Transmembrane Organization of Mouse P-glycoprotein Determined by Epitope Insertion and Immunofluorescence*

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P-glycoprotein (P-gp) is an integral membrane protein that causes multidrug resistance when overexpressed in tumor cells. Efforts to identify the position and polarity of its 12 putative transmembrane (TM) domains have so far failed to yield a consistent topological model. Recently, we have described a method for topology mapping based on the insertion of a small antigenic peptide epitope (YPYDVPDYA) in predicted intra- or extracellular loops of the protein. The tagged proteins are then functionally expressed in Chinese hamster ovary cells, and the polarity of the inserted tag with respect to plasma membrane is deduced by immunofluorescence in intact or permeabilized cells. We previously localized segments between TM1 and TM2, and TM5 and TM6 as extracellular and segments between TM2 and TM3 and downstream of TM6 as intracellular (Kast, C., Canfield, V., Levenson, R., and Gros, P. (1995) Biochemistry 34, 4407–4411). We have now inserted single epitope tags at positions 207, 235, 276, 741, 782, 797, 815, 849, 887, 961, and 1024; double epitope tags at positions 736, 849, and 961; and a triple epitope tag at position 849. Insertions of epitopes at positions 235, 736, 741, 849, 887, 961, and 1024 resulted in functional proteins, whereas insertions at positions 207, 276, 782, 797, and 815 abrogated the capacity of P-gp to confer multidrug resistance. The epitope tags inserted at positions 736, 849, and 961 were localized extracellularly, whereas tags at positions 235, 887, and 1024 mapped intracellularly. These results indicate that the intervening segments separated by TM4-TM5, TM10-TM11, and downstream of TM12 are cytoplasmic; segments delineated by TM7-TM8, TM9-TM10, and TM11-TM12 are extracellular. Our combined analysis of the amino- and carboxyl-terminal halves of P-gp supports a 12-TM domain topology with intracellular amino and carboxyl termini and ATP binding sites and an extracellular glycosylated loop (TM1-TM2) in agreement with hydropathy prediction. These results are clearly distinct from those obtained by the analysis of truncated P-gps in vitro and in heterologous expression systems.

Overexpression of P-glycoprotein (P-gp) is associated with the onset of multidrug resistance in cancer cells in vivo and in cultured cells in vitro (Gottesman and Pastan, 1993; Shustik et al., 1995). P-gps are integral membrane phosphoglycoproteins that can directly bind photoactivatable analogs of ATP (Cornwell et al., 1987; Schurr et al., 1989) and drug molecules (Cornwell et al., 1986; Safa et al., 1986; Safa, 1993) and use the energy of ATP hydrolysis to reduce intracellular drug accumulation in resistant cells through an active efflux mechanism (Gottesman and Pastan, 1993; Ruetz and Gros, 1994). P-gps are encoded by a small gene family that comprises three members in rodents (mdr1, mdr-2, mdr-3) (Gros et al., 1986, 1988; Devault and Gros, 1990) and two members in humans (MDR1, MDR2) (Chen et al., 1986; Van der Bliek et al., 1986). Primary amino acid sequence analysis from cloned cDNAs (Gros et al., 1986) indicates that P-gps are formed by two sequence homologous halves, each encoding six putative transmembrane (TM) domains and one nucleotide binding domain (Walker et al., 1982). Furthermore, P-gps contain a putative glycosylated loop between predicted TM1 and TM2, with a cluster of N-linked glycosylation signals (Schinkel et al., 1993). The P-gp family is also a member of a much larger family of structurally and functionally related membrane proteins that together form the ATP-binding cassette (ABC) superfamily of transport proteins (for review, see Higgins (1992)). The majority of the members of this ATP-binding cassette superfamily share a similar structure consisting of 12 TM domains and two nucleotide binding domains, either linearly joined in the same molecule (P-gp, CFTR, STE-6; Gros et al., 1988; Riordan et al., 1989; McGrath and Varshavsky (1989)) or expressed as half-molecules in the form of heterodimers (TAP1/TAP2, Trowsdale et al. (1990), Spies et al. (1990); ALDP/PMP70, Kamijo et al. (1990), Mosser et al. (1993)).

