Isoform I (mdr3) is the major form of P-glycoprotein expressed in mouse brain capillaries

Evidence for cross-reactivity of antibody C219 with an unrelated protein

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P-glycoprotein (P-gp) is expressed in various non-cancerous tissues such as the endothelial cells of the blood–brain barrier. We used several monoclonal antibodies (mAbs) and isoform-specific polyclonal antibodies to establish which P-gp isoforms are expressed in isolated mouse brain capillaries. P-gp class I isoform was detected in capillaries with a Western immunoblotting procedure using a specific antiserum. No immunoreactivity was observed with either class II- or class III-specific antisera. Immunoreactivity was observed with mAb C219. However, this antibody detected two distinct immunoreactive proteins (155 and 190 kDa) in the isolated brain capillaries. These two proteins comigrated as a broad band when the samples were submitted to heat prior to gel electrophoresis. The glycoprotein nature of these two antigens was evaluated by their sensitivity to N-glycanase treatment. Following this treatment, the size of the proteins was reduced from 190 and 155 kDa to 180 and 120 kDa, respectively. Triton X-114 phase-partitioning studies showed that the 190 kDa immunoreactive protein was poorly solubilized by Triton X-114, while the 155 kDa protein was partitioned in the detergent-rich phase. In labelling experiments, only the 155 kDa protein was photolabelled with [^{125}I]iodoarylazidoprazosin. These results show that a 190 kDa protein detected by antibody C219 is an antigen unrelated to the three P-gp isoforms presently known. Cross-reactivity of C219 with an unrelated protein emphasizes the fact that more than one antibody should be used in the assessment of P-gp expression in cell lines and tissues.

INTRODUCTION

Brain microvascular endothelial cells possess a unique structure which is responsible for their anatomical barrier properties. These capillaries possess tight interendothelial cell junctions, sparse pinocytic vesicular transport and an absence of pores or fenestrations [1]. Also, a thin basal lamina supports the ablumenal surface of the endothelial cells. Thus, substances entering or leaving the brain must pass through the endothelial cells and their luminal and abluminal membranes. The blood–brain barrier (BBB) has multiple physiological functions [2,3]. The BBB is a dynamic conduit for the transport between blood and brain of nutrients, peptides, proteins, or immune cells that have access to specific transport systems localized within the capillary membranes. This barrier protects the brain from many exogenous toxins and sudden fluctuations in the levels of systemic substances, such as neurotransmitters. The BBB also excludes a large number of xenobiotics and potentially therapeutic agents from the brain. This may be responsible for the low efficacy of chemotherapeutic treatments of brain tumours [4,5]. This intrinsic chemoresistance of the brain tissue may be due, at least in part, to the overexpression of P-gp at BBB sites.

P-gp, the product of *mdr* genes, is an active efflux pump for some antitumour agents in multidrug-resistant cancer cell lines [6,7]. P-gp is also expressed in non-cancerous tissues such as the endothelial cells of BBB capillaries [8–12]. Recent studies performed with cultured brain endothelial cells have shown that P-gp in BBB capillaries transports vincristine and interacts with progesterone [11,13]. Interactions of P-gp with lipophilic compounds suggest that this glycoprotein may contribute to the barrier function of the brain capillaries. However, a physiological role is still to be determined for P-gp expressed in BBB endothelial cells.

In rodents, P-gp is encoded by a small gene family comprising three *mdr* genes [14–17]. All currently identified P-gp isoforms can be grouped into three classes (see Table 1). Only isoforms I and II are capable of multidrug transport [14]. In the studies performed so far, it has never been clearly established which P-gp isoform is expressed in brain capillaries. Northern-blot analyses of *mdr* RNA from mouse brain have shown that P-gp class I and II isoforms are both present in this tissue [18]. These

Table 1 Classification of the murine P-gp genes (adapted from [14–17])

	P-glycoprotein		
Species	Class I	Class II	Class III
Hamster	P-gp1	P-gp2	P-gp3
Mouse	mdr3 (mdr1a)	mdr1 (mdr1b)	mdr2

Abbreviations used: BBB, blood–brain barrier; CHO, Chinese hamster ovary; IAAP, [^{125}I]iodoarylazidoprazosin; mAb, monoclonal antibody; MC, mouse brain capillary; P-gp, P-glycoprotein; PB, physiological buffer; TBS, Tris-buffered saline; MRP, multidrug-resistance associated protein; ECL, enhanced chemiluminescence.

