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IDENTIFICATION OF P-GLYCOPROTEIN IN RENAL BRUSH BORDER MEMBRANES

Debra M. Lieberman[1], Reinhart A.F. Reithmeier*[1,2], Victor Ling[3], Jeffrey H.M. Charuk[1], Howard Goldberg[1], and Karl L. Skorecki[1]

MRC Group in Membrane Biology, [1]Department of Medicine, [2]Department of Biochemistry, Ontario Cancer Institute, Princess Margaret Hospital and [3]Department of Medical Biophysics, University of Toronto, Toronto, Ontario, CANADA M5S 1A8

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SUMMARY: A monoclonal antibody (C219) that recognizes the P-glycoprotein (M_r = 170,000) in plasma membranes of multidrug-resistant Chinese hamster ovary (CHO) cell lines was used to assay renal brush border membrane (BBM) and basolateral membrane (BLM) fractions for the presence of a cross-reactive polypeptide. The C219 antibody bound to a 155,000 dalton protein in immunoblots of rat BBM but not BLM proteins resolved by sodium dodecyl sulfate gel electrophoresis. The corresponding human kidney BBM and dog kidney BBM proteins had molecular weights of 170,000 and 160,000 respectively. The glycoprotein nature of the renal protein was shown by its sensitivity to N-glycanase treatment which reduced the apparent molecular weight of the dog protein to 120,000. In addition, dog P-glycoprotein could be bound to and eluted from immobilized wheat germ agglutinin. The molecular weight, antibody crossreactivity, glycosidase sensitivity and lectin binding show that this protein is a normal kidney analogue of the P-glycoprotein induced in multidrug resistant cell lines.

INTRODUCTION: Cells grown in culture and selected for cross-resistance to drugs overexpress a multidrug transport protein, P-glycoprotein (M_r = 170,000), in their plasma membrane (1-6). The increased production of the protein is due to amplification of the mdr gene and increased synthesis of a 4.5 - 5.0 kb mRNA (7-16). The mouse (17) and human (18) P-glycoproteins consist of 1,276 and 1,280 homologous amino acid sequences respectively with twelve predicted transmembrane segments and potential glycosylation sites (20) near

*To whom all correspondence should be addressed: MRC Group in Membrane Biology, Department of Medicine, Room 7307, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

Abbreviations: BBM, brush border membrane; BLM, basolateral membrane; CHO, Chinese hamster ovary; CH^RC5, Chinese hamster ovary cell line 200 fold more resistant to colchicine than the parental cell line (27).

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the amino terminus. The deglycosylated P-glycoprotein had a molecular weight of 120,000 (9). The protein contains a large internal repeat and is homologous to an Escherichia coli membrane protein that is responsible for the export of the hemolysin protein (17-19). The P-glycoprotein also contains two consensus sequences for nucleotide-binding domains (17-19) and it can be photo-labelled with 8-azido-ATP confirming its ability to bind ATP (21). The P-glycoprotein has been purified from multidrug-resistant cell lines using Ricinus communis lectin (22) or antibody (23) affinity chromatography and it shows ATPase activity. Membrane vesicles from multi-drug resistant cells bind vinblastine (24) and photo-affinity analogues of vinblastine label the P-glycoprotein (25,26). Resistant cell lines accumulate far less drug than their parental counterparts (1,2,27,28) and verapamil causes an increase in ATP consumption in multidrug resistant tumor cells (29). Membrane vesicles from drug-resistant human KB carcinoma cells can take up vinblastine in an ATP-dependent manner (30). Single amino acid changes in MDR-1 can alter the substrate specificity (eg. colchicine versus vinblastine) (31). The above facts suggest that the P-glycoprotein is an ATPase that catalyzes the active transport of drugs from resistant cells.

The gravid uterus, adrenal gland, kidney, liver and intestine often express P-glycoprotein mRNA (32-36). Immunohistochemical studies have localized the P-glycoprotein to the biliary surface of hepatocytes, the apical membrane of renal proximal tubule cells (37) and to the secretory epithelium of the gravid uterus (36). The P-glycoprotein may act to secrete cytotoxic compounds from the body (1,37). In this paper we have used the monoclonal antibody C219 to identify the P-glycoprotein in kidney membrane fractions and have studied the glycosidase sensitivity and lectin binding properties of the P-glycoprotein from renal brush border membranes.

