Membrane Topology and Glycosylation of the Human Multidrug Resistance-associated Protein*

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The membrane topology of the human multidrug resistance-associated protein (MRP) was examined by flow cytometry phenotyping, immunoblotting, and limited proteolysis in drug-resistant human and baculovirus-infected insect cells, expressing either the glycosylated or the underglycosylated forms of this protein. Inhibition of N-linked glycosylation in human cells by tunicamycin did not inhibit the transport function or the antibody recognition of MRP, although its apparent molecular mass was reduced from 180 kDa to 150 kDa. Extracellular addition of trypsin or chymotrypsin had no effect either on the function or on the molecular mass of MRP, while in isolated membranes limited proteolysis produced three large membrane-bound fragments. These experiments and the alignment of the MRP sequence with the human cystic fibrosis transmembrane conductance regulator (CFTR) suggest that human MRP, similarly to CFTR, contains a tandem repeat of six transmembrane helices, each followed by a nucleotide binding domain, and that the C-terminal membrane-bound region is glycosylated. However, the N-terminal region of MRP contains an additional membrane-bound, glycosylated area with four or five transmembrane helices, which seems to be a characteristic feature of MRP-like ATP-binding cassette transporters.

Overexpression of the multidrug transporter proteins, P-glycoprotein (MDR1)1 or the multidrug resistance-associated protein (MRP) provides the molecular basis of the multidrug resistance phenotype in tumor cells. The possible clinical importance fuels an intensive research activity toward a better understanding of the molecular structure and mechanism of action of these membrane transporters (1–3).

Both P-glycoprotein and MRP, together with several other bacterial and eukaryotic transporters, are members of the ABC-transporter (ATP-binding cassette) protein family. These proteins share a common molecular architecture, i.e. they contain two large transmembrane domains and two cytoplasmic ATP utilization (ABC) units (4). Due to the difficulty of crystallizing large membrane proteins, no detailed three-dimensional structure of any members of these transporters is currently available, and empirical prediction methods are used to obtain molecular models of their structure, especially to predict the locations and numbers of the membrane-spanning helices. In most cases, these methods identify six short transmembrane segments in each of the two transmembrane domains (1–4).

The relevance of the prediction for the membrane topology of CFTR has been confirmed experimentally by insertional mutagenesis (5), thus proving the 2 × 6 transmembrane helix model. The same arrangement of transmembrane helices has been suggested in the case of P-glycoprotein (6, 7), and a large body of experimental data strongly favors this model (8, 9). On the other hand, Ling and co-workers (10, 11), by suggesting an alternative 6- and 4-helix conformation, raised the possibility that P-glycoprotein may exist in two different topological forms in the cell membrane.

When the multidrug resistance-associated protein (MRP) was cloned and sequenced, analysis of its primary amino acid sequence revealed that MRP is more closely related to CFTR than to P-glycoprotein (3). Cole et al. (3) predicted a unique transmembrane topology for MRP, with eight N-terminal and four C-terminal transmembrane segments. In the present experiments, we have examined the membrane topology of MRP by immunodetection with flow cytometry in intact and permeabilized cells, by limited proteolysis of isolated membranes with trypsin and chymotrypsin, and by immunoblotting of the proteolytic fragments with antipeptide antibodies, reacting either with the N-terminal or the C-terminal half of the protein. By using glycosylated and underglycosylated forms of MRP, its major sites of glycosylation could be determined. We have compared the experimental findings with a newly developed membrane topology model of MRP, based on the experimentally confirmed transmembrane topology of CFTR. According to our results, the transmembrane topology of MRP does not follow the model predicted by Cole et al. (3), but this protein has a characteristic, triple membrane-bound domain structure.