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two P-gp isoforms have overlapping but distinct substrate specificities [14]. Determination of which P-gp isoform is predominantly expressed in brain capillaries is important for a better understanding of the biochemical properties and the physiological roles of P-gp in this tissue.

In this paper, we have used several monoclonal antibodies (mAbs) and specific polyclonal antibodies raised against each P-gp isoform to clearly establish that P-gp class I isoform is predominantly expressed in brain capillaries. We also report that, in our studies, the mAb C219 cross-reacted with two distinct proteins from brain capillaries. Biochemical characterization of these two immunoreactive proteins revealed that only one of these is likely to be a P-gp isoform, the identity of the other antigen remaining unknown. Therefore, mAb C219 should be used with caution, especially in clinical studies where this antibody is often used as a diagnostic tool for the detection of P-gp in tumours.

MATERIALS AND METHODS

Chemicals

Dextran T-70 was obtained from Pharmacia LKB Biotechnology (Montréal, Québec, Canada). Reagents for electrophoresis were products of Bio-Rad (Mississauga, Ontario, Canada). mAbs C219, C494 and JSB-1 were purchased from ID Labs (London, Ontario, Canada) and membrane fractions of the colchicine-resistant Chinese hamster ovary (CHO) cell line $CH^{R}C5$ were purchased from Centocor Diagnostics (Malvern, PA, U.S.A.). Specific antibodies for the three P-gp isoforms encoded by the mouse *mdr*1 (antiserum 61), *mdr*2 (antiserum 2080) and *mdr*3 (antiserum 2037) genes were kindly provided by Dr. Philippe Gros (McGill University, Montréal, Québec, Canada). Horseradish peroxidase-conjugated rabbit anti-(mouse IgG), horseradish peroxidase-conjugated goat anti-(rabbit IgG) and enhanced chemiluminescence (ECL) reagents were from Amersham (Oakville, Ontario, Canada). Endoglycosidase F/glycopeptidase F mixture was obtained from Boehringer (Laval, Québec, Canada). $[^{125}I]$Iodoarylazidoprazosin (IAAP, 2200 Ci/mmol) was purchased from Dupont-New England Nuclear (Markham, Ontario, Canada). All other reagents were from Sigma (St. Louis, MO, U.S.A.

Isolation of brain capillaries

Capillaries were isolated from C57BL/6 male or female mice. The brains were cleared of meninges, superficial large blood vessels and choroid plexus. The cerebral cortices were homogenized in five volumes of physiological buffer (PB) composed of 147 mM NaCl/4 mM KCl/3 mM $CaCl_2$/1.2 mM $MgCl_2$/5 mM glucose/15 mM Hepes/Tris, pH 7.4, with a Polytron homogenizer (Brinkman Instruments, Rexdale, Ontario, Canada). 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. [19]. The final pellets containing isolated microvessels were resuspended in PB and stored in liquid nitrogen until use. γ-Glutamyltranspeptidase enrichment of the capillary preparations was 12-fold that of the homogenate. Protein content was determined in all experiments using the method of Bradford [20], except for the fractionation studies using Triton X-114 where the protein content was determined with the bicinchoninic acid method [21].