METHODS: Preparation of membrane fractions- Colchicine (10 ug/ml)-resistant CHO cell line $CH^{R}C5$ were grown as described previously (27) and a detergent extract was prepared as described by Gerlach et al. (38) and stored at -70°C until further use. The BBM fraction was isolated from kidney cortex by the Mg^{2+} precipitation method of Booth and Kenny (39,40). The BBM membranes were typically enriched 15-20 fold for alkaline phosphatase over the crude membrane fraction. The BLM fraction was prepared as described by Sacktor et al. (41) and was enriched 20-fold in Na^{+}/K^{+}ATPase over the crude membrane extract.

Electrophoresis and immunoblotting- Sodium dodecyl sulfate gel electrophoresis was performed according to Laemmli (42). Proteins were transferred electrophoretically (43) to Immobilon or nitrocellulose sheets at room temperature. The transfers were

incubated with a 1:2500 dilution of monoclonal antibody C219 (5) at 1 mg/ml for 24 h. The transfers were then incubated with a 1/350 dilution of alkaline phosphatase-conjugated anti-mouse IgG for 1 h at room temperature. Alkaline phosphatase activity was detected by incubating the blots with 5-bromo-4-chloroindoxyl phosphate and nitroblue tetrazolium in 0.1 M Tris-HCl, pH 9.2 for up to 30 min at 22°C (44).

Glycosidase treatment- BBM or C5 membranes were solubilized with 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol at room temperature for 10 min and then diluted 4-fold with 1% octaethylene glycol mono-n-dodecyl ether ($C_{12}E_8$), 4 mM EDTA, 30 mM sodium phosphate, pH 8.0. Endoglycosidase F/glycopeptidase F mixture (Boehringer-Mannheim) was added at 3 U/mg protein and the digestion was allowed to proceed for 16 h at room temperature. Laemmli sample buffer (42) was then added without boiling.

Lectin binding- Dog kidney BBMs were solubilized in 1% $C_{12}E_8$, 0.2 M NaCl, 10 mM Tris-HCl, pH 7.5 (binding buffer) to a final protein concentration of 2 mg/ml. Solubilized BBM (1 ml) was added to 0.5 ml of wheat germ agglutinin resin and was gently shaken at 4°C for 2 h. The supernatant was removed and an aliquot was boiled with an equal volume of 2 x concentrated Laemmli sample buffer (42). The resin was washed 3 times with binding buffer and then the bound protein was eluted with 1 ml of 1 M N-acetyl-D-glucosamine in binding buffer. The resin was washed as above and extracted with an equal volume of 2 x Laemmli sample buffer to release tightly-bound proteins. The N-acetyl-D-glucosamine elute was made 1% in sodium dodecyl sulfate, dialyzed against 0.1% sodium dodecyl sulfate, 50 mM ammonium bicarbonate, pH 7.6 and lyophilized. Laemmli sample buffer (100 ul) was added to the dried sample. A control experiment using Sepharose 4B under identical conditions was also performed. Equal aliquots (40 ul) of the solubilized BBM (diluted 1:1 with Laemmli sample buffer), unbound fraction, N-acetyl-D-glucosamine elute and tightly-bound fraction were resolved by sodium dodecyl sulfate gel electrophoresis. Small scale binding assays using 50 ul aliquots of Concanavalin A-, peanut- or Ricinus communis lectin-Sepharose were also performed.

Protein concentrations were determined according to Lowry et al. (45) in the presence of 1% sodium dodecyl sulfate.

RESULTS AND DISCUSSION: The monoclonal antibody (C219) used in this study is specific for the P-glycoprotein and is able to detect this protein on immunoblots of plasma membrane proteins prepared from multidrug resistant hamster, mouse and human cell lines (5). The antibody binds to a cytoplasmic epitope within 200 amino acids of the carboxyl terminus of the P-glycoprotein (8). This antibody was used to determine the presence of a crossreactive protein in BBM and BLM fractions prepared from the kidney cortex of a number of different animal species. A detergent extract prepared from a colchicine-resistant CHO cell line (CH^RC_5) was used as a control (27). Figure 1 shows an SDS gel and the corresponding immunoblot of the CHO extract and the BBM and BLM fractions prepared from rat kidney cortex. A protein with a molecular weight of 170,000 could be readily detected in the C5 plasma membrane extract (Fig. 1 lane 3). This protein corresponds to the P-glycoprotein that is greatly

