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P-glycoprotein is a plasma membrane protein that functions as a drug transporter and is responsible for multidrug resistance in some breast cancers. In the past one year, we have generated two site-specific antibodies and used them to determine the topologies of P-glycoprotein in multidrug resistant cancer cells. We found that P-glycoproteins in the plasma membrane of mammalian cells express at least two alternate topologies. This observation is consistent with our previous study using cell-free expression system. The more than one topology feature of P-glycoprotein may be responsible for its multifunctional nature. We have also been able to express the transmembrane domains of P-glycoprotein in bacteria. The success in this study will allow us to map the drug-binding domain in P-glycoprotein and study the drug-P-glycoprotein interactions.

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4. TABLE OF CONTENTS:

5. INTRODUCTION:

5.1. Background and Nature of the Problem: Despite the improvement in early detection and treatment of breast cancer, the mortality rate of women with breast cancer remained high. One major obstacle for curing breast cancer is the development of **multidrug resistance** in breast cancer cells which reduces the effectiveness of chemotherapy. Breast cancer is often intrinsically drug resistant, and sometimes acquires resistance following chemotherapy. Although there are conflicting reports, **P-glycoprotein** (Pgp)-mediated multidrug resistance seems to be the most frequent mechanism of drug resistance in breast cancer (Sugawara *et al.*, 1988; Schneider *et al.*, 1989; Ro *et al.*, 1990; Goldstein *et al.*, 1989; Keith *et al.*, 1989; Salmon *et al.*, 1989; Merkel *et al.*, 1989; Sanfilippo *et al.*, 1991; Verrelle *et al.*, 1991; Wishart and Kaye, 1991; Charpin *et al.*, 1994; Bates *et al.*, 1995).

Pgp is a plasma membrane protein which functions as an efflux pump for anticancer drugs (Gottesman and Pastan, 1993). Overexpression of Pgp reduces the accumulation of cytotoxic drugs in breast cancer cells which, therefore, will survive the chemotherapeutic treatment. Pgp has been shown to interact directly with various anticancer drugs, for example, adriamycin (Bushe *et al.*, 1989), vinblastine (Cornwell *et al.*, 1986; Safa *et al.*, 1986), and colchicine (Safa *et al.*, 1989). Pgp expressed in the plasma membrane has been covalently labeled by photosensitive drug analogues (Safa *et al.*, 1987). Greenberger *et al.* (1991) and Bruggemann *et al.* (1989, 1992) suggested that the [^{3}H]azidopine and [^{3}H]vinblastine cross-linked to fragments including TM6 and TM12 of mouse and human Pgp, respectively. Despite the success of these drug-labelling studies, it is still not known if these domains directly bind drugs or the azido groups of these drugs are simply in a close proximity to the labelled sites. The detailed interaction between Pgp and drugs is unknown.

Using a cell-free expression system, I have previously shown that Pgp has at least two distinct topological structures in microsomal membranes (Zhang and Ling, 1991; Zhang *et al.*, 1993). Pgp molecules have also been suggested to function as chloride channels as well as drug transporters and these two functions are separable (Valverde *et al.*, 1992; Gill *et al.*, 1992; Higgins, 1992; Alternberg *et al.*, 1994). Indeed, drug transport requires ATP hydrolysis whereas the channel function can be supported by non-hydrolyzable ATP analogues (Gill *et al.*,, 1992). I have previously proposed that these two functions may be carried out by the two different topological structures of the molecule; while molecules with one structure may function as a chloride channel, molecules with the other structure may function as a drug-efflux pump. However, one question remains to be answered is whether Pgp in multidrug resistant cancer cells have two alternate topologies.

5.2. Purpose of the Present Work: The current project is designed to (1) determine the topological structure of Pgp in multidrug-resistant mammalian cells and (2) map the drug-binding site in Pgp.

5.3. Methods of Approaches: To determine the topological structure of Pgp in multidrug resistant cancer cells, site-specific antibodies will be produced against relevant domains of Pgp. We will use immunocytochemistry and proteolysis/membrane protection assay to map the membrane

orientation of the relevant domains of Pgp. To map the drug-binding domain, different domains of Pgp will be expressed in bacteria in large quantity and used to study its drug binding property.

6. BODY:

6.1. Methods Used:

6.1.1. Generation and characterization of site specific antibodies:

(a), production of fusion proteins: cDNA fragments encoding relevant Pgp domains were generated using PCR technology and cloned into a bacterial expression vector (pGEX series, Pharmacia LKB Biotechnology). The final DNA products were sequenced to ensure the correct reading frame and to eliminate any possible mutations introduced by Taq DNA polymerase. The pGEX vectors provide all three possible reading frames in the multiple cloning site for generating fusion proteins with glutathione S-transferase at the N-terminus and inserted foreign protein at the C-terminus. All vectors also supply stop codons in all three reading frames for translation termination. Fusion protein was prepared from transformed bacteria JM109 and purified by separation through an affinity column of glutathione-conjugated Sepharose-4B (Smith and Johnson, 1988). The glutathione S-transferase can be cleaved off from the fusion protein using factor Xa or thrombin and removed by separation on a second glutathione-conjugated Sepharose-4B column. The purified Pgp peptides were used to raise antibodies.