Detection of P-gp

P-gp was detected by Western-blot analysis. SDS/PAGE was performed according to the method of Laemmli [22]. Capillaries were resuspended in sample buffer to a final protein concentration of 1 mg/ml and loaded on 6.0 or 7.5% acrylamide/bis-acrylamide (29.2:0.8) gels, with or without prior heating. The proteins were transferred electrophoretically to 0.45 μm pore-size Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). Blots were blocked overnight at 4 °C, with 5% (w/v) non-fat powdered milk in Tris-buffered saline (TBS) containing 50 mM Tris/150 mM NaCl/0.3% (w/v) Tween-20, pH 7.0. The membranes, washed three times with TBS, were incubated with the mAbs C219 (200 ng/ml), C494 (200 ng/ml) or JSB-1 (1:100 dilution) for 2 h at 37 °C. The rabbit polyclonal anti-mdr1 (antiserum 61), anti-mdr3 (antiserum 2037) and the purified anti-mdr2 (antiserum 2080) antibodies were all used at a 1:100 dilution, as described by Buschman et al. [23]. All antibodies were diluted in TBS containing 1% (w/v) BSA. Horseradish peroxidase-conjugated rabbit anti-(mouse IgG) and horseradish peroxidase-conjugated goat anti-(rabbit IgG) were used as secondary antibodies. Detection was carried out with ECL reagents according to the manufacturer's instructions. The blots, exposed to Fuji films, were scanned with an LKB Ultroscan XL densitometer. Molecular-mass determination was performed using the following standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa) and ovalbumin (43 kDa).

Electrophoretic analysis

Electrophoretic mobility of P-gp was analysed by the method of Laemmli [22]. Laemmli sample buffer was added to the capillary protein and the samples were boiled for 3 min, or the capillary proteins were solubilized in 0.5% (w/v) SDS for 30 min, centrifuged (8000 ***g***, 10 min), and the Laemmli sample buffer was added to the supernatant. The proteins were separated using a 6.0% polyacrylamide gel. The immunodetection using mAb C219 was performed as described previously.

Glycosidase treatment

The enzymic reactions were carried out in the presence of a cocktail of protease inhibitors: aprotinin (2 μg/ml), pepstatin A (10 μg/ml) and bacitracin (100 μg/ml). Brain capillaries were solubilized with 0.5% (w/v) SDS and 1% (v/v) 2-mercaptoethanol at room temperature for 5 min and then diluted 4-fold with 1% (w/v) octyl β-D-glucopyranoside/20 mM Tris/HCl, pH 8.0. Endoglycosidase F/glycopeptidase F mixture was then added at 2 units/mg of protein and the digestion was allowed to proceed for 16 h at room temperature. Laemmli sample buffer was added, without boiling. Western blots were carried out as described previously.

Fractionation with Triton X-114

Fractionation of brain capillary proteins with Triton X-114 phase-partitioning was performed as described by Vachon et al. [24]. Capillary proteins (5 mg/ml, determined with the bicinchoninic acid method [21]) were solubilized at 4 °C for 20 min in PB containing 2% (w/v) Triton X-114. The mixture was centrifuged at 100000 ***g*** for 30 min at 4 °C and the pellet was resuspended in PB containing 1% (w/v) SDS. The supernatant was layered over a cushion composed of 250 mM sucrose (1/10 volume of the sample) and incubated for 10 min at 30 °C. The phase separation was completed by centrifuging the turbid mixture at 2000 ***g*** for 5 min at 30 °C. The upper detergent-poor phase was separated from the lower detergent-rich phase with a Pasteur pipette. The different fractions were then mixed with Laemmli sample buffer and electrophoresed on a 7.5% SDS–

polyacrylamide gel. Immunoblot detection of P-gp was then performed using mAb C219, as described previously. The immunoreactive bands were evaluated with an LKB Ultroscan XL densitometer to evaluate their relative distribution and enrichment factor. The relative distribution in each fraction was expressed as a percentage of the total amount of the immunoreactive protein present in the capillaries before solubilization. The enrichment factors were the ratio of the relative area of the immunoreactive bands detected in each fraction and in the unfractionated brain capillaries.