Some of the major topological features of P-gp, including the cytoplasmic localization of the two ATP binding sites, the extracellular location of the TM1-TM2 glycosylated loop, and the intracellular position of the carboxyl terminus, have been verified by biochemical and immunological methods (Kartner et al., 1985; Yoshimura et al., 1989; Bruggermann et al., 1989; Schinkel et al., 1993). In addition, localization studies of the epitope for monoclonal anti-Pgp antibody MRK-16 in intact cells revealed that the short protein segments located between predicted TM1 and TM2 and between TM7 and TM8 are indeed extracellular and in close proximity (discontinuous epitope), in agreement with the topological model proposed above by hydropathy analysis (Georges et al., 1993). The exact number and proposed topology of individual TM domains of P-gp have been analyzed by several groups, but a clear consensus has yet to emerge. Two strategies have been used to map the topology of individual TM domains: (a) in vitro methods in which truncated P-gps are fused to an indicator molecule, and polarity is
deduced biochemically after insertion into microsomes or heterologous expression systems; and (b) in vivo methods in which discrete tags are inserted into key locations. In this case, the full-length mutant cDNAs are expressed in mammalian cells, and the tags are mapped using epitope-specific antibodies or labeling reagents. These approaches have resulted in contradictory topological models for P-gp. Methods using truncated proteins fused to indicator molecules suggested topological models different from hydrophathy prediction, whereas methods using the full-length functional P-gp suggested a topological model in agreement with hydrophathy prediction.

We have used an alternative strategy for topology mapping of the membrane-associated portion of P-gp (Kast et al., 1995). A nine-amino acid hemagglutinin peptide derived from influenza virus and recognized by the monoclonal antibody 12CA5 was inserted within individual predicted intracellular and extracellular loops of P-gp. The mutant proteins were expressed in mammalian cells to test their capacity to confer multidrug resistance. Immunofluorescence with 12CA5 using light microscopy in normal or permeabilized cells was then used to deduce the polarity of the tag with respect to the plasma membrane (intracellular versus extracellular). In addition, confocal microscopy was used to monitor proper targeting to the plasma membrane of mutants showing intracellular localization of the tag. Using this approach, we previously mapped the topology of loops connecting TM1-TM2 and TM5-TM6 as extracellular and that connecting TM2-TM3 and the segment downstream of TM6 as intracellular (Kast et al., 1995). Here we report a more complete topological map of Mdr3 localizing three additional intracellular and three additional extracellular loops of P-gp. Our results are in agreement with the topology predicted by hydropathy analysis and recent results obtained with the cysteineless MDR1 mutant (Loo and Clarke, 1995a). In contrast, our findings contradict topological models deduced from the study of truncated P-gps fused to reporter genes and expressed in heterologous in vitro or in vivo systems (Zhang and Ling, 1991, 1993; Zhang et al., 1993; Bibi and Béja, 1994; Béja and Bibi, 1995).

**Experimental Procedures**

**Materials—Geneticin (G418) was obtained from Life Technologies, Inc. Vinblastine and calicheic were from Sigma, Adriamycin and actinomycin-D were a generous gift of Dr. C. Shustik (Royal Victoria Hospital, Montreal, Quebec). All restriction enzymes were obtained from New England Biolabs or Pharmacia Biotech Inc. The protein assay was from Bio-Rad, the dNTP nucleotide-directed in vitro mutagenesis system (version 2.1, RPN 1523) from Amersham Canada, and the monoclonal anti-hemagglutinin antibody 12CA5 was purchased from Babco Laboratories (Richmond, CA).

**Site-directed Mutagenesis—** The procedure for inserting epitope tags was as described previously (Kast et al., 1995). A list of the dNTP nucleotides used, including nucleotide sequences and positions in the primary amino acid sequence of Mdr3, is shown in Table I. The hemagglutinin tags contained the nine amino acids (YPDYVPDYA) derived from influenza virus hemagglutinin A or the hemagglutinin tag with an additional serine (YPDYVPDYAS) and were inserted at the positions shown in Fig. 1. For insertional mutagenesis in the amino-terminal half of P-gp, a full-length cDNA for mouse mdr3 cloned into the plasmid pGEM7Zf (Promega) was digested with Sphl (polylinker) and Smal (nucleotide position 1767), and the resulting 1.7-kilobase 5' half-fragment was cloned in the corresponding sites of M13mp19. For insertional mutagenesis of the carboxyl-terminal half, a 1.7-kilobase mdr3 cDNA fragment (SmaI, position 9767 to PstI, position 9516) was cloned in the corresponding sites of M13mp18. Mutagenesis was carried out using a commercially available kit (Amersham; version 2.1, RPN 1523). The integrity of the entire mutagenized mdr3 cDNA inserts was verified by nucleotide sequencing using the dyeoxy chain termination method of Sanger et al. (1977). The mutant cDNA inserts were then reconstructed back into the full mdr3 cDNA in plasmid pGEM7z before subcloning in the mammalian expression vector pcB6 (Canfield and Levenson, 1993).