(b), production of polyclonal antibodies: Rabbits were immunized subcutaneously with fusion proteins in complete Freund's adjuvant, followed by periodic boosting with peptide in incomplete Freund's adjuvant, and then bled for serum. The polyclonal antibodies were characterized against their fusion peptide antigens using ELISA or Western blot analysis (Harlow and Lane, 1988). The specificity of these antibodies for Pgp was assessed against plasma membrane fractions containing Pgp isolated from MDR cells (e.g. CH^rB30 which has 10% Pgp in its total plasma membrane proteins) using Western blot and immunofluorescence. Polyclonal antibodies were affinity-purified on a MAC-25 cartridge using purified fusion peptide as previously described (Zhang and Nicholson, 1994).

6.1.2. Proteolysis/membrane protection assay of isolated membrane vesicles: Membrane vesicles were prepared from MDR cells as previously described (Lever, 1977) and subjected to complete protease digestion using trypsin or proteinase K. The digested fragments were then separated on SDS-PAGE and detected by using site-specific polyclonal antibodies on Western blot. Size of the fragments resistant to protease digestion and detected by each specific antibody were analyzed according to the two alternative topologies (Zhang *et al.*, 1993).

To determine the potential conformational change of Pgp in its catalytic cycle, inside-out membrane vesicles containing Pgp were preincubated for 30 min at room temperature with 5 mM ATP, ADP, or its non-hydrolyzable ATP analogous (AMP-PNP) in the presence of 5 mM $MgCl_2$, followed by trypsin digestion in the presence of these ligands. The trypsin digestion was then stopped after two-hour incubation at 37°C by addition of trypsin inhibitor and PMSF. The membrane fraction was then be separated from the soluble trypsin fraction by centrifugation at 4°C

and immediately solubilized in SDS-PAGE sample buffer. The Pgp fragments containing the loop 8 was detected on Western blot using the mAb MD-7 which has an epitope in the loop 8 (Zhang *et al.*, 1996).

6.1.3. Immunocytochemistry staining: The polyclonal antibodies generated in 6.1.1. were used to label fixed or fixed and permeabilized cells. The cells were fixed with paraformaldehyde (fixed, but not permeabilized) or fixed and permeabilized with acetone/methanol as previously described (Kartner *et al.*, 1985). This method of fixation and permeabilization does not affect the antigenicity of mAb C219 epitope. The labelling was detected using secondary antibody conjugated with peroxidase and the staining was viewed under a microscope.

6.1.4. Expression of a domain including TM11-TM12 of Pgp and drug labelling: Engineering the cDNA fragments into bacterial expression vectors and preparation of fusion protein have been described in detail elsewhere (Zhang *et al.*, 1996). Briefly, cDNA fragments encoding the relevant domains of Pgp were amplified by PCR and cloned into the multiple cloning site of a bacterial expression vector (pGEX series, Pharmacia LKB Biotechnology). The pGEX vectors provide all three possible reading frames in the multiple cloning site for generating fusion proteins of glutathione S-transferase (GST) at the N-terminus and inserted foreign Pgp at the C-terminus. The vectors also supply stop codons in all three reading frames for translation termination at the C-terminus of the fusion protein. The constructs encoding fusion proteins of N-terminal GST and various C-terminal Pgp sequences were introduced into *E. coli* (e.g. JM109, BL-21) using the $CaCl_2$ transformation technique (Perbal, 1984). The transformed cells were selected by ampicillin and positive clones were screened by DNA restriction mapping and confirmed by DNA sequencing.

Expression of fusion proteins was induced by IPTG (Isopropyl b-D-Thiogalactopyranoside). Bacteria clones expressing the fusion protein were lysed using sonication and the membrane fraction of the lysate were prepared by centrifugation at 100,000 g for 1 hr. For purification, fusion proteins in the membrane were solubilized with detergent and the fusion proteins were isolated with an affinity column of glutathione-conjugated Sepharose-4B. The size and purity of the fusion protein were determined on an SDS-PAGE stained with Commassie blue.

The drug binding was performed using photoactive drug analogous [^{3}H]azidopine. The membranes containing fusion protein were incubated with photosensitive [^{3}H]azidopine in the dark, followed by UV irradiation. The drug-linked peptides were analyzed by SDS-PAGE. After separation, the gel was fixed, treated with Amplify^{TM}, and exposed to x-ray film for fluorography. To eliminate the role of GST portion of the fusion protein in binding drugs, we removed the GST from the fusion protein by thrombin digestion. For competitive inhibition of the drug binding, vinblastine was used.

To purify the expressed drug-binding domain of Pgp, the fusion protein in bacterial membranes wasill be solubilized with detergent. The detergent will then be removed by dialysis and the protein will be allowed to refold at 4°C as described previously (Fiermonte *et al.*, 1993). Glutathione-conjugated Sepharose will then be added to precipitate the fusion protein. Alternatively, we will use classical methods, such as gel-filtration and ion-exchange

chromatography to purify the fusion protein as described in D.2.D.2. Preparation of large amount of the purified Pgp domains will allow us to investigate the detailed interaction between Pgp and its substrates at 3-D level. With large amount of drug-binding domains of Pgp, we will be able to address how Pgp can interact with such a wide variety of drug substrates.