Photoaffinity labelling

Isolated brain capillaries were incubated with the prazosin analogue IAAP (final concentration 20 nM) 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 for 1 h at 25 °C in the dark and followed by cross-linking under a Spectroline u.v. lamp (Fisher Scientific, Montréal, Québec, Canada) for 5 min at 4 °C. The unincorporated radioactive prazosin analogue was removed by centrifugation (8000 *g*, 10 min). The labelled P-gps were recovered by immunoprecipitation with mAb C219. Immunoprecipitation was carried out for 16 h at 4 °C in 0.25 ml of buffer containing 50 mM Tris/150 mM NaCl/0.1 % (w/v) SDS/1 % (w/v) Triton X-100/0.5 % (w/v) deoxycholate, pH 7.4. Immune complexes were isolated by incubation for 2 h at 25 °C with protein A–Sepharose beads, followed by two washes in buffer containing 50 mM Tris/150 mM NaCl/0.1 % (w/v) SDS/1 % (w/v) Triton X-100/0.5 % (w/v) deoxycholate, pH 7.4, one wash in buffer containing 50 mM Tris/150 mM NaCl/0.01 % (w/v) SDS/0.1 % (w/v) Triton X-100/0.05 % (w/v) deoxycholate, pH 7.4, and two final washes in buffer containing 50 mM Tris/150 mM NaCl, pH 7.4. The beads were then resuspended in Laemmli sample buffer and electrophoresed on a 6.0 % SDS–polyacrylamide gel. Unlabelled protein samples from isolated brain capillaries were also loaded on the same gels. The proteins were transfered electrophoretically to Immobilon-P membranes and exposed to Kodak films with an intensifying screen (Picker, Montréal, Québec, Canada) at −80 °C for 2 weeks. Immunoblot detection using mAb C219 was then performed, as described previously.

RESULTS

Detection of P-gp isoforms

The mAb C219 has been shown to be directed against peptide epitopes located at N-terminal position 568–574 (Val-Gln-Ala-Ala-Leu-Asp or Val-Gln-Val-Ala-Leu-Asp) and C-terminal position 1213–1219 (Val-Gln-Glu-Ala-Leu-Asp) in the amino acid sequence of P-gp [25]. The mAb C494 is directed against the peptide epitope located at C-terminal position 320–327 (Lys-Pro-Asn-Thr-Leu-Glu-Gly-Asn-Val) in the amino acid sequence of human and hamster P-gp class I isoform [25]. The mAb JSB-1 has been shown to recognize an internal cytoplasmic determinant of P-gp class I isoform which has not yet been characterized [26]. These different antibodies were used in Western-blot analyses to determine which P-gp isoform is expressed in mouse brain capillaries (MCs). A detergent extract prepared from a CHO cell line (CH^{R}C5) was used as control [27]. The three antibodies showed strong reactivity with P-gp in the plasma membrane of CH^{R}C5 cells. In mouse capillaries, antibody C219 detected a protein migrating as a broad band of molecular mass 190 kDa (Figure 1). Antibodies C494 and JSB-1 showed weak reactivity with a protein of molecular mass 170 kDa.

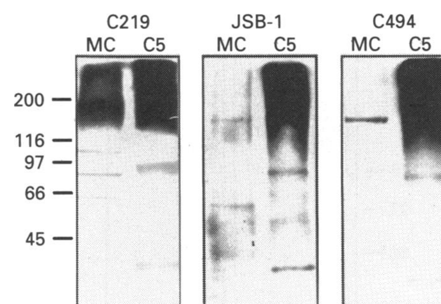

Figure 1 Detection of P-gp in brain capillaries using mAbs

Immunoblots were performed as described in the Materials and methods section. Protein samples (10 μg) from MCs (MC) and a membrane preparation of the CH^{R}C5 cell line (C5) were resolved by SDS/PAGE using a 7.5% polyacrylamide gel. mAbs C219, JSB-1 and C494 were used as primary antibodies. Horseradish peroxidase-conjugated rabbit anti-(mouse IgG) was used as secondary antibody and detection was carried out with ECL reagents ($n = 2$).

P-gp-related proteins in brain capillaries were further investigated using specific antibodies raised against each mouse P-gp isoform [23]. A purified membrane fraction from drug-resistant LR73 transfectants overexpressing the *mdr1/mdr2* chimeric cDNA containing the *mdr2* linker region inserted in *mdr1* were used as control (kindly provided by Dr. Philippe Gros, McGill University, Montreal). Both mdr1- and mdr2-specific antisera reacted strongly with this clone, as expected. However, these antibodies detected no cross-reactive protein in isolated MCs (Figure 2). The mdr3-specific antiserum, which was unreactive with the clone expressing mdr1 and mdr2, reacted with a 170 kDa protein, indicating that it is the P-gp class I isoform that is expressed in brain capillaries. However, the protein detected by the mdr3-specific antiserum had a size (170 kDa) different from that detected by mAb C219 (190 kDa). Further experiments were aimed at solving this ambiguity.