**Cell Culture—** Wild type and mutant mdr3 cDNAs were doned into the mammalian expression vector pcB6, which uses the promoter and enhancer elements of human cytomegalovirus to direct high level expression of cloned cDNAs and also contains a Tn5 transposon that allows selection of transfected cell clones in G418. Drug-sensitive L73 Chinese hamster ovary cells and L929 mouse fibroblasts) were transfected by the calcium phosphate cocoprecipitation method and grown in α-minimal essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml). Two days later, cells were subcultured at a 1:5 dilution, and stable transfecants were selected in G418 (final concentration 500 μg/ml). Mass populations of G418-resistant transfecants were then plated in the MDR drugs vinblastine (final concentration 50 ng/ml) or actinomycin-D (final concentration 25 ng/ml) to select for cells overexpressing the various tagged Mdr3 proteins.

**Membrane Preparation and Western Blotting—** Crude membranes of transfected L73 cells were isolated as described (Devault and Gros, 1990). Protein concentration was determined by the method of Bradford (1976) using a commercially available reagent (Bio-Rad). Proteins (6 μg/lane) were resolved on a SDS-containing 7.5% polyacrylamide gel and transferred by electroblotting to nitrocellulose membranes. The membranes were incubated with the isoform-specific rabbit anti-mouse Mdr3-specific polyclonal antibody B2037 (Devault and Gros, 1990) or the anti-epitope monoclonal antibody 12CA5 (Kim et al., 1983) used at a dilution of 1:200 or 1:1,000, respectively. Specific immunocomplexes were revealed by a second goat anti-rabbit antibody (1:3,000) coupled to alkaline phosphatase (Amersham).

**Cytotoxicity Assay—** 7.5 × 10^5 drug-sensitive L73 control cells or cells from mass populations of vinblastine-resistant transfecants expressing individual tagged proteins were seeded in 96-well titer plates in α-minimal essential medium containing increasing concentrations of either vinblastine, adriamycin, actinomycin-D, or colchicine (Tang-Wai et al., 1993). The cells were incubated at 37°C for 2 h, fixed in 17% trichloroacetic acid in phosphate-buffered saline, and cellular protein was stained for 10 min at room temperature with 0.4% sulforhodamine B in a 1% acetic acid solution. The plates were washed with water, dried, and the stain dissolved in 0.2 ml of 10 mM Tris, pH 9. Quantification was done using an automated enzyme-linked immunosorbent assay plate reader (Bio-Rad model 450) at a wavelength of 490 nm.

**Immunofluorescence and Confocal Microscopy—** For immunofluorescence (Canfield and Levenson, 1993), mass populations of vinblastine-resistant L73 cells transfected with tagged cDNAs, and control drug-sensitive L73 cells were grown on glass coverslips. Nonpermeabilized L73 cells were incubated with the monoclonal antibody 12CA5 (1:250) in α-minimal essential medium supplemented with 10% fetal calf serum and 20 mM Hepes, pH 7.4, for 1 h at 4°C. The cells were fixed in 4%
paraformaldehyde in phosphate-buffered saline and permeabilized and blocked with 0.05% Nonidet P-40 in phosphate-buffered saline, 5% goat serum, and 1% bovine serum albumin at room temperature for 15 min. A secondary antibody (rhodamine-conjugated rabbit anti-mouse IgG; Jackson ImmunoResearch Labs., Inc.) was then applied in the same buffer (dilution of 1:400). In experiments with permeabilized cells, the cells were fixed, permeabilized, and blocked as described above and then incubated with the first antibody (1:500) for 1 h at room temperature before exposure to the secondary antibody (1:400). Immunofluorescence microscopy was performed using standard epifluorescence optics (Zeiss Axiophot) or by confocal laser scanning microscopy using a Zeiss LSM 210 confocal microscope.