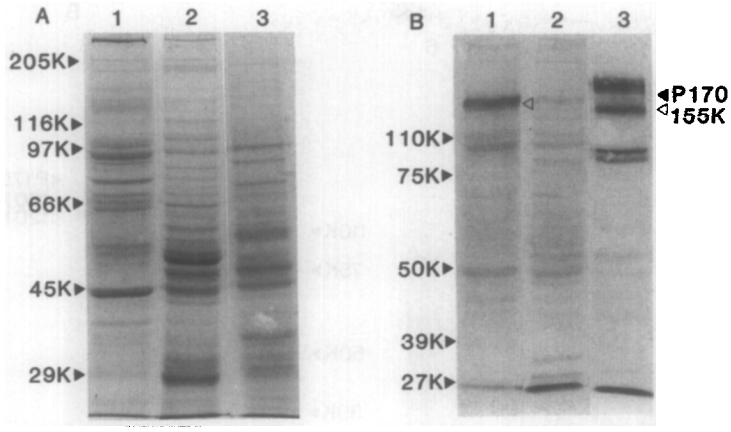

Figure 1. Monoclonal antibody C219 binding to the P-glycoprotein in BBM isolated from rat kidney cortex. 40 ug samples of proteins from rat kidney BBM (lane 1), rat kidney BLM (lane 2) and a detergent extract of the multidrug-resistant CHO cell line $CH^{R}C5$ (lane 3) were resolved by sodium dodecyl sulfate gel electrophoresis using a 7.5% polyacrylamide gel. (A), Coomassie Blue-stained gel, (B) corresponding immunoblot (Immobilon). Antibody binding was detected using goat anti-mouse IgG conjugated to alkaline phosphatase. P170 (closed arrow) indicates position of the P-glycoprotein from the CHO cells, 155K (open arrow) indicates the position of the immunoreactive protein in rat kidney BBM. The molecular weight markers indicated to the left of the figure are myosin (205K), B-galactosidase (116K), phosphorylase B (97K), bovine serum albumin (66K), ovalbumin (45K) and carbonic anhydrase (29K). The prestained markers to the left of panel B are phosphorylase B (110K), bovine serum albumin (75K) and ovalbumin (50K), carbonic anhydrase (39K) and soybean trypsin inhibitor (27K).

enriched in the plasma membrane of this drug resistant cell line (4,5). An immunologically related protein of similar molecular weight (M_r = 155,000) could be detected in the BBM isolated from rat renal cortex (Fig.1, lane 1). It was necessary to overdevelop the blot with respect to the P-glycoprotein in the CHO cells in order to readily detect the cross-reacting protein in the kidney BBM. The 155,000 dalton renal protein was localized to the BBM membrane. Based on the staining with the antibody the crossreactive protein is much less abundant in the BLM fraction than the BBM fraction (Fig. 1, lane 2). The small amount of cross-reactive material in the BLM fraction can be accounted for by a low level of contamination of the BLM with BBM.

A crossreacting protein could also be detected in BBM prepared from kidney cortex of human and dog kidney. The molecular weights as determined by sodium dodecyl sulfate gel electrophoresis were 170,000 for the human kidney protein and 160,000 for the dog homologue. The localization of the P-glycoprotein to the BBM is in agreement with the immunolocalization of the P-glycoprotein to the apical surface of the epithelial cells of the proximal tubule of human kidney (37).