Characterization of immunoreactive proteins

The physicochemical properties of the antigen detected by mAb C219 were investigated. Electrophoresis was performed according to Laemmli [22]. As described earlier, when the samples were

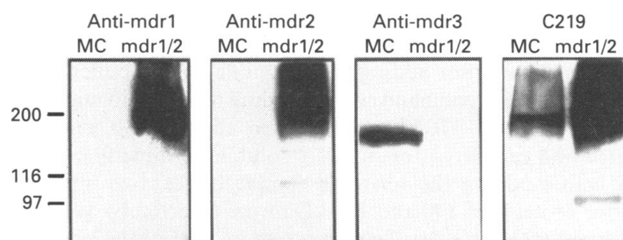

Figure 2 Detection of P-gp using isoform-specific antibodies

Protein samples (20 μg) from MCs (MC) and a purified membrane fraction from drug-resistant LR73 transfectants overexpressing the mdr1/mdr2 chimeric cDNA (mdr 1/2) were separated by SDS/PAGE using a 7.5% polyacrylamide gel. Immunoblots were performed as described in the Materials and methods section. The immunoblots were incubated with either mdr1-specific antiserum 61 (anti-mdr1), mdr2-specific antiserum 2080 (anti-mdr2), mdr3-specific antiserum 2037 (anti-mdr3) or mAb C219 (C219). Horseradish peroxidase-conjugated antibodies were used as secondary antibodies and detection was carried out using ECL reagents ($n = 2$).

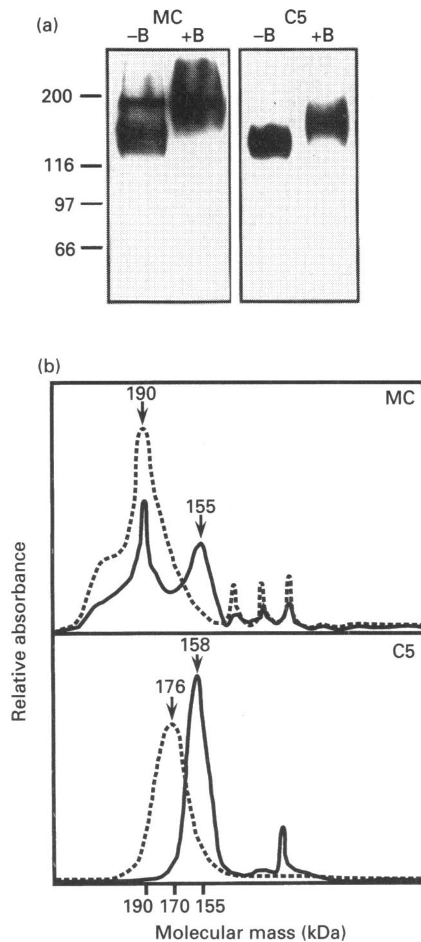

Figure 3 Effect of boiling on the electrophoretic mobility of P-gps

(**a**) Protein samples (20 μg) from MCs (MC) or membrane preparations (5 μg protein) of the CH^{R}C5 cell line (C5) were resolved by SDS/PAGE using a 6.0% polyacrylamide gel. Samples were either solubilized in 0.5% (w/v) SDS for 30 min (−B) before adding the Laemmli sample buffer or boiled for 3 min after adding the Laemmli sample buffer (+B). Immunoblots were performed with mAb C219 as described in the Materials and methods section. The immune complex was detected using a horseradish peroxidase-conjugated rabbit anti-(mouse IgG) and ECL reagents. (**b**) The migration profiles of the immunoreactive bands were evaluated with an LKB Ultroscan XL densitometer. The broken lines represent samples that were boiled for 3 min after adding the Laemmli sample buffer and the solid lines represent the samples that were not boiled before electrophoresis ($n = 2$).