**RESULTS**

Construction of Epitope-tagged Mutant P-gps—Hydropathy

Analysis of P-gp predicts two large hydrophobic regions (positions 51–345 and 707–990) and two hydrophilic regions corresponding to the cytoplasmic ATP binding folds. The two hydrophobic regions encode 12 putative TM domains, possibly forming six extracellular (EC) loops and four intracellular (IC) loops with the amino and carboxyl termini localized intracellularly (Fig. 1A). Previously, we analyzed the membrane topology of the amino-terminal hydrophobic region of mouse Mdr3 by epitope insertion (Kast et al., 1995). To complete the topology of the rest of the amino-terminal region and to map more precisely the carboxyl-terminal region, we inserted the epitope tag YPYDVPDYA in various predicted EC and IC loops. In the amino-terminal half, epitopes were inserted in the second predicted EC loop at position 207 (mutant 7) and in the second predicted IC loop at positions 235 (mutant 8) and 276 (mutant 9). In the carboxyl-terminal half, epitopes were inserted in the fourth, fifth, and sixth predicted EC loops at amino acid positions 741, 849, and 961 (mutants 11, 15, 19, respectively), within the third and fourth predicted IC loops at positions 782, 797, 815 (mutants 12, 13, 14), and 887 (mutant 18) and at position 1024 (mutant 21) within the loop that contains the second ATP binding site (Fig. 1, B and C). Since single epitopes added to the predicted EC loops of the carboxyl-terminal half of the protein were not accessible to the anti-epitope antibody (see below), we also introduced tandem epitope tags at positions 736 (mutant 10), 849 (mutant 16), and 961 (mutant 20), as well as a triple epitope tag at position 849 (mutant 17).

Stable Transfection of LR73 Cells with the Mutant mdr3 Constructs—Wild type and mutant mdr3 cDNAs encoding tagged proteins (7–21) cloned into the mammalian expression vector pCB6 were transfected into drug-sensitive LR73 Chinese hamster ovary cells, and stable transfectants were selected in G418. Pools of G418-resistant colonies were then plated in medium containing vinblastine (50 ng/ml). In the case of constructs 8 (predicted EC5) and 10 (predicted IC4), colonies were observed at a frequency similar to that of cells transfected with wild type mdr3. For constructs 16 (predicted EC6) and 18 (predicted IC4), colonies were observed at a lower frequency and appeared after 7 days (constructs 16 and 18) or 21 days of selection (construct 8). Vector-transfected cells yielded no colonies within 4 weeks of vinblastine selection. For mutant 17 (predicted EC5) we were able to select drug-resistant colonies in medium containing...
mutant or wild type cDNA showed a specific immunoreactive mass populations of drug-resistant colonies transfected with (second ATP binding fold). Crude membranes prepared from native mutants 8 (predicted IC2), 10 (predicted EC4), 17 (predicted EC5), 15 (predicted EC5), 16 (predicted EC5), and 19 (predicted IC4) expressed significantly higher amounts of protein. The high level expression detected in mutants 8 and 18 suggests a decreased activity of these two mutants, necessitating higher level of expression than wild type Mdr3 to survive the same level of drug selection.

Drug Survival Characteristics of Biologically Active Mutants—Mass populations of cells expressing the Mdr3 mutants were plated in increasing concentrations of vinblastine, adriamycin, colchicine, and actinomycin-D (Fig. 3), and the drug concentration required to reduce the plating efficiency of each mass population by 50% (IC50) was calculated. We noted three phenotypic consequences of the tag insertion on Mdr3 function. In the first case, exemplified by mutant 21 (tag inserted at position 1024 in the loop containing the second ATP binding site), no effect on P-gp function was observed, and the drug resistance profile of mutant 21 was identical to that of wild type (for similar level of protein expression; Fig. 2). Second, the insertion of tags at certain locations caused an overall reduction in activity of the protein as exemplified by mutants 8 (position 235, predicted IC2) with severe reduction in resistance to all drugs (in particular adriamycin, colchicine, actinomycin-D) despite higher level of protein expression in these cells compared with wild type mdr3 control cells. A similar situation was observed for mutant 17 (triple tag at position 849, predicted EC5) (Fig. 3). Third, certain mutants showed a wild type level of resistance for certain drugs and impaired resistance to other drugs. For example, mutant 18 (predicted IC4) showed wild type activity against vinblastine, colchicine, and adriamycin but severely reduced activity against actinomycin-D; conversely, mutant 10 (predicted EC4) showed wild type or near wild type activity against vinblastine and actinomycin-D but reduced activity against colchicine and adriamycin. Nevertheless, the observation that mutants 8, 10, 17, 18, 20, and 21 were found in the membrane fraction of transfected cells (Fig. 2) together with their ability to confer resistance to at least some MDR drugs suggests that they are expressed in a functional state at the plasma membrane.