6.2. Results Obtained:

6.2.1. The site-specific polyclonal antibodies generated against fusion proteins detect specifically P-glycoprotein from multidrug-resistant cells We have generated site-specific polyclonal antibodies against fusion proteins containing the loop linking TM4 and TM5 (loop 4) and the loop linking TM8 and TM9 (loop 8). These antibodies are designated αPgp-L4 and αPgp-L8, respectively. Fig. 1 shows a Western blot of membranes isolated from sensitive and drug-resistant CHO cells detected by monoclonal antibody (mAb) C219 as well as polyclonal antibody (pAb) αPgp-L4 and αPgp-L8. The mAb C219 (lane 2), pAb αPgp-L4 (lane 5) and pAb αPgp-L8 (lane 8) specifically detected the 180-kDa Pgp. This protein was not detected in the sensitive Aux B1 cells (lanes 1, 4 and 7). It has been shown previously that hamster Pgp can be cleaved into two halves by mild protease digestion and both halves have a mAb C219 epitope (Georges *et al.*, 1991). As shown in lane 3, two half molecules of Pgp were produced by trypsin digestion and both react with the mAb C219. However, as expected, the pAb αPgp-L4 reacted only with the N-terminal half (lane 6), whereas the pAb αPgp-L8 reacted only with C-terminal half (lane 9) of Pgp.

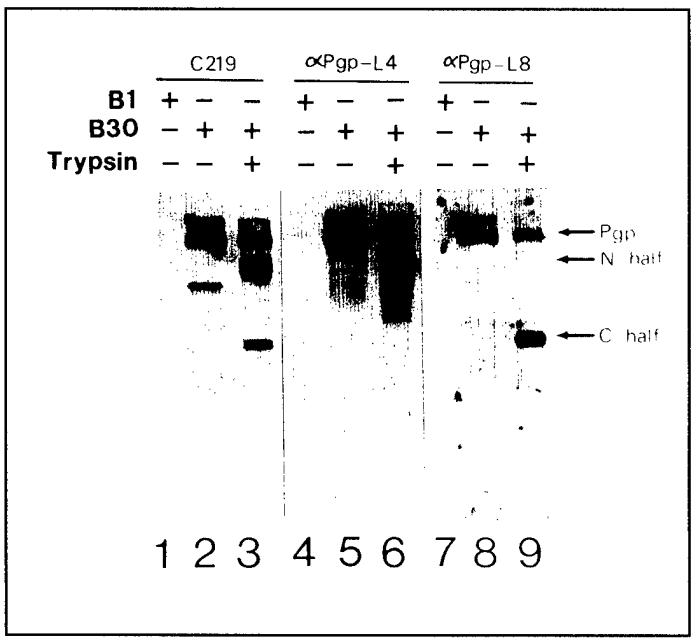

Figure 1. Characterization of the specificity of αPgp-L4 and αPgp-L8 antibodies to P-glycoprotein of CH^RB30 cells. Crude membranes were isolated from parental Aux B1 (lanes 1, 4 and 7) and multidrug-resistant CH^RB30 cells (lane 2, 5 and 8). Monoclonal antibody C219 as well as the polyclonal antibodies αPgp-L4 and αPgp-L8 specifically detected the 180-kDa Pgp and its aggregated and degraded products (lanes 2, 5 and 8). No protein from Aux B1 cells was detected by any of these antibodies. C219 detects both N- and C-terminal half fragments (lane 3). As expected, only the N-terminal half fragment was recognized by αPgp-L4 (lane 6) whereas the C-terminal half was recognized by αPgp-L8 (lane 9).

To confirm whether pAb αPgp-L4 and αPgp-L8 specifically detect Pgp on plasma membranes, we labelled fixed and permeablized multidrug-resistant cells with mAb C219, pAb αPgp-L4 and αPgp-L8, and detected the binding with FITC-conjugated secondary antibodies and

viewed on a confocal-fluorescence microscope. As shown in Fig. 2, mAb C219 (panel A), as well as pAb αPgp-L4 (panel C) and αPgp-L8 (panel E) stained predominantly the plasma membranes. No signal was detected on plasma membranes in control experiments with either normal mouse IgG (panel B), or preimmune sera of αPgp-L4 (panel D) or αPgp-L8 (panel F).

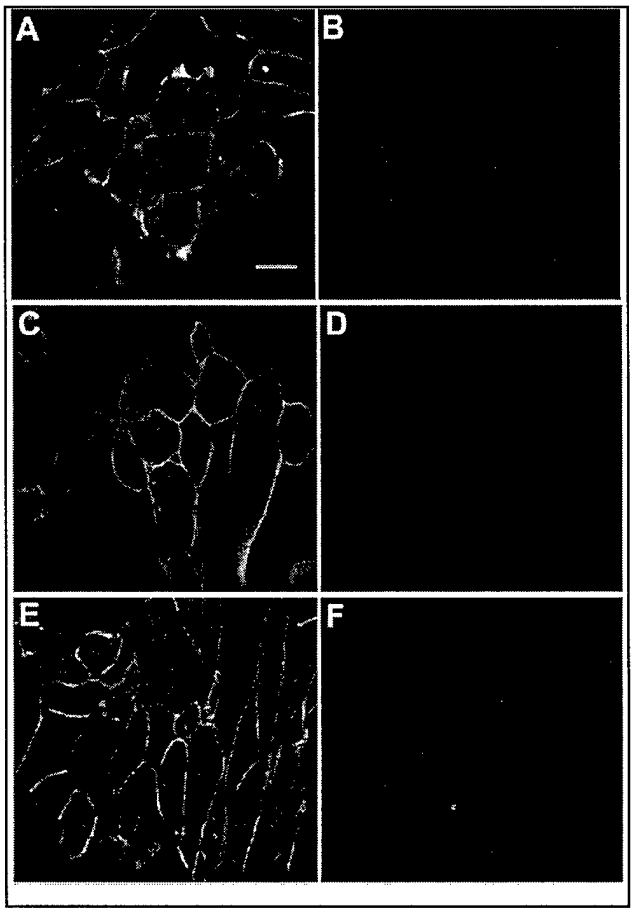

Figure 2. Immunofluorescence labelling of CH^RB30 cells using αPgp-L4 and αPgp-L8. Multidrug-resistant CH^RB30 cells were fixed and permeabilized with acetone/methanol and labelled with mAb C219 (panel A), pAb αPgp-L4 (panel C), pAb αPgp-L8 (panel E). The labeling was detected using FITC-conjugated secondary antibodies and by confocal fluorescence microscopy. In the controls, cells were labelled using normal mouse IgG (panel B) and preimmune sera of αPgp-L4 (panel D) and αPgp-L8 (panel F). All three antibodies labelled predominantly on the plasma membranes. The bar in panel A denotes 10 μm. All photographs were taken with the same magnification.