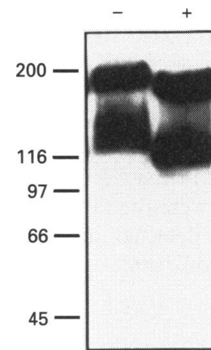

Figure 4 Effect of N-glycanase treatment on the electrophoretic mobility of the immunoreactive proteins

Isolated MCs were solubilized in 0.5% (w/v) SDS/1% (v/v) 2-mercaptoethanol and then diluted 4-fold with 20 mM Tris/HCl/1% (w/v) octyl β-D-glucopyranoside, pH 8.0, and incubated at 25 °C for 16 h. The samples were incubated with (+) or without (−) 2 units/mg protein endoglycosidase F/glycopeptidase mixture. Immunoblots were performed with mAb C219 as described in the Materials and methods section. All lanes contained 20 μg of protein ($n = 2$).

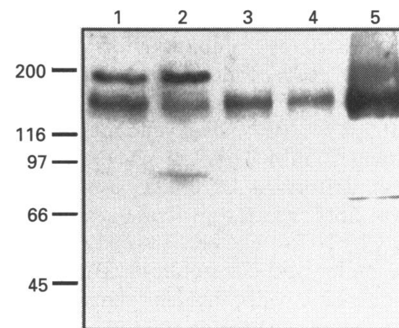

Figure 5 Triton X-114 phase-partitioning of immunoreactive proteins

Fractionation of brain capillary proteins with Triton X-114 was performed as described in the Materials and methods section. Immunoblot detection using mAb C219 was performed on unfractionated brain capillaries (lane 1), pellet (lane 2), supernatant (lane 3), detergent-poor fraction (lane 4) and detergent-rich fraction (lane 5). All lanes contained 10 μg of protein ($n = 2$).

boiled for 3 min after adding the Laemmli sample buffer, mAb C219 detected a broad band corresponding to a protein migrating between 220 and 170 kDa. But when the heating step was omitted and capillary proteins were solubilized directly in 0.5% SDS before adding the Laemmli sample buffer, two immunoreactive proteins of 190 and 155 kDa were detected by Western-blot analysis (Figure 3a). The migration profiles of the immunoreactive bands were evaluated with a laser densitometer. The heat-treatment induced a shift in the electrophoretic mobility of the 155 kDa protein which migrated as a diffuse band of molecular mass 170–180 kDa (Figure 3b). The migration of a 190 kDa protein was not affected by this procedure. Boiling the samples prior to gel electrophoresis also altered the electrophoretic mobility of the P-gp expressed in CH^{R}C5 cells. The immunoreactive protein (158 kDa) migrated as a 176 kDa protein when the samples were boiled.

The level of glycosylation of the two antigens detected by mAb C219 was also investigated. MCs were solubilized and incubated with a mixture of endoglycosidase F and glycopeptidase F (N-glycanase). Proteins were resolved using a 6% polyacrylamide gel to enhance the separation of the two proteins. Following this treatment, the size of the 190 kDa protein was only reduced to 180 kDa, while the size of the 155 kDa protein was reduced to 120 kDa (Figure 4).

Triton X-114 phase-partitioning was used to fractionate the two proteins on the basis of their degree of hydrophobicity. Following this treatment, 85% of the 190 kDa immunoreactive protein remained associated with the pellet containing the insoluble material, where it was enriched 3-fold (Figure 5). On the other hand, 78% of the 155 kDa protein was extracted by Triton X-114 and partitioned mostly into the detergent-rich fraction, where it was enriched 4- to 5-fold. When the proteins contained in the detergent-rich phase were boiled before gel electrophoresis, the 155 kDa protein migrated as a broad band of

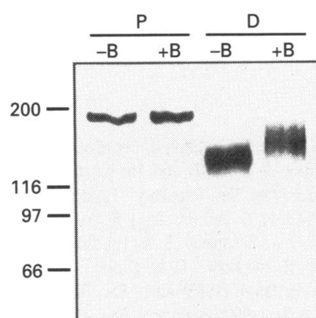

Figure 6 Effect of boiling on Triton X-114-fractionated proteins

Fractionation of brain capillary proteins with Triton X-114 and immunoblot detection were performed as described in Figure 5. The proteins (10 μg) contained in the pellet (P) and in the detergent-rich fraction (D) were either not boiled (−B) or boiled for 3 min (+B) before electrophoresis, as described in Figure 3 ($n = 2$).