Localization of the Epitope Tags—The polarity of the tag with respect to its intracellular versus extracellular localization was determined by immunofluorescence with the anti-hemagglutinin epitope-specific antibody 12CA5 in all mutants analyzed but was not present in cells transfected with wild type mdr3 or in nontransfected LR73 cells (Fig. 2B). Taken together, these results confirm that mutants 8, 10, 17, 18, 20, and 21 are functional and present within the membrane-enriched fraction. Cells transfected with mutants 10 (predicted EC4), 17 (predicted EC5), 20 (predicted EC6), and 21 (second ATP binding fold) expressed levels of Mdr3 similar to those measured in vinblastine-resistant cells expressing wild type Mdr3, whereas cells transfected with mutants 8 (predicted IC2) and 18 (predicted IC4) expressed significantly higher amounts of protein. The high level expression detected in mutants 8 and 18 suggests a decreased activity of these two mutants, necessitating higher level of expression than wild type Mdr3 to survive the same level of drug selection.

actinomycin-D at 25 ng/ml but not vinblastine (25 or 50 ng/ml). These results indicate that constructs 8, 10, 11, 15, 16, 17, 18, 19, 20, and 21 retain biological activity. On the other hand, mass populations of G418-resistant clones transfected with mutants 7 (predicted EC2), 9 (predicted IC2), 12 (predicted IC3), 13 (predicted IC3), and 14 (predicted IC3) yielded no drug-resistant colonies at standard (50 ng/ml), reduced vinblastine concentration (25 ng/ml), or in medium containing other MDR drugs, such as adriamycin, actinomycin-D, and colchicine. Therefore, epitope insertions at positions 207 (predicted EC2; mutant 7), 276 (predicted IC2; mutant 9) or 782, 797, and 815 (predicted IC3; mutants 12-14) produced nonfunctional P-gps.

The expression of mutant P-gps in drug-resistant cell clones was analyzed by immunoblotting with an isoform-specific anti-mouse Mdr3 antibody (Devalt and Gros, 1990) or the mouse anti-hemagglutinin epitope monoclonal antibody 12CA5 (Niman et al., 1983). As detailed below, mutants 11 (predicted EC4), 15 (predicted EC5), 16 (predicted EC5), and 19 (predicted EC6) were nondetectable by immunofluorescence analysis. Therefore, immunoblotting results are shown only for informative mutants 8 (predicted IC2), 10 (predicted EC4), 17 (predicted EC5), 18 (predicted IC4), 20 (predicted EC6), and 21 (second ATP binding fold). Crude membranes prepared from mass populations of drug-resistant colonies transfected with mutant or wild type cDNA showed a specific immunoreactive band of molecular mass ~160 kDa, which was absent in untransfected LR73 cells (Fig. 2A). The same band of molecular mass ~160 kDa was also recognized with the hemagglutinin epitope-specific antibody 12CA5 in all mutants analyzed but was not present in cells transfected with wild type mdr3 or in nontransfected LR73 cells (Fig. 2B). Taken together, these results confirm that mutants 8, 10, 17, 18, 20, and 21 are functional and present within the membrane-enriched fraction. Cells transfected with mutants 10 (predicted EC4), 17 (predicted EC5), 20 (predicted EC6), and 21 (second ATP binding fold) expressed levels of Mdr3 similar to those measured in vinblastine-resistant cells expressing wild type Mdr3, whereas cells transfected with mutants 8 (predicted IC2) and 18 (predicted IC4) expressed significantly higher amounts of protein. The high level expression detected in mutants 8 and 18 suggests a decreased activity of these two mutants, necessitating higher level of expression than wild type Mdr3 to survive the same level of drug selection.

FIG. 2. Expression of the mouse Mdr3 fusion proteins 8, 10, 17, 18, 20, and 21 in LR73 cells. LR73 Chinese hamster ovary cells were transfected with either wild type (WT) or mutant mdr3 cDNAs containing inserted hemagglutinin epitopes and selected in drug-containing medium. Crude membrane fractions were isolated from mass populations of transfected cells. Proteins (6 μg/lane) were resolved on 7.5% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with the isoform specific anti-Mdr3 polyclonal antibody B2037 (panel A) or the mouse monoclonal anti-hemagglutinin epitope antibody 12CA5 (panel B). The positions of the molecular mass markers (in kDa) are shown to the left.  