6.2.2. Expression of multiple topologies of Pgp in multidrug resistant cells: Two methods were used to determine the membrane orientation of Pgp in drug-resistant cells: proteolysis/membrane protection assay and immunocytochemistry as described in 6.1.2. Fig. 3 shows the results of proteolysis/membrane protection assay detected by various antibodies.

(a), the loop 8 of Pgp in inside-out membrane vesicles is resistant to trypsin digestion: To confirm that the loops 4 and 8 of Pgp in MDR cells were located extracellularly, we performed proteolysis/membrane protection assay and Western blot of isolated membrane vesicles. We used the human multidrug-resistant SKOV/VLB cells (Bradley et al., 1989), from which all vesicles isolated are inside-out (data not shown) as determined using acetylcholinesterase (extracellular marker). This is confirmed by another marker Na^+/K^+-ATPase (cytoplasmic side marker) (data not shown). Meanwhile, we obtained a mAb MD-7 which has an epitope in the loop 8 (Shapiro et al., 1996).

Fig. 3A shows the trypsin-digestion profile of Pgp detected by the mAb MD-7. Under mild conditions, peptides indicated as C (C-half) were detected (lanes 2 and 3, Fig. 3A). More extensive trypsin digestion generated smaller fragments labeled as X, Y, and Z, respectively (lanes 4 and 5, Fig. 3A). All these fragments were presumably derived from the C-half molecule with the epitope for the mAb MD-7. The smallest trypsin-resistant fragment detected by the mAb MD-7 has an apparent molecular mass of 17 kDa (peptide Z). It is possible that the generation of these fragments represents the progressive digestion of the C-terminal half molecule of Pgp and the peptide Z represents the minimum membrane-protected fragment containing the loop 8 with the mAb MD-7 epitope.

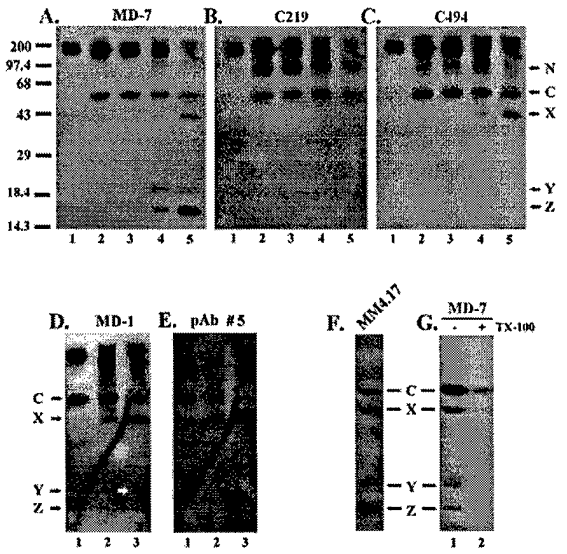

Figure 5. Proteolysis profile of P-glycoprotein. (A-C), trypsin cleavage profile of human P-glycoprotein. 10 mg of SKOV/VLB cell membranes were incubated in the presence of 0.2 mg (lanes 2 and 3), 3 mg (lane 4), and 6 mg (lane 5) trypsin at 37°C. The reaction duration was 15 min (lane 2), 30 min (lane 3), and 2 hrs (lanes 4 and 5), respectively. Lane 1 is a control of 3 mg undigested SKOV/VLB cell membranes. Panels A-C are the same blot probed with different antibodies. A=MD-7, B=C219, C=C494. (D and E), panels D and E are the same blot as A-C, but only the corresponding lanes 3-5 were shown. D=MD-1, E=pAb #5. Separate experiments have also been performed with MD-1 and pAb #5 and the results are consistent with the data shown here. However, to be consistent with the panels A, B, C, only the stripped blot was shown. (F), 10 mg of SKOV/VLB cell membranes were incubated in the presence of 3 mg trypsin at 37°C for 2 hrs (same as lane 4, panel A). The blot was probed with MM4.17. (G), 10 mg of SKOV/VLB cell membranes were incubated in the presence of 3 mg trypsin at 37°C for 2 hrs (same as in lane 4, panel A) in the absence (lane 1) or presence (lane 2) of 1% Triton X-100. The blot was probed by MD-7.

To determine the likelihood of the above possibility, we stripped the same blot and probed it with other mAbs or site-specific pAbs. Fig. 3B shows the same blot from Fig. 3A probed with the mAb C219. Only two peptide fragments indicated as N and C were detected by C219 (lanes 2-5, Fig. 3B). The peptide N did not react with the mAb MD-7 and represents the N-terminal half molecule (lane 2-5, Fig. 3A). The mAb C494, on the other hand, detected the peptide C, (lanes 2-5, Fig. 3C). Together, these results suggest that the peptides N and C represent the N- and C-terminal half molecules, respectively. These results are consistent with the previous studies which showed that the mAb C219 has two epitopes, one in each ATP-binding domain, and the mAb C494 has only one epitope in the C-terminal half molecule (Georges et al., 1990; see also Fig. 1) and Pgp can be cleaved into two halves by trypsin (Georges et al., 1991).