MATERIALS AND METHODS

Culturing of the Human Cells—This was performed under standard conditions (12, 13). S1 (SW1353 cells) and S1MRP (MRP-transfected S1 cells reselected 51 cells reselected and cloned in ADR-containing media) were described in Ref. 13. The HL60 ADR (ADR-selected HL60) cells were gifts of Dr. M. Center (see Ref. 14). Where indicated, the cells were pretreated with 10 μg/ml tunicamycin for 72 h. This tunicamycin treatment slightly slowed down cell growth but did not significantly decrease cell viability. S19 (Spodoptera frugiperda ovarian cells) were cultured and infected with a baculovirus as described in Ref. 15. Recombinant baculoviruses, carry-
ing the human MRP cDNA, were generated by using the BaculoGold Transfection Kit (PharMingen), according to the manufacturer's suggestions. Baculovirus transfer vector containing the human MRP cDNA was constructed as follows: a SalI-BamHI fragment was removed from pl31-MRP vector (13); this segment contains 115 base pairs of the 5'-nontranslated region and the 1-840 region of the human MRP cDNA. It was subcloned into M13mp18, and a new XbaI site was introduced by site-directed mutagenesis at position -5 of MRP cDNA (16), and the entire insert was sequenced. The fragment containing the mutation was inserted back into its original position in pl31-MRP. The MRP cDNA was isolated from this modified plasmid by digestion with XbaI and NotI and subcloned into pVL1393 baculovirus transfer vector (Invitrogen).

Membrane Preparation and Proteolysis—For membrane preparation and proteolysis, the human tumor cells and the virus-infected Sf9 cells were harvested, their membranes were isolated and stored, and the membrane protein concentrations were determined, as described in Ref. 17. For proteolytic digestion, the membranes (100–300 μg of protein) were incubated in a reaction buffer of MOPS (50 mm), KCl (50 mm), EGTA (0.5 mm), dithiothreitol (0.5 mm), pH 7.0, in a final volume of 400 μl, in the presence of 5–50 μg of trypsin (type XIX, Sigma) or 5–50 μg of a-chymotrypsin (type VII, Sigma) for 10 min at 4 °C. Thereafter, the reaction was stopped with excess soybean trypsin inhibitor (Sigma, type 1-S) or trypsin-chymotrypsin inhibitor (Sigma, Bowman-Birk, from soybean) and the membranes were washed twice with 1 ml of the incubation buffer with 1-min centrifugations at 12,000 × g. The pellet was dissolved in 100 μl of the electrophoresis buffer, and the samples (25 μl) were run on 6%, 7.5%, or 10% Laemmli-type gels, thereafter electroblotted onto polyvinylidene difluoride membranes.

Immunoblotting—Immunoblotting was performed by dissolving and sonicating the isolated membranes in a dissociation buffer (17). Detection of human MRP with the R1 (rat mAb, 500 × diluted) and M6 (mouse mAb, 200 × diluted) anti-MRP monodonal antibodies (see Ref. 18) were carried out as described in Ref. 18, except that polyvinylidene difluoride membranes were used for electroblotting. The second antibodies were obtained from Jackson Immunoresearch (anti-rat or anti-mouse, peroxidase-conjugated donkey IgG, 20,000 × diluted). Peroxidase-dependent luminescence on the blots (ECL, Amersham) was determined by autoradiography.

Immunofluorescence Staining—For immunofluorescence staining, 106 cells were resuspended in PBS, fixed with 2% formaldehyde, and then incubated either with or without 1% Triton X-100 for 5 min. Cells were incubated for 45 min in PBS + 1% bovine serum albumin medium, and then labeling was performed in the same medium with the monoclonal antibody R1 (100 × diluted), which reacts with an N-terminal epitope of the human MRP protein (18) or with rat IgG2a isotype control mAb, at 4 °C for 45 min. The cells were then washed twice with PBS containing 1% bovine serum albumin and once with PBS, while the permeabilized cells were washed twice with PBS containing 1% Triton X-100. Thereafter, an anti-rat-fluorescein isothiocyanate antibody conjugated (Sigma, 300 × diluted) was applied to the first antibody. Finally, the cells were resuspended in PBS. Cellular fluorescence was measured with a Becton Dickinson FACSCalibur flow cytometer, and data were analyzed by the WinList software (Verity Software House, Inc.).