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but no signal in intact cells (Fig. 4, C, I, and M), indicating an intracellular localization of the epitope tag (see Fig. 6). A third class of mutants was uninformative about the extracellular or intracellular localization of the epitope tag. Although mutants 11 (predicted EC4 loop), 15 (predicted EC5 loop), and 19 (predicted EC6 loop) could readily confer drug resistance to transfected cells (data not shown), the single tag inserted in these mutants could not be detected by immunofluorescence in either intact or permeabilized cells (data not shown). The fact that the corresponding proteins could be detected in vinblastine-resistant selected cells by immunoblotting with rabbit anti-mouse Mdr3 isoform-specific antibody and with the anti-tag antibody 12CA5 suggested that the mutants were properly expressed in the membrane of transfected cells, but that single tags in these carboxyl-terminal extracellular loops were inaccessible to the antibody in intact cells. In the case of the predicted EC4 loop (mutant 10) and EC6 loop (mutant 20), this problem was alleviated by introducing a tandem tag while exposing the predicted EC5 loops to the antibody required the insertion of a triple tag (mutant 17). To confirm that the inability to detect the epitope tags in mutant 8 (predicted IC2), 18 (predicted IC4), and 21 (second ATP binding fold) in intact cells does not reflect inappropriate processing/maturation or targeting of the mutant proteins to a site other than the plasma membrane, we used confocal microscopy to determine more accurately the subcellular localizations of mutants 8, 18, and 21. Results of these experiments are shown in Fig. 5. Confocal microscopic analysis of permeabilized cells expressing these mutants and exposed to 12CA5 showed a very uniform fluorescence pattern with a ring-like distribution with no intracellular staining at mid-cell depth. These results clearly indicate proper targeting of mutants 8, 18, and 21 to the plasma membrane and confirm the cytoplasmic localization of tags inserted in predicted IC loops 2 and 4 and the second predicted ATP binding fold (Fig. 6).

**DISCUSSION**

Since the membrane-associated regions of P-gp are responsible for recognition and interaction with its various substrates and inhibitors (Greenberger et al., 1991; Zhang et al., 1995), much effort has been directed toward the elucidation of the number, location, and polarity of individual TM domains. Hydrophathy profiling of the primary amino acid sequences of the murine and human P-gp families (Devault and Gros, 1990; Chen et al., 1986) has consistently led to a proposed structure of 12 TM domains grouped into two symmetrical and sequence homologous halves, with the amino and carboxyl termini intracellular (Fig. 7A). Such a model would position the two predicted nucleotide binding sites intracellularly and the predicted glycosylated loop located between predicted TM1 and TM2 extracellularly, two predictions verified experimentally by epitope mapping (Kartner et al., 1985; Georges et al., 1993). However, despite several attempts by different groups using unrelated experimental strategies, a clear topological picture for this region of the protein has yet to emerge. A summary of the current topological models for the membrane-associated portion of P-gp is shown in Fig. 7. Studies of in vitro translated truncated P-gp molecules containing an epitope tag whose polarity with respect to the membrane was monitored after translocation into pancreatic microsomes suggested that P-gp may also exist in a different conformation with each hydrophobic domain spanning the membrane four rather than six times. In this model, predicted TM5 and TM8 were extracellular, and predicted TM3 and TM10 were located intracellularly (Fig. 7B; Zhang and Ling, 1991, 1993; Zhang et al., 1993). Independent studies of truncated P-gp molecules fused to a prolactin re-
Membrane Topology of P-gp

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**Fig. 5. Subcellular localization of the epitope-tagged Mdr3 fusion proteins by confocal laser microscopy.** Transfected cells stably expressing Mdr3 fusion protein 8 (panel A), 18 (panel B), and 21 (panel C) were permeabilized, and localization of the tagged proteins was revealed by immunofluorescence with anti-hemagglutinin antibody 12CA5. Laser confocal microscopy was done on optical sections scanned through cells at ~3–5 μm above the surface of the coverslip.

The results of our study differ from those deduced from the in vitro analysis of truncated molecules (Zhang and Ling, 1991, 1993, Zhang et al., 1993) or from studies of fusion proteins in E. coli (Bibi and Béja, 1994; Béja and Bibi, 1995). Whereas the results of Zhang et al. (1993) predicted that the intervening segment between TM4 and TM5 would be extracellular, our results with mutant 8 (insertion at position 235) indicate that this segment is in fact intracellular (Fig. 7B). Likewise, these authors predicted that the intervening segment between predicted TM9 and TM10 would be intracellular (Fig. 7B), whereas the results obtained here with mutant 17 (insertion of triple tag at position 849) clearly indicate that it would be positioned extracellularly. On the other hand, Bibi and Béja proposed a

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**Fig. 4. Detection of epitope-tagged Mdr3 fusion proteins by immunofluorescence.** Control LR73 cells and transfectants stably expressing mutant P-gps 8, 10, 17, 18, 20, and 21 were exposed to the mouse monoclonal anti-hemagglutinin epitope antibody 12CA5 with (permeabilized cells, right column) or without pretreatment (intact cells, left column) with 0.05% Nonidet P-40. Cells were then incubated with a second goat anti-mouse antibody conjugated to rhodamine, and the cells were photographed using a fluorescence microscope. The hemagglutinin epitopes in constructs 8 (panels C and D), 18 (panels I and J), and 21 (panels M and N) were only detected in permeabilized cells, whereas the hemagglutinin epitopes in constructs 10 (panels E and F), 17 (panels G and H), and 20 (panels K and L) were detected in both permeabilized and nonpermeabilized cells.