In addition to the peptide C, the mAb C494 also reacted with the peptide X (lanes 4 and 5, Fig. 3C) which was not detected by the mAb C219 (lanes 4 and 5, Fig. 3B). This peptide is likely a further degradation product from the peptide C. Thus, after Pgp was cleaved into two halves, the C-terminal half molecule was further digested and the ATP-binding domain containing the mAb C219 epitope was removed, resulting in peptide X. However, the C494 epitope is still attached to the truncated C-terminal half molecule (peptide X). Both the mAb C219 and C494 did not react with peptides Y and Z, suggesting that these two peptides are further degradation products and have lost epitopes for both C219 and C494.

To determine whether the peptide Z contains the linker region and the loop 10, we probed the same stripped blot again with a mAb MD-1 (Shapiro et al., 1996) and a site-specific pAb #5 (Greenberger et al., 1991). The mAb MD-1 is specific to the minilinker region whereas the epitope for the pAb #5 is in the loop 10. Both antibodies are specific to the C-terminal half molecule as shown by their reactivity with the peptide C, but not the peptide N (lanes 1 and 2 in Fig. 3D and Fig. 3E). Both antibodies also reacted with the peptide X (lanes 2 and 3 in Fig. 3D and Fig. 3E), suggesting that the peptide X still contains the epitope for both antibodies. The mAb MD-1 detected, in addition, the peptide Y (lane 3, Fig. 3D) which, however, was not detected by the pAb #5 (lane 3, Fig. 3E). Therefore, the peptide Y lost the epitope for the pAb #5, but still retains the epitope for the mAb MD-1. The mAb MD-1, however, did not detect the peptide Z albeit the amount of peptide Z is higher than the peptide Y (compare lane 5 in Fig. 3A with lane 3 in Fig. 3D). The peptides Y and Z are still on membrane after stripping as determined using MD-7 (data not shown). These results suggest that the peptide Z is a final trypsin-resistant fragment which only retains the epitope for the mAb MD-7 and does not contain the epitopes for the mAbs C219, C494, MD-1, nor the site-specific pAb #5.

To prove that the peptide Z also contains the loop 7 (linking TM7 and TM8) in addition to the loop 8 (linking TM8 and TM9), we probed the digested products with another mAb MM4.17 of which the epitope is in the loop 7 (Cianfriglia et al., 1994). As expected, the mAb MM4.17 reacted with all the peptides C, X, Y, and Z (Fig. 3F). To confirm that the trypsin resistance of the peptide Z was due to membrane protection, we performed a digestion in the presence of Triton X-100 to permeabilize the membrane. As shown in Fig. 3G, the trypsin-resistant peptide Z was produced in the absence of Triton X-100 (lane 1, Fig. 3G), but was completely digested in the presence of Triton X-100 (lane 2, Fig. 3G). The above results showed that the domain containing loop 7 and loop 8 (with mAb MM4.17 and MD-7 epitopes) in inside-out vesicles is resistant to trypsin digestion whereas the loop 10 (linking TM10 and TM11 with pAb #5 epitope) and the C-terminal ATP-binding domain (containing mAb C219 and C494 epitopes) are sensitive to trypsin digestion. Thus, the segment containing both the loop 7 and loop 8 is likely located in the lumen of the isolated inside-out membrane vesicles (extracellular location), consistent with the alternative topology.

(b), the loop 4 of human Pgp in inside-out membrane vesicles is protected from trypsin digestion To determine whether the loop linking the putative TM4 and TM5 (loop 4) is also resistant to trypsin digestion in inside-out vesicles of MDR cells (SKOV/VLB), we again stripped the blot shown in Fig. 3A and probed it with the pAb aPgp-L4. This antibody specifically detected the peptide N (lanes 2-5, Fig. 4). In addition, further digested products X', X", Y' (a doublet), and Z'

were also detected (lanes 4 and 5, Fig. 4). The peptide Z', with an apparent molecular mass of 14.5 kDa, is the smallest trypsin-resistant fragment from the N-terminal half molecule. It presumably represents the membrane-protected fragment containing the loop 4 with the epitope for the pAb aPgp-L4 whereas the peptides X', X", and Y' are incompletely digested products of the peptide Z'. In the presence of Triton X-100, these peptides were also completely digested (data not shown). Therefore, by analogy to the study of loop 8 in the C-terminal half, it is likely that the loop 4 of Pgp is also located in the lumen of the inside-out membrane vesicles (extracellular location).

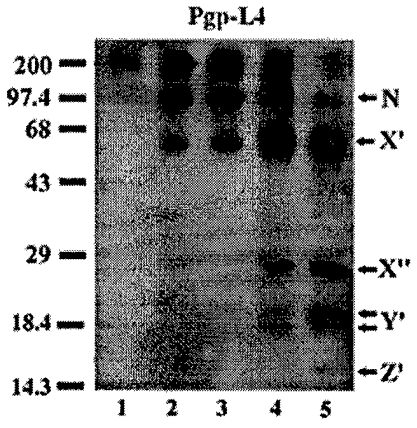

Figure 4. Membrane protection of loop 4. The same blot from Fig. 3A-3C (membranes from the SKOV/VLB cells) was stripped and probed with the pAb αPgp-L4. The minimum peptide fragment Z' is the protected loop 4 of Pgp.