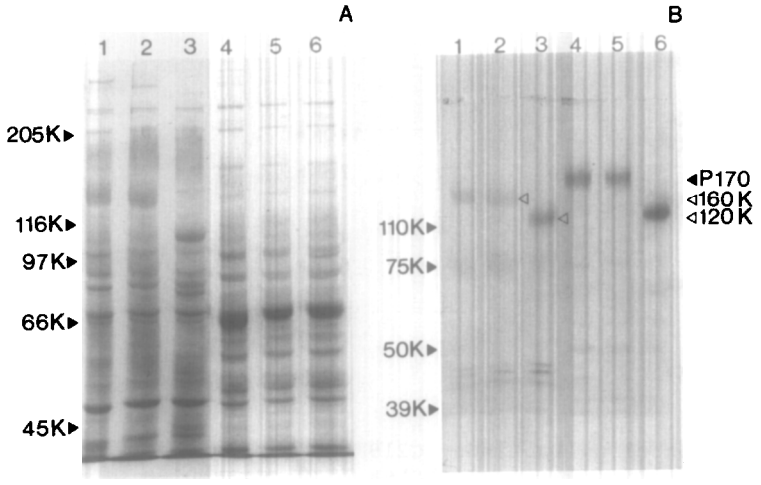

Figure 2. Effect of endoglycosidase F treatment on the electrophoretic mobility of the P-glycoprotein. All lanes contained 20 ug of protein. Lane 1, dog kidney BBM. Lane 2, dog kidney BBM solubilized in 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol and then diluted-4 fold with 30 mM sodium phosphate, pH 7.0, 4 mM EDTA, 1% n-dodecyl-octaethylene glycol and incubated at 22°C for 18 h. Lane 3, dog kidney BBM solubilized as in lane 2 but incubated with 3 U/mg protein endoglycosidase F/glycopeptidase mixture at 22°C for 18 h. Lane 4, detergent extract of multidrug-resistant CH^RC5 CHO cells. Lane 5, detergent extract of CH^RC5 cells incubated as in lane 2. Lane 6, detergent extract of CH^RC5 cells treated with Endoglycosidase F as in lane 3. (A), sodium dodecyl sulfate polyacrylamide gel (6%) stained with Coomassie Blue. (B), corresponding immunoblot (nitrocellulose). P170 (closed arrow) indicates the position of the P-glycoprotein from CHO cells, 160K (open arrow) indicates the position of the immunoreactive protein from dog kidney BBM and 120K (open arrow) indicates the position of the enzymatically deglycosylated proteins. Molecular weight standards were as described in Figure 1.

In order to confirm the glycoprotein nature of the antigen detected in the renal BBM fraction, the proteins of the dog kidney BBM and the plasma membrane of the C5 cell line were solubilized and treated with a mixture of endoglycosidase F and glycopeptidase F (N-glycanase). These enzymes remove the carbohydrate moieties from N-linked glycoproteins (46). Figure 2 shows that this treatment changes the mobility of the C5 P-glycoprotein and its renal counterpart. The deglycosylated proteins both have a molecular weight of 120,000 as determined by sodium dodecyl sulfate gel electrophoresis. Treatment of rat BBM with endoglycosidase F also reduced the molecular weight of the P-glycoprotein from 155,000 to 120,000. Enzymatic deglycosylation of the P-glycoprotein from C5 cells was previously shown to reduce the apparent molecular weight of the protein by 20,000 (20). This is similar to the decrease in molecular weight of the P-glycoprotein from multidrug-resistant J774.2 mouse macrophage-like cells that has been enzymatically

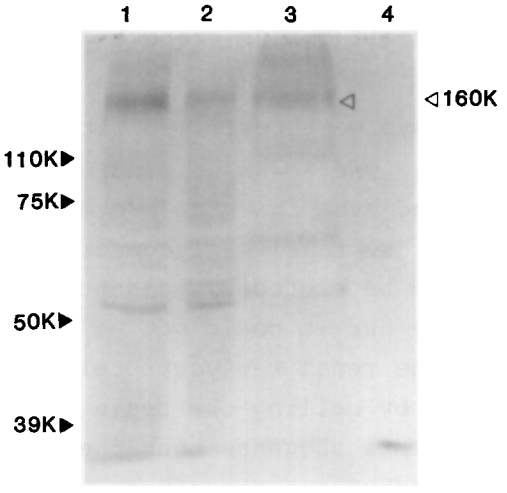

Figure 3. Immunoblot showing binding of the P-glycoprotein of dog kidney BBM to immobilized wheat germ agglutinin lectin. Solubilized BBM (1 ml at 2 mg protein/ml) were incubated with 0.5 ml of immobilized lectin resin at 4°C for 2 h. Lane 1, solubilized BBM; lane 2, unbound fraction; lane 3, N-acetyl-D-glucosamine elute and lane 4, Laemmli sample buffer extract of sugar-eluted resin. All samples (40 ul) were first resolved by sodium dodecyl sulfate gel electrophoresis, then the proteins were transferred to nitrocellulose and the blots were incubated with monoclonal antibody C219 and visualized as in Fig. 1. The molecular weight markers indicated to the left of the figure are as in Fig. 1B. The open arrow indicates the position of the renal P-glycoprotein.

deglycosylated or produced in cell cultures in the presence of tunicamycin (9).