Calcein Fluorescence—Calcein fluorescence was measured by incubating 2.5–106 cells/ml in HPM1 medium (19, 20), containing 0.25 μM calcein-AM (Molecular Probes, Eugene, OR) at 37 °C with gentle stirring in a Hitachi F-4000 fluorescence spectrophotometer (excitation and emission wavelengths for calcein were 493 and 515 nm, respectively, with a band width of 5 nm).

Prediction of Membrane Topology of Human MRP—The amino acid sequences of human MRP, human CFTR, and yeast YCF1 (Swissprot accession numbers P33527, P13569, and P39109, respectively) were aligned by using CLUSTAL V. Hydrophobicity plots were generated by the method of Kyte and Doolittle (21) with a window of 9 residues. Gaps were introduced into the hydrophobicity plots according to gaps in the sequence alignment.

RESULTS AND DISCUSSION

In these experiments, we have used two drug-resistant human cell lines, the adriamycin-selected HL60 leukemia cells (14) and the MRP-transfected S1 lung tumor cells (13), both expressing MRP but not MDR1 (22–25). We have also generated an SF9 cell-baculovirus system producing human MRP (see "Materials and Methods"). In human tumor cells, MRP is known to be extensively glycosylated, with an apparent molecular mass of about 180–190 kDa (3, 13, 14, 18). As shown in Fig. 1A, in an immunoblot MRP is well recognized at the expected molecular mass both in the HL60 ADR and the S1MRP cells by the two monoclonal MRP-specific antibodies, R1 and M6, generated against peptide segments 192–360 (located at the N-terminal half), and two fused sequences from the C-terminal half (1294–1430 plus 1497–1531), respectively (18) (these peptide segments are darkened on the model in Fig. 4B). As also shown in Fig. 1A, when the HL60 ADR or S1MRP cells are pretreated with tunicamycin (which prevents post-transla-
The rate is greatly increased by the addition of vinblastine. As sensitive parent cell lines, and in the MRP-expressing cells, this increase in cellular calcein fluorescence is much slower in the tumor cells, and this extrusion is blocked by cytostatic drugs or chemotherapeutic agents. As shown in Fig. 2, the functional assay with HL60 ADR cells pretreated with tunicamycin (+Tun); lanes 7–9, membranes from Sf9 cells expressing human MRP. Lanes 1, 4, and 7, nondigested membranes; lanes 2, 5, and 8, membranes digested with trypsin; lanes 3, 6, and 9, membranes digested with chymotrypsin. On 10% running gels, each lane contained 10 μg of original membrane protein.

According to the membrane topology model of MRP by Cole et al. (3), the 192–360 peptide segment, the binding site of the R1 mAb, is localized extracellularly. However, as noted by Flens et al. (18), this mAb recognizes MRP only in permeabilized cells. Flens et al. (18) suggested that the lack of reaction of R1 with MRP in intact cells might be due to an epitope-shielding effect of glycosylation or to a complex conformation of this epitope in the matured MRP protein. As shown in Fig. 1B, in flow cytometry experiments with HL60 ADR cells, both the fully glycosylated and the underglycosylated (tunicamycin-treated) MRP was recognized by R1 mAb when the cells were permeabilized by Triton X-100, while there was no mAb recognition in the intact cells, even when MRP was underglycosylated. These experiments exclude the possibility that a large carbohydrate side-chain shields the epitope of R1 in intact cells and suggest that the peptide region recognized by this mAb is located intracellularly.