6.2.3. Conformational changes of Pgp in its catalytic cycle.

(a), MgATP and its nonhydrolyzable analogous affect the generation of the membrane protected TM7-loop7-TM8-loop8-TM9 of Pgp in inside-out vesicles: To determine whether the binding of MgATP to Pgp causes any structural change, we used trypsin sensitivity of the loop 8 as an indicator and the change of trypsin sensitivity was determined using the mAb MD-7 on Western blot. If there is any structural change of Pgp, the loop 8 of Pgp may become either more or less sensitive to trypsin due to the change in topological structure. As shown in Fig. 5A, the peptide Y was generated in large quantity relative to the peptide Z in the presence of MgATP (lane 3) as compared with the control digestion (lane 2, Fig. 5A).

To determine whether Mg^{++} is required for ATP to stimulate the generation of the peptide Y, we performed a trypsin digestion of Pgp in the presence of ATP without Mg^{++}. As shown in Fig. 5B, ATP without Mg^{++} stimulated little the generation of the peptide Y (lane 1, Fig. 5B). Mg^{++} alone did not stimulate the generation of the peptide Y (lane 2, Fig. 5B). Therefore, the complex of Mg^{++} with ATP is apparently required to stimulate the generation of the peptide Y. The non-hydrolyzable analogues MgATPgS (lane 4, Fig. 5B) and MgAMP-PNP (lane 5, Fig. 5B) did not stimulate the generation of the peptide Y. However, more peptide C was observed in the presence of MgATPgS and MgAMP-PNP. These results suggest that different conformational states of Pgp may exist and can be generated by the binding and/or hydrolysis of MgATP.

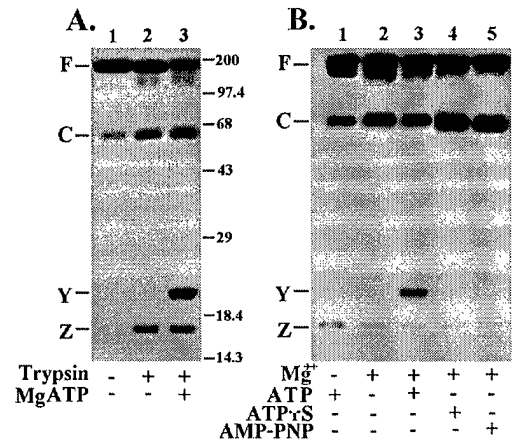

Figure 5. Trypsin digestion of Pgp in inside-out membrane vesicles and MgATP effects. (A), effects of MgATP on trypsin digestion of Pgp. 30 mg of membrane proteins were pre-treated without (lane 2) or with 5 mM MgATP (lane 3) for 30 min at room temperature, followed by digestion with 6 mg trypsin at 37°C for 2 hrs. The reaction was stopped by using PMSF and soybean trypsin inhibitor. The membrane fraction was pelleted by centrifugation, separated by SDS-PAGE and transferred onto a PVDF membrane. Pgp and its fragments were detected by the mAb MD-7. Lane 1 is control membranes not digested by trypsin. (B), requirement of Mg^{++} for ATP-induced digestion of Pgp. 30 mg of membrane proteins were pre-treated for 30 min at room temperature with 5 mM ATP (lane 1), 5 mM $MgCl_2$ (lane 2), or in the presence of 5 mM each MgATP (lane 3), MgATPgS (lane 4), and MgAMP-PNP (lane 5). 6 mg trypsin was then added and the digestion was performed for 2 hrs at 37°C. Pgp and its fragments were detected using the mAb MD-7. F=full length. C=carboxyl half. Y=peptide Y. Z=peptide Z.

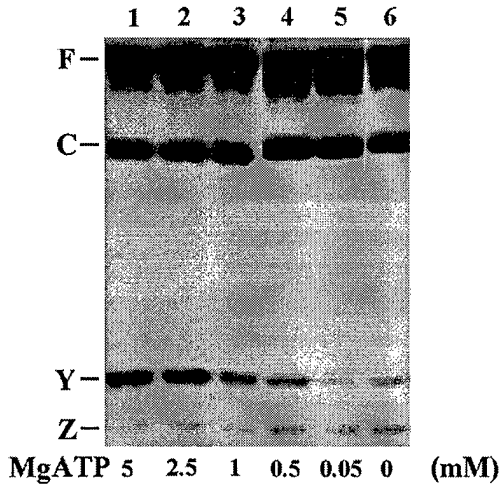

Figure 6. Concentration dependence of MgATP effects. 30 mg of membrane proteins were pre-treated for 30 min at room temperature with MgATP at 5 mM (lane 1), 2.5 mM (lane 2), 1 mM (lane 3), 0.5 mM (lane 4), or 0.05 mM (lane 5). Lane 6 is a control reaction pre-treated without any nucleotides. Trypsin digestion, SDS-PAGE, and Western blot were performed as described in Fig. 5.

(b), concentration dependence of MgATP effects on trypsin digestion of Pgp: As shown in Fig. 6, 1 mM MgATP was sufficient to stimulate the generation of the peptide Y (compare lanes 1-5 with lane 6, Fig. 6). This value corresponds nicely to the estimated K_m (MgATP) value of 0.6-1.4 mM (Doige *et al.*, 1992; Al-Shawi *et al.*, 1993; Shapiro and Ling, 1994; Rao, 1995).