porter gene and expressed in Xenopus laevis oocytes suggested six TM domains in the amino-terminal half and four TM domains for the carboxyl-terminal half of the protein (Fig. 7C; Skach et al., 1993; Skach and Lingappa, 1993, 1994). Finally, topological studies of truncated P-gps fused to alkaline phosphatase and expressed in Escherichia coli proposed a six-TM domain topology for the amino-terminal half of the protein, but with a different arrangement than predicted by hydropathy: TM4 was extracellularly located and replaced by a novel TM4 mapping in a further carboxyl-terminal segment (Fig. 7D; Bibi and Béja, 1994). The reasons for discrepancies between topological models derived from the study of truncated proteins and that predicted by hydropathy are not clear, but they have prompted the search for methods that would allow topology mapping in intact and active proteins. Recently, Loo and Clarke (1995a) inserted single cysteine residues in the backbone of a cysteineless but biologically active human P-gp. Cysteines inserted in the third, fourth, or sixth predicted EC loop were biotinylated in intact cells by low concentrations of biotin maleimide, suggesting an extracellular location of these resi-
new location for TM4 being flanked by residues 245 (out) and 266 (in), based on the analysis in E. coli of various P-gps fused to alkaline phosphatase and on the extracellular localization of an alkaline phosphatase (~50 kDa) inserted between positions 226 and 227 in P-gp that retains activity in E. coli (Fig. 7D). Our intracellular localization of the epitope inserted at position 235 (mutant B) supports the initial positioning of TM4 between positions 210 (in) and 229 (out) as predicted by hydropathy analysis. The study of alkaline phosphatase fusions in E. coli (intracellular at positions 683, and 736; extracellular at position 720; Fig. 7D) also led to the proposition that TM7 may span the membrane twice (Béja and Bibi, 1995). However, our extracellular localization of a tandem epitope inserted at position 736 strongly suggests that TM7 spans the membrane only once and that the intervening segment between predicted TM7 and TM8 is extracellular (Fig. 7F). This conclusion is also supported by the independent observations that (a) a cysteine inserted in a cysteineless P-gp mutant at position 745 maps extracellularly (Loo and Clarke, 1995a) and (b) the protein segment spanning positions 740–747 is recognized in intact cells by the monoclonal antibodies MRK-16 (Georges et al., 1993) and MM4.17 (Cianfriglia et al., 1994). There may be
various reasons for the observed discrepancies between topologies deduced from truncated proteins in vitro or expressed in E. coli and our results. First, truncated proteins may lack carboxyl-terminal sequence determinants important for correct protein folding and membrane insertion of the full-length peptide (Traxler et al., 1993). Second, the insertion of a small epitope tag of 9–19 amino acids in P-gp (this study) may be less detrimental to proper membrane insertion and function than the insertion of a ~50-kDa alkaline phosphatase (Manoil and Beckwith, 1985). Third, the expression systems selected in these studies (X. laevis oocytes, intact E. coli cells) may only incompletely reveal P-gp function. On the other hand, the advantages of the method we have used are 4-fold. The topology was always assessed in the context of the full-length protein. Expression of the tagged proteins was in an intact mammalian cell background where proper post-translational modification possibly important for folding and targeting was expected to be preserved. All proteins analyzed were functional as they conferred drug resistance in transfected cells, strongly suggesting proper folding and targeting to the membrane. The use of normal optics and confocal microscopy to localize the tag ensures maximal confidence for plasma membrane targeting of the protein. This direct visualization of plasma membrane association distinguishes our method from all previous topological studies on P-gp (Fig. 7, B–E). Finally it is interesting to note that independent topology mapping studies performed in the intact full-length proteins in human cysteineless P-gp mutant (Fig. 7E) and those reported by our group in the mouse Mdr3 isoform (Kast et al., 1995; this study; summarized in Fig. 7F) are in agreement with each other and with the membrane topology initially proposed by hydrophathy analysis. Studies in both systems were complementary: although EC5 could not be mapped in the cysteineless P-gp (Loo and Clarke, 1995a), it could be localized in our study (Fig. 7F); on the other hand, although IC3 could not be localized by epitope tagging, it was successfully assigned in the cysteineless MDR1 mutant (Fig. 7E).