The P-glycoprotein from the C5 cell line binds the lectin RCA-1 from Ricinus communis (22). The lectin binding properties of the P-glycoprotein from kidney were determined by incubating solubilized BBMs with immobilized lectins. The lectins tested include Concanavalin A, wheat germ agglutinin, peanut lectin and Ricinus communis agglutinin. Figure 3 shows that the P-glycoprotein from dog BBM binds to wheat germ agglutinin and can be eluted by N-acetyl-D-glucosamine. The content of P-glycoprotein in various fractions was assayed by sodium dodecyl sulfate gel electrophoresis and immunoblotting with the C219 monoclonal antibody. The fractions assayed include the solubilized BBM preparation, the unbound fraction, the proteins bound to immobilized wheat germ agglutinin and eluted with N-acetyl-D-glucosamine and finally, tightly-bound proteins extracted from the resin with Laemmli sample buffer. The unbound fraction (Fig. 3, lane 2) had a lower content of P-glycoprotein compared to the solubilized BBM (Fig. 3, lane 1) showing binding of the P-glycoprotein to this lectin. N-acetyl-D-glucosamine (1 M) was able to elute all the bound P-glycoprotein (Fig. 3, lane 3) since no P-glycoprotein could be

detected in the final Laemmli buffer extract of the resin (Fig. 3, lane 4).

Similar binding assays were performed with other immobilized lectins (data not shown). There was no binding of the P-glycoprotein to peanut lectin which suggests that the protein has terminal sialic acid. It was found that the renal P-glycoprotein also bound Ricinus communis lectin and could be eluted by galactose. The protein bound to Concanavalin A tightly but it could not be eluted by 1 M α-methyl mannoside. Elution of the renal P-glycoprotein from Concanavalin A-Sepharose was effected by boiling the resin in the presence of Laemmli sample buffer. This suggests that the protein contains a high mannose type of carbohydrate structure.

These findings suggest the presence of a protein in the BBM fraction of kidney cortex that is recognized by a monoclonal antibody specific for the P-glycoprotein of multidrug-resistant cells. The glycoprotein nature of this protein was shown by its sensitivity to N-glycanase treatment and its lectin binding properties. Of note, the deglycosylated protein from dog and rat BBM has the same molecular weight as the deglycosylated P-glycoprotein from CHO cells. The difference in molecular weight of the protein (160,000 in dog BBM, 155,000 in rat BBM and 170,000 in human kidney BBM and CHO cells) is therefore likely due to differences in glycosylation.

The P-glycoprotein in multidrug-resistant cell lines is an ATP-dependent enzyme responsible for transporting a broad spectrum of drugs out of the cell. The protein found in the BBM fraction from kidney cortex likely plays a similar role. The kidney is an important organ for the elimination of endogenous products of metabolism as well as exogenous toxins. In this regard, the proximal tubule of the kidney serves as a site for secretion of organic anions and cations and other compounds, but the identity of the transporters involved for all of these compounds has not been established. The protein we have detected in the BBM might catalyze the active transport of xenobiotics across the apical membrane of epithelial cells in the proximal tubule of kidney. These compounds could enter the epithelial cell from the blood by diffusion or by facilitated transport across the antiluminal membrane. The P-glycoprotein of kidney may play an important role in the elimination of drugs and metabolites from the body. The presence of P-glycoprotein in BBM vesicles may permit transport studies that will elucidate the normal function of this protein in the kidney. Also, regulation of P-glycoprotein synthesis in kidney proximal tubule cells may occur as

in multidrug resistant cell lines. This can be tested by studying the effect of chemotherapeutic agents and other drugs on the level of this protein in kidney cell lines and in animal models.

Finally, we have recently reported that the widely used immunosuppressive drug, cyclosporine is a substrate for P-glycoprotein and that the pharmacological blockade of P-glycoprotein in renal epithelial cell line augments the intracellular accumulation of cyclosporine (47). Nephrotoxicity is the major problem which limits the use of cyclosporine. Therefore the presence of P-glycoprotein in the apical membrane of the renal proximal tubule may be of importance in cyclosporine toxicity.

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