(c), the trypsin digestion profile of Pgp is also altered by vinblastine, but not by methotrexate: Limited trypsin digestion of inside-out vesicles from human SKOV/VLB cells was performed in the absence (lane 1, Fig. 7) or presence of 5 mM (lane 2), 50 mM (lane 3), and 100 mM (lane 4) methotrexate (MTX) or 5 mM (lane 5), 50 mM (lane 6), and 100 mM (lane 7) vinblastine (VLB). It is clear that more peptide Z was produced in the presence of vinblastine whereas no change was observed in the presence of MTX. The fact that 5 mM VLB enhanced the

proteolysis of Pgp to generate the peptide Z is consistent with the Kd(VLB) of ~2 mM (Cornwell *et al.*, 1986). It is also interesting to note that the generation of the peptide Y was enhanced by MgATP whereas the generation of the peptide Z was enhanced by VLB. This suggests that different structural changes may be elicited by MgATP and VLB respectively

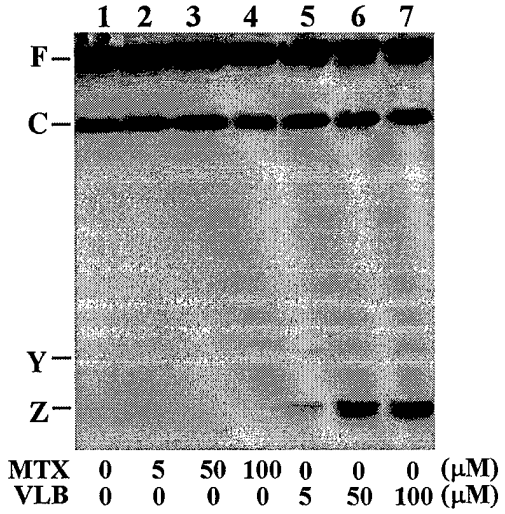

Figure 7. Effects of drug substrates on trypsin digestion of Pgp. 30 min at room temperature with methotrexate (MTX, lanes 2-4) or vinblastine (VLB, lanes 5-7) at various concentrations as indicated. Lane 1 is 30 mg of membrane proteins were pre-treated for a control reaction pre-treated without drugs. Trypsin digestion, SDS-PAGE, and Western blot were performed as described in Fig. 5.

6.2.4. Expression of a drug-binding domain of Pgp

(a), expression and drug binding of Pgp fragment as a fusion protein in bacterial membranes: It has been shown previously that a domain including TM11-TM12 of Pgp binds photoactive drug analogous (Safa *et al.*, 1987; Greenberger *et al.*, 1991; Bruggemann *et al.*, 1989; 1992). We have engineered a Pgp cDNA fragment encoding the TM11-TM12 with the C-terminal ATP-binding domain into the GST fusion protein expression vector pGEX-2T. The expression of a 65-kDa fusion protein in bacterial BL-21 membranes was induced by IPTG and was detected by commassie blue staining (lane 2, Fig. 8A) and by monoclonal antibody C219 (lane 1, Fig. 8C). Thrombin digestion cleaved off the GST portion from the fusion protein and the Pgp portion of ~40 kDa was detected (lane 3, Fig. 8A and lane 2, Fig. 8C).

To determine if the fusion protein binds drugs, we incubated the bacterial membranes with photoactive [^{3}H]azidopine and the crosslinking was activated by exposing the reaction mixture to UV light. The labeled proteins were then separated on an SDS-PAGE which was then fixed in acetic acid/methanol, treated with amplify^{TM}, and dried before exposing to an x-ray film for fluorography. As shown in lane 1 (Fig. 8B), the fusion protein was apparently crosslinked to [^{3}H]azidopine. Using thrombin digestion, it was also shown that the Pgp portion of fusion protein can bind [^{3}H]azidopine (lane 2, Fig. 8B). The binding of azidopine to the fusion protein was inhibited by the competition with vinblastine (Fig. 9). Therefore, it is likely that the Pgp fragment alone containing TM11, TM12, and the C-terminal ATP-binding domain can bind drugs. This study shows that the drug binding to Pgp does not require the full-length Pgp molecule and that the fusion protein expression system can be used to investigate molecular interaction between drug substrates and Pgp.

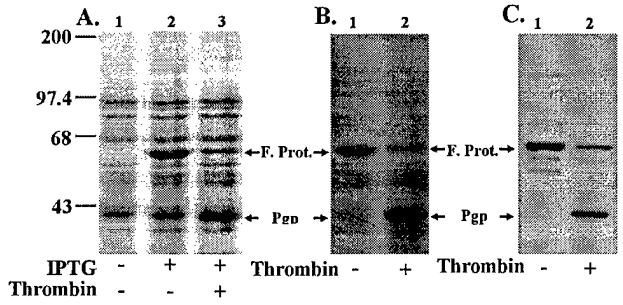

Figure 8. Binding of [^{3}H]azidopine to Pgp fragments expressed in bacteria membranes. (A), expression of TM11,TM12-NBD. The cDNA encoding the TM11,TM12-NBD of human MDR1 Pgp was cloned into the pGEX-2T expression vector, and transformed into bacteria BL-21. The expression of the fusion protein was induced by IPTG (lane 2). Thrombin digestion cleaved the GST off the fusion protein and the Pgp portion remained with the membrane (lane 3). Lane 1 is a control membrane prepared from cells that were not induced by IPTG. The proteins were detected by commassie blue staining. (B), [^{3}H]azidopine binding to the fusion protein. Membranes containing fusion protein (lane 1) or Pgp portion (lane 2) were incubated with [^{3}H]azidopine at room temperature for 30 min and the labeling was activated using a UV Stratalinker (Stratagene). The proteins were then fractionated on an SDS-PAGE and the labeled proteins were recorded on an x-ray film. (C), Western blot of the fusion protein. Membrane preparations containing fusion protein or Pgp portion were separated on an SDS-PAGE, and transferred onto a PVDF membrane. The blot was probed with the Pgp-specific monoclonal antibody C219.