We noted a non-random distribution for tag insertion sites that were either completely tolerated by the protein versus those that caused a complete loss of function. In general, insertion of epitopes in the predicted EC loops was much better tolerated than insertion in predicted IC loops. Although only two of the nine insertion sites selected for typing EC loops proved to yield nonfunctional proteins (both are clustered in the TM3–TM4 interval, see discussion below), half of the sites selected in predicted IC loops (5 out of 10) resulted in complete loss of function. Interestingly, insertion of single epitopes in all three predicted EC loops of the carboxyl-terminal half (positions 741, 849, and 961) produced active proteins, but the tag at these locations could not be identified by immunofluorescence in the corresponding transfecants (although the tag was detected by immunoblotting). Additional copies of the tag at these sites were well tolerated to retain function and were required to allow detection by immunofluorescence (Fig. 3). Hydropathy analysis predicts that these loops should be fairly short at 21, 2, and 16 residues in length, respectively, and therefore may be tightly packed with the TM domains in a membrane-embedded bundle. Insertion of multiple epitopes in this context, although not detrimental to helix packing and protein function, may be required to reveal possible flexible extracellular loops. We note that predicted EC2 loop defined by the TM3–TM4 segment did not tolerate epitope insertion (positions 206 and 207), and the resulting proteins were nonfunctional. On the other hand, insertion of single cysteine residues in this loop in the P-gp cysteineless backbone (positions 209, 211, and 215), although producing active P-gps, failed to yield an identifiable location, suggesting inaccessibility of the inserted cysteine to the maleimide reagent (Loo and Clarke, 1995a). Together these results suggest that (a) this short loop (four residues) may not be exposed to the surface of the cell and may be in a much more compact structure; and (b) as opposed to its carboxyl-terminal counterparts, this loop does not tolerate alterations to maintain proper folding, maturation, or activity of P-gp.

Predicted IC loops were often more sensitive to tag insertion than EC loops. This was most evident for IC loops delineated by TM4–TM5 and TM8–TM9, where two out of three insertions in the former and all three tested in the latter seemingly inactivated the protein (Fig. 6). The sequence of these segments is fairly well conserved among the different isoforms of P-gps and among the same isoform in different species. Therefore, the integrity of the segments may be crucial for the mechanism of action of P-gp, as they may be important for drug transport, in particular for signaling to and from the drug binding site(s) located within the membrane portion of the protein and the ATP binding sites. Alternatively, these regions may play an important role in protein folding or proper targeting to the plasma membrane. Indeed, it has been observed that mutations in these regions often resulted in partially glycosylated proteins often retained in the endoplasmic reticulum, probably because of misfolding of the protein (Loo and Clarke, 1994). Therefore, amino acids in these regions may be responsible for proper protein folding because of their interaction with molecular chaperons, such as shown recently for calnexin (Loo and Clarke, 1995b).

In our study, some of the epitope insertions resulted in apparent alterations in the substrate specificity of P-gp, with the more pronounced ones associated with insertions in predicted IC loops (mutants 2, 8, and 18). In general, mutations in P-gp affecting substrate specificity map in the TM portion of the protein (Choi et al., 1988; Gros et al., 1991; Loo and Clarke, 1993a, 1993b) where they have been found to affect drug binding or drug release from the protein (Safa et al., 1990). A few mutations in certain IC loops (Currier et al., 1992; Loo and Clarke, 1994) or near the ATP binding sites (Hoof et al., 1994; Beaudet and Gros, 1995) have also been found to alter the profile of drug resistance encoded by the mutant P-gps. Such mutations have been proposed to impair a signal from the TM regions of the protein to the ATP binding sites, which normally underlies the drug-stimulatable ATPase activity characteristic to P-gp. In the case of a Gly to Val substitution at position 185 within the predicted IC loop defined by the TM2–TM3 interval of the human MDR1 P-gp, it was observed that the alteration in substrate specificity is associated with a change in the drug-stimulatable ATPase activity of the protein (Rao, 1995). Therefore, it is possible that the altered drug specificity caused by insertions in IC loops observed in some of our mutants may be mediated by alterations in this aspect of P-gp ATPase activity.

In conclusion, our epitope mapping studies (Fig. 7F), together with the parallel analysis by Loo and Clarke of single Cys mutants in the human MDR1 protein (Fig. 7E), provide a complementary and unambiguous topology for all TM domains of P-gp. This topology is strikingly close to that predicted by hydropathy analysis of the primary amino acid sequence of the protein but is in disagreement with data obtained with truncated proteins, suggesting that these methods are not optimal to establish the topology of P-gp.

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