In order to examine the possible effect of glycosylation on the functional structure of MRP, we have studied the expression of calcein AM from S1MRP and HL60 ADR cells. As demonstrated earlier, calcein AM is actively extruded both from MDR1-expressing (12, 19, 20) and MRP-expressing (25, 26) tumor cells, and this extrusion is blocked by cytostatic drugs or by drug-resistance reversing agents. As shown in Fig. 2, the increase in cellular calcein fluorescence is much slower in the HL60 ADR (A) or in the S1MRP (B) cells than in the drug-sensitive parent cell lines, and in the MRP-expressing cells this rate is greatly increased by the addition of vinblastine. As shown previously (25, 26), the decreased formation of intracellular free calcein (restored to the control rate, for example, by vinblastine) in the MRP-expressing cells closely correlates with the drug extrusion function of MRP. Fig. 2 demonstrates that this calcein AM extrusion (that is the lower rate of free calcein production, restorable to the control value by vinblastine) is not significantly different in the HL60 ADR or S1MRP cells cultured for 72 h without or with tunicamycin, respectively (tunicamycin, which slightly interferes with calcein AM extrusion, was removed by washing before the experiments). It has been demonstrated earlier that neither the HL60 ADR nor the S1MRP cells contain any measurable amount of MDR1 (13, 14, 18). Thus, the function of MRP, at least according to this fluorescent dye extrusion assay, is unimpaired in the tumor cells expressing an underglycosylated protein, as compared to its fully glycosylated form (see Fig. 1A). This is in line with previous findings for the human MDR1 protein, which is also fully functional in an underglycosylated form (1, 2, 27).

The flow cytometry experiments with R1 mAb and the above functional assay altogether strongly suggest that the N-linked glycosylation of MRP does not result in a major conformational alteration in its molecular structure. It is worth noting that tunicamycin, when present in the assay, inhibits calcein AM extrusion and increases the cytotoxic effect of Adriamycin, thus probably directly inhibits MRP function.

When the cultured HL60 ADR or S1MRP cells were treated with up to 2 mg/ml trypsin or chymotrypsin for 10 min at room temperature, there was no visible change in the immunoblot pattern of MRP. The transport function of the protein, as measured by calcein AM extrusion, remained intact as well under
these conditions (data not shown). Thus, MRP has no easily accessible proteolytic cleavage sites on its extracellular surface. In the following experiments, we have performed limited proteolysis experiments in the isolated membranes of three different MRP-expressing cells, i.e., in human tumor cells expressing the native, glycosylated MRP, the same cells grown in the presence of tunicamycin, and in insect cells (Sf9), the latter both expressing an underglycosylated MRP. By using low concentrations of trypsin and chymotrypsin at 4°C, we obtained relatively large proteolytic fragments of MRP which could be distinguished clearly by immunoblotting with the two monoclonal anti-peptide antibodies, R1 and M6.

As shown in Fig. 3, trypsin digestion of the glycosylated MRP, with an original molecular mass of about 180 kDa (lane 1), resulted in the predominant formation of a 110-kDa band, as detected by the N-terminal R1 mAb (A, lane 2), and in the formation of a wide 70-kDa band, as detected by the C-terminal M6 mAb (B, lane 2). Chymotrypsin digestion (lanes 3 on both panels) produced a fuzzy, 35-45-kDa band, as seen by the N-terminal mAb (A), and a predominant 140-150-kDa band, as seen by the C-terminal mAb (B, with further chymotrypsin digestion the bands corresponding to the trypsin digestion also appeared).

In the case of the underglycosylated MRP, as expressed in the membranes of HL60 ADR + tunicamycin cells, and of Sf9 cells, the original 150-kDa MRP band (lanes 4 and 7 on both panels), after trypsin digestion yielded a 90-kDa band with the N-terminal R1 mAb (A, lanes 5 and 8), while a 50-55-kDa band (in some cases a doublet in this region) with the C-terminal M6 mAb (B, lanes 5 and 8). Chymotrypsin digestion in these cases produced a sharp 30-kDa band as seen by the N-terminally located mAb (A, lanes 6 and 9), and a predominant 120-kDa band, as seen by the C-terminally located mAb (B, lanes 6 and 9, again, with further chymotrypsin digestion the bands corresponding to the trypsin digestion also appeared).

