resolved by BN-polyacrylamide gel electrophoresis, and immunoblots were performed using pAb α-mdr (Ab-1), as described in the Experimental Procedures (n = 3).

expressed in cancer cell lines following deglycosylation (Greenberger et al., 1987; Ichikawa et al., 1991), which is slightly smaller than the size (140 kDa) calculated from the amino acid sequence deduced from the nucleotide sequence of the *mdr* genes (Chen et al., 1986; Gros et al., 1986). Since the molecular size determined by radiation−inactivation is independent of the sugar content of a glycoprotein, the TS measured in the present study suggest that P-gp exists as a dimer in renal BBMs and brain capillaries.

The fragmentation TSs were also determined using the photoaffinity probe IAAP to detect P-gp. The fragmentation sizes estimated with this method were greater (204−211 kDa) than the monomeric size of P-gp calculated from its amino acid sequence. These results are in agreement with those obtained in Western blot experiments which suggested that P-gp exists as an oligomeric complex in biological membranes. However, these fragmentation sizes (average of 208 kDa) were about 4/5 of the TS determined in Western blot experiments (average of 259 kDa). The complexity of the technical procedure, which included solubilization and immunoprecipitation steps, could be responsible for the smaller TS evaluated in the photolabeling studies.

The oligomeric state of P-gp was also evaluated according to its electrophoretic migration on nondenaturing gels in the presence of Coomassie blue. In contrast with those measured with the radiation−inactivation method, the size of a protein evaluated from its migration on a native gel can be influenced by the glycosylation state of a glycoprotein. In this system, P-gp from brain capillaries and renal BBMs migrated as a 331 and 265 kDa protein, respectively. This is approximately twice the size of the glycosylated monomeric subunit of P-gp estimated from its electrophoretic mobility on SDS−polyacrylamide gels followed by Western blot analysis (Table 1). This tissue difference in the size of P-gp is likely to be due to differences in the carbohydrate content of the protein since it was shown that P-gp expressed in both tissues migrates as a 120 kDa protein following N-glycanase treatment (Lieberman et al., 1989; Jetté et al., 1995b). The large sizes measured with this technique is unlikely to be due to aggregation of proteins or formation of large lipid−

protein−detergent complexes since solubilization was performed in the presence of a high concentration of Triton X-100 (Helenius & Simons, 1975), and similar apparent molecular sizes were found when solubilization was performed using *n*-dodecyl *β*-D-maltoside as detergent. The presence of 6-aminocaproic acid and the absence of divalent cations in the solubilization medium and the gel buffer, plus the addition of Coomassie blue to the samples also prevented aggregation of proteins.

Oligomerization of P-gp in normal tissues is in agreement with other studies showing that this protein exists as an oligomeric structure in cancer cell lines. The characterization of the discontinuous epitope recognized by the monoclonal antibody MRK-16 has suggested that P-gp may exist as a dimer to form a pore structure (Georges et al., 1993). Experiments using the inactivation radiation method on isolated membranes have estimated a target size of 250 kDa for P-gp expressed in CH^RC5 cells (Boscoboinik et al., 1990). Chemical cross-linking experiments (Naito & Tsuruo, 1992) have demonstrated the existence of P-gp as a 340 kDa glycosylated complex in a leukemia cell line. These results suggest that P-gp exists as a dimer in cancer cell lines. However, mono-, di-, tri-, and tetrameric forms of P-gp have been detected from human and hamster MDR cell lines in a study which used sucrose gradient sedimentation velocity (Poruchynsky & Ling, 1994). Mono-, tri-, and tetrameric forms of P-gp were not detected in the present study using native gel electrophoresis. The radiation−inactivation experiments demonstrated that the fragmentation sizes (TS) of P-gp correspond to the size of the dimeric forms of this protein. These results suggest that, if tri- and tetrameric forms of P-gp are present in biological membranes, the subunits are not associated closely enough to allow energy transfer between the three or four subunits and cause the fragmentation of the entire oligomer. It is also possible that P-gp exists mainly as a dimer in biological membranes, and that the presence in much smaller amount of the mono-, tri-, and tetrameric forms of the protein could not be detected in the present study.

The oligomeric structure required for the binding of IAAP was investigated by submitting the samples to ionizing radiation before incubation with IAAP. The radiation−inactivation sizes estimated from the loss of IAAP binding activity (215−222 kDa) corresponded approximately to the size of the dimeric form of P-gp calculated from its amino acid sequence, suggesting again that P-gp dimers probably represent the dominant complexes of P-gp in the membrane.

PSC is one of the most potent reversal agents, being at least 10 times more potent than CsA (Boesch et al., 1991; Twentyman, 1992). It causes a persistent inhibition of P-gp transport function in MDR cells (Boesch & Loor, 1994) and of IAAP binding to P-gp expressed in renal BBMs (Jetté et al., personal communication). A similar inactivation size for P-gp was obtained in renal BBMs treated in the presence or absence of PSC. The size of P-gp, which is overexpressed in renal BBMs isolated from CsA- and PSC-treated animals (Jetté et al., 1996; Jetté et al. personal communication), was similar to the one detected in membranes isolated from control rats, as determined by native gel electrophoresis and immunodetection of the protein. These results suggest that RMA, such as CsA and PSC, do not alter P-gp function by modifying its oligomeric state. Modification of P-gp conformation by PSC has been proposed to explain its strong RMA activity since the binding of a specific antibody to P-gp is altered following exposure of resistant cells to this compound (Jachez et al., 1994). This suggests that PSC could modify the spatial organization of the dimeric P-gp complexes without altering its oligomeric state. Whether PSC is an allosteric modulator of P-gp remains to be established.

In conclusion, our results suggest that P-gp exists mainly as a dimer in renal BBMs and brain capillaries. Furthermore, regulation of P-gp function by CsA and PSC does not seem to involve an alteration of the oligomeric state of this transporter. However, P-gp oligomerization may be implicated in other aspects of its function such as substrate specificity. Further studies are still required to understand the physiological significance of P-gp multimerization.

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