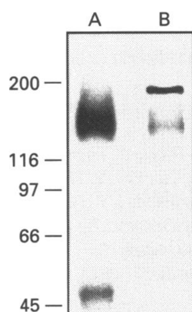

Figure 7 Photoaffinity labelling of P-gp with IAAP

Protein samples (50 μg) from MCs were incubated with IAAP (20 nM), cross-linked with u.v. light, and P-gp was immunoprecipitated with antibody C219. Unlabelled protein samples (10 μg) from brain capillaries were also loaded on the same gel. The proteins were transferred electrophoretically to an Immobilon-P membrane which was exposed to Kodak films for 2 weeks (lane A). Immunoblot detection was then performed using antibody C219 (lane B), as described in the Materials and methods section ($n = 2$).

170 kDa (Figure 6). In contrast, the 190 kDa protein remaining in the pellet was not affected by the heat treatment.

Photoaffinity labelling with IAAP

Photoaffinity labelling experiments were performed to confirm that P-gp class I isoform is expressed in MCs. Buschman et al. [23] have shown that only the P-gp class I isoform, but not the class III, could bind the photoactivatable drug analogue IAAP. These experiments were also performed to see if the two immunoreactive proteins detected by mAb C219 by Western-blot analysis could each be photolabelled with IAAP. Brain capillaries were incubated with IAAP, exposed to u.v. light, followed by immunoprecipitation with mAb C219 and SDS/PAGE. mAb C219 immunoprecipitated a photolabelled protein of 155 kDa (Figure 7). The immunodetection by Western blot with mAb C219 showed that the photoaffinity-labelled protein corresponded to the 155 kDa protein. The 190 kDa protein detected by C219 in the immunoblot was not photolabelled with IAAP. These results confirm that the 155 kDa protein detected by mAb C219 is the P-gp class I isoform, which was detected by Western-blot studies using the mouse mdr3-specific antiserum. The 190 kDa protein recognized by mAb C219 is likely to be an antigen which is not part of the known P-gp family.

DISCUSSION

The mAb C219 used in this study is able to detect the three P-gp isoforms on immunoblots of plasma-membrane proteins prepared from multidrug-resistant mouse, hamster and human cell lines [26]. We have detected the presence of a cross-reacting protein by Western-blot analysis in isolated MCs. This antigen migrated as a broad band of 190 kDa when the samples were subjected to heat before electrophoresis. Antibodies C494 and JSB-1 showed weak reactivity with MCs. The antibodies C494 and JSB-1 are specific probes for the detection of human and hamster P-gp class I isoforms [26–28]. Although the basic P-gp structure is conserved among species, there are structural differences between the P-gps of different species or tissues [29]. The internal cytoplasmic epitope recognized by mAb JSB-1 has not yet been characterized. In humans and hamsters, mAb C494 binds to the peptide epitope Lys-Pro-Asn-Thr-Leu-Glu-Gly-Asn-Val located at the C-terminus of P-gp, with Thr-323 and Glu-325 as the critical residues [26]. In the mouse, the critical residue, Thr-323, is replaced by a methionine residue at the same position in the amino acid sequence of the P-gp class I isoform (mdr3) [14]. This substitution may explain the weak reactivity of mAb C494 observed with MCs.

Specific polyclonal antibodies raised against each mouse P-gp isoform have been used to identify the P-gp isoform expressed in brain capillaries. In our studies, the mdr3-specific antiserum reacted with a 170 kDa protein. The mdr1- and mdr2-specific antisera showed no reactivity with the capillary proteins. These results clearly indicate that the P-gp isoform, mainly expressed in isolated MCs, is isoform I. However, under the same experimental procedures, mAb C219 and the mdr3-specific antiserum recognized proteins of different molecular masses. This difference in antigen recognition was further investigated by studying the electrophoretic properties of these proteins. When the brain capillary proteins were unheated prior to electrophoresis, mAb C219 detected two very distinct proteins of molecular mass 155 and 190 kDa. Heat treatment altered the electrophoretic mobility of the lower molecular-mass protein. In contrast, the electrophoretic mobility of the 190 kDa protein detected by mAb C219 was not altered by the heat treatment. Thus, the two immunoreactive proteins seemed to comigrate when the samples were heated before gel electrophoresis. It has been shown that boiling the samples before Laemmli gel electrophoresis decreases the mobility of the P-gp expressed in cancerous cell lines by an amount equivalent to approximately 15 kDa [30]. From these results, it is clear that mAb C219 detected two distinct antigens from isolated MCs. Since the 190 kDa protein detected by mAb C219 was not recognized by any of the mouse mdr-specific antisera, it seems improbable that it corresponds to a P-gp isoform.