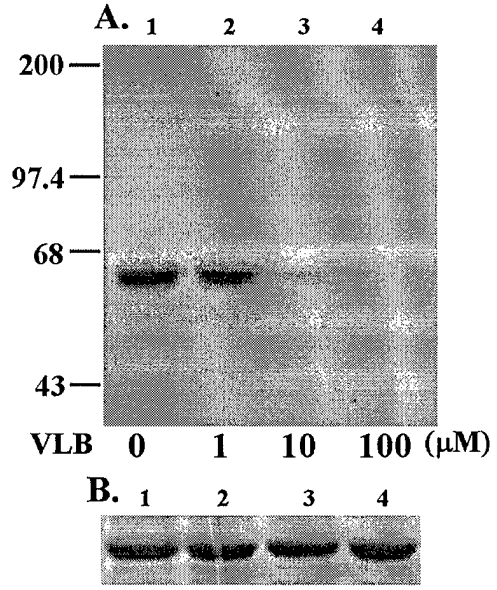

Figure 9. Vinblastine competition of the azidopine binding to the fusion protein. (A), equal amount of fusion proteins in membranes were pretreated with 0 (lane 1), 1 (lane 2), 10 (lane 3), or 100 (lane 4) mM vinblastine, followed by addition of 0.25 mM [^{3}H]azidopine and incubation for 30 min at room temperature. The crosslinking was then activated by UV light. The proteins were separated on an SDS-PAGE, dried, and the labeled proteins were recorded on an x-ray film. (B), after recording the signal of labeled proteins on x-ray film, the gel in panel A was rehydrated and stained with commassie blue. The results confirmed the equal amount of fusion protein used in each reaction.

(b), solubilization of the fusion protein including TM11, TM12 and the nucleotide-binding domain: We have attempted to solubilize the fusion protein of Pgp expressed in bacterial membranes using various detergents and chaeotropic reagents. We have worked out a method to purify the fusion protein partially. As shown in Fig. 10, membranes containing the fusion protein was treated with 1% b-D-maltoside to remove most membrane proteins. The pellet was then treated with 0.4% N-lauroylsarcosine including 25 mM DTT to solubilize the fusion protein. In the second step, at least 50% of the solubilized proteins are the fusion protein.

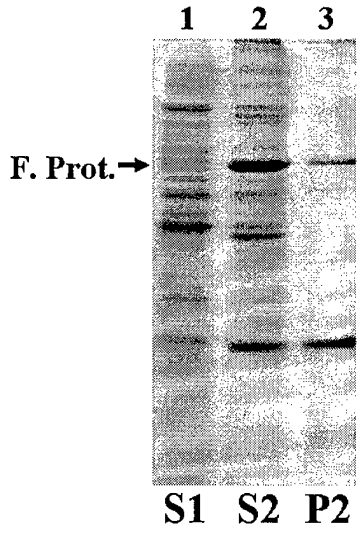

Figure 10. Solubilization of fusion proteins containing TM11, TM12, and their following sequences of Pgp. 15 mg of membranes containing the fusion protein of GST and Pgp fragment were first treated with 1% b-D-maltoside in PBS for 30 min on ice. The soluble and insoluble fractions were separated by centrifugation. The soluble (S1) fraction was used for SDS-PAGE (lane 1) and the pellet was resuspended in 0.4% N-lauroylsarcosine in PBS containing 25 mM DTT and treated for 30 min on ice. The soluble (S2) lane 2) and insoluble (P2) (lane 3) fractions were separated again by centrifugation and were analyzed on SDS-PAGE. It is clear that the fusion protein was insoluble in 1% b-D-maltoside, but soluble in 0.4% N-lauroylsarcosine. Majority of other proteins were removed by the first treatment with b-D-maltoside.

7. CONCLUSIONS:

In the two and half years, we have determined the topologies of P-glycoprotein in multidrug resistant cells using site-specific antibodies. We found that P-glycoproteins in the plasma membrane of mammalian cells express at least two alternate topologies. This observation is consistent with our previous study using cell-free expression system. The more than one topology feature of Pgp may be responsible for its multifunctional nature.

We have also determined the potential conformation changes of Pgp in its catalytic cycle. Pgp is an ATPase that hydrolyzes ATP even in the absence of drug substrates. We showed that the trypsin digestion profile of Pgp was altered in the presence of ribonucleotide ligands. This suggests that the conformation of Pgp was altered by the binding of various nucleotides.

Furthermore, we were able to express the transmembrane domain of Pgp in bacteria for drug binding studies. The putative TM11 and TM12 have been suggested to be drug-binding site (Zhang *et al.*, 1995b). By expressing the drug-binding domain alone in bacterial will provide an opportunity to prepare large quantity for further structure and function studies. For example, the 3-dimensional (3-D) fold of the drug-binding peptides can be analyzed using x-ray crystallography and NMR technology. Studies of 3-D structure will help define the group(s) of drugs that interact with the drug binding peptides. Alteration of the groups of these drugs may lead to a design of new agents that will overcome the MDR, but still have therapeutic effects.

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10. LIST OF PERSONNEL WHO RECEIVED PAY

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11. APPENDIX

Reprints of the published articles were appended in previous reports.