Based on these limited proteolysis experiments we can conclude that there are two preferentially accessible proteolytic sites on the cytoplasmic surface of the MRP protein, and that all three large fragments obtained are membrane-inserted (they are not removed by washing of the membranes after proteolysis). Moreover, the C-terminal and the N-terminal fragments are both glycosylated in the human HL60 cells, while the central fragment, based on the additive molecular mass values, is probably not glycosylated (we have no direct antibody detection for this fragment). As shown earlier for MDR1 and CFTR, these proteins are also preferentially proteolyzed at the large cytoplasmic loop between the two major membrane-inserted portions (1, 2, 28); thus, limited proteolysis provides a useful tool for examining the membrane topology of these ABC transporters.

The above described experimental findings have been compared to a newly developed membrane topology model of MRP. The model described here is based on the comparison of the corrected amino acid sequence of MRP (3, 29) with two mem-
bers of the ABC protein family: the human CFTR, which has a close position to MRPs on the relative similarity dendogram (3, 30), and whose membrane topology is experimentally established (5), and with a recently cloned yeast cadmium resistance protein, YCF1, which seems to be the closest relative of MRP (30). We found that when the CFTR and MRP sequences were aligned, the hydrophobicity analysis of the aligned sequences yielded a close matching of the transmembrane segments, thus suggesting a 6 + 6 transmembrane helix topology for MRP as well (Fig. 4A). However, MRP contains an additional N-terminal segment of about 200 amino acids, which has no counterpart in CFTRs or MDRs, but closely resembles the N-terminal region of the YCF1 (30). We suggest that the mostly hydrophobic N-terminal segments of both YCF1 and MRP are membrane-embedded, and the Kyte-Doolittle analysis (21) of this region predicts several transmembrane helices for both proteins.

On the basis of these above described predictions, our membrane topology model for MRP, as shown in Fig. 4B, proposes three major membrane-inserted regions, separated by large cytoplasmic loops. Potential N-linked glycosylation sites in this model can be found on the extracellular portions of all three membrane-bound regions. In the N-terminal membrane-bound region, extracellular glycosylation sites could be obtained by two possible transmembrane helix predictions based on the hydrophobicity analysis (versions I and II in the model of Fig. 4B). Glycosylation of the second membrane-bound domain may not be probable, as a consensus site closer to the membrane than about 10 amino acids, is unlikely to be glycosylated in naturally expressed membrane proteins (see Ref. 31). This model is strongly supported by our limited proteolysis data, producing three large, membrane-bound fragments of MRP, and experimentally confirming the glycosylation of both the N-terminal and the C-terminal membrane-spanning regions.

The original prediction of Cole et al. (3) for the membrane topology of MRP identified two major membrane-bound segments, the N-terminal part with eight and the C-terminal region with four transmembrane helices. The first major trypsin cleavage site found in our experiments coincides with the predicted "linker" region of this model, C-terminally located from the first ABC domain. However, in this model (3), the whole 192–360 peptide region faces the extracellular space, with two possible N-glycosylation sites, while there is no glycosylation site further N-terminal from this region. In contrast, the membrane topology model described here with three membrane-bound domains suggests that a large portion of the 192–360 peptide region is intracellularly located and predicts an extra glycosylation site at the first N-terminal extracellular loop. Our model is strongly supported by (i) the lack of reactivity of R1 mAb with MRP in intact, nonpermeabilized cells, independently of the glycosylation status of MRP, (ii) preferential, intracellularly located proteolytic cleavage sites in this region, (iii) glycosylation of the protein further N-terminal from this proteolytic cleavage site. Altogether, the experimental findings are fully compatible with the model presented here for the membrane topology of MRP.

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Addendum—While this manuscript was under editorial review, Paulusma et al. (32) reported the discovery of an MRP homolog protein (cMOAT), expressed in the liver canalicular membranes, and probably involved in the excretion of organic anions. The authors, based on the homology with MRP (3), predicted a membrane topology for this protein with 8 + 4 transmembrane helices. Alignment of the cMOAT and the MRP sequences (not shown here) indicates that the membrane topology model described above for MRP may be valid for cMOAT as well, thus suggesting a special structural feature for these proteins among ABC transporters.