The biochemical properties of the two antigens detected by mAb C219 were investigated further by N-glycanase treatment. These enzymes, which remove the carbohydrate moieties from N-linked glycoproteins [31], induced a small shift (10 kDa) in the electrophoretic mobility of the 190 kDa protein detected by C219. After the same treatment, the molecular mass of the 155 kDa immunoreactive protein was reduced to 120 kDa. A similar size (120 kDa) was reported for the deglycosylated form of P-gp expressed in the CH^RC5 cell line [30].

Triton X-114 phase-partitioning [24,32] showed that the 190 kDa protein detected by mAb C219 was poorly solubilized

by Triton X-114 since it remained associated with the pellet which contains the insoluble material, such as the cytoskeleton proteins. However, the 155 kDa protein detected by mAb C219 fractionated in the detergent-rich phase. These results are consistent with the hydrophobic nature of P-gp [33].

P-gp has been identified as a specific acceptor of IAAP [34]. Photoaffinity labelling studies using this compound have shown that the 155 kDa protein detected by mAb C219 reacted with IAAP. These results confirmed that the 155 kDa protein is a P-gp class I isoform since the class III isoform does not bind this photolabelling probe [23]. The 190 kDa protein detected by mAb C219 did not react with any of the mdr-specific antisera and was not photolabelled with IAAP. These results strongly suggest that the 190 kDa protein cross-reacting with mAb C219 is an antigen unrelated to any of the three presently known P-gp isoforms.

It was shown recently that the multidrug resistance-associated protein (MRP) gene, which is associated with a non-P-gp multidrug resistance, encodes a 190 kDa membrane-bound protein [35]. The 190 kDa antigen detected by mAb C219 in our experiments is unlikely to be the product of the MRP gene since the deduced sequence of the MRP protein does not contain the amino acid sequence that is recognized by mAb C219 [36,37].

Cross-reactivity of C219 with proteins other than P-gps, and unrelated immunoreactivity of this antibody preparation caused by contamination of reagent lots, have already been reported [10,38]. mAb C219 should be used with caution, especially in immunohistochemical and cytochemical studies where it would be more difficult to eliminate non-specific immunoreactivity of the antibody. Studies are currently being performed to evaluate the presence of the 190 kDa unrelated protein in other tissues. Preliminary results have shown that this protein can be detected in liver homogenate and in some tumour samples. This cross-reactivity is important since mAb C219 is often used as a tool to diagnose the presence of P-gp in tumours from cancer patients. Therapeutic approaches used to treat these patients are influenced by the expression of the MDR phenotype. MDR modulators, such as verapamil and cyclosporin A, are administered to patients to enhance the drug sensitivity of P-gp-positive cancer cells to chemotherapeutic agents [39]. Therefore, the presence of unrelated proteins in normal or cancerous tissue samples cross-reacting with mAb C219 may result in the administration of an unnecessary or inappropriate chemotherapeutic regimen to the patients. Since other antibodies, such as JSB-1 and MRK-16, have been found to cross-react with unrelated proteins [10,40], it has been suggested that tissue samples should be considered P-gp positive only if reactivity is observed with at least three different antibodies [40].

In conclusion, we report here that P-gp class I isoform (mouse mdr3) is the dominant, if not the single isoform, expressed in MCs. We also report that mAb C219 cross-reacted with a 190 kDa protein. The biochemical and physicochemical properties of this protein suggest that it is unlikely to be a P-gp isoform. In view of this unexpected cross-reactivity of mAb C219, it needs to be re-emphasized that the use of more than one antibody in the assessment of P-gp expression in clinical specimens should be standard practice.

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