Location of a Protease-hypersensitive Region in the Multidrug Resistance Protein (MRP) by Mapping of the Epitope of MRP-specific Monoclonal Antibody QCRL-1

David R. Hipfner, Kurt C. Almqvis, Brenda D. Stride, Roger G. Deele, and Susan P. C. Cole

Cancer Research Laboratories and Departments of Pathology [D. R. H., K. C. A., R. G. D., S. P. C. C.] and Biochemistry [B. D. S., R. G. D.], Queen’s University, Third Floor, Botterell Hall, Kingston, Ontario, K7L 3N6 Canada

ABSTRACT

Multidrug resistance protein (MRP) is a Mr, 190,000 integral membrane phosphoglycoprotein which has been shown by transfection studies to confer multidrug resistance. We have previously raised and characterized a panel of MRP-specific monoclonal antibodies (MAbs) which detect distinct epitopes in the MRP molecule (D. R. Hipfner et al., Cancer Res., 54: 5788—5792, 1994), and, in the present study, we have identified the epitope of one of these, MAB QCRL-1. Immunoblot analysis of MRP fragments generated by digestion with formalic acid or trypsin suggested that the MAB QCRL-1 epitope was located in the region connecting the two halves of MRP. Subsequent analyses of a series of truncated bacterial glutathione S-transferase fusion proteins containing segments of human MRP further localized the MAB QCRL-1 epitope to a region encompassing amino acids 903—956. Similar experiments with an analogous segment of murine MRP demonstrated that MAB QCRL-1 was highly specific for the human protein. The reactivity of MAB QCRL-1 with a series of overlapping hexapeptides and heptapeptides within this region identified the human MRP-specific heptapeptide SSYSGDI (corresponding to amino acids 918—924) as the epitope, and this peptide was shown to specifically inhibit MAB QCRL-1 binding to MRP. The results of these studies confirm that this epitope has a cytosplasmic localization consistent with the topology of MRP predicted from hydrophobicity analyses. These experiments also revealed the presence of a number of protease-sensitive sites on either side of the MAB QCRL-1 epitope in the cytoplasmic domain connecting the two halves of MRP. Future epitope-mapping studies with other MRP-specific MAbs will provide additional insights into the topology of MRP, and may help to identify functionally important regions of this protein. Moreover, definition of the epitope recognized by MAB QCRL-1 as well as the other MAbs will facilitate the use of these reagents for immunohistological studies of MRP expression in drug-resistant tumors.

INTRODUCTION

Cancer chemotherapy is often unsuccessful because of the simultaneous development of resistance to both the drug(s) used in treatment and other functionally and structurally unrelated agents. This resistance, termed multidrug resistance, has been shown to be mediated by at least two different proteins in vitro: the well-characterized P-glycoprotein (1, 2) and the more recently discovered MRP (3—5). MRP and P-glycoprotein are integral membrane phosphoglycoproteins belonging to the ABC superfamily of transport proteins, and both confer resistance to a similar spectrum of hydrophobic drugs (4—7).

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2 To whom requests for reprints should be addressed. Phone: (613) 545-6507; Fax: (613) 545-6830.

3 The abbreviations used are: MRP, multidrug resistance protein; ABC, ATP-binding cassette; LTR, leukemia C; NBD, nucleotide-binding domain; CFTR, cystic fibrosis transmembrane conductance regulator; MAB, monoclonal antibody; CHAPS, 3-(3-cholamidopropyl)dimethyl ammonium propane sulfonate; TBS-T, Tris-buffered saline with Tween 20, GST, glutathione S-transferase.

4 Unpublished observations.

5 Unpublished results.

P-glycoprotein, which is encoded by the MDR1 gene, is thought to confer multidrug resistance by binding drugs and pumping them out of the cell in an ATP-dependent manner, thus preventing their accumulation to cytotoxic levels within the cell (1, 2). The mechanism by which MRP mediates multidrug resistance is not yet clear. Although drug accumulation is decreased and drug efflux is increased in MRP-transfected cells (6, 7), the direct binding of drugs to MRP has not been shown (6, 8). However, membrane vesicle studies have shown MRP to be an ATP-dependent export pump for the cytotoxic phospholipids LTC4 and several other glutathione conjugates (9—12), but also as for certain steroid glucuronide conjugates (13). Furthermore, we have shown that direct transport of unmodified vincristine by MRP-enriched vesicles occurs in the presence of physiological concentrations of glutathione (12).

Very little is known about the secondary structure of MRP. Current evidence suggests it differs from the usual six-plus-six arrangement typical of a number of eukaryotic ABC transport proteins (14). The topological model originally proposed for MRP was based on computer-assisted hydrophy analysis and alignment with the predicted structure of Leishmania ItgPA, which was then the closest known relative of MRP (3, 15). This model suggested that the NH2-terminal half of MRP was composed of eight transmembrane segments and a NBD, while the COOH-terminal half was comprised of only four transmembrane segments and a NBD. However, subsequent studies have indicated that this model is probably not correct. An antisera raised against a peptide from the proposed secondary extracellular domain of MRP reacted with intact MRP-overexpressing cells only when the cells were permeabilized (4). Similarly, a MAB raised against a fusion protein containing this same predicted extracellular domain reacted only with permeabilized cells (16). These data suggested that at least a portion of this domain was cytosolic. We recently proposed a second model for MRP aided by alignment of the human and mouse MRP sequences with the sequences of several additional closely related ABC transporters that were described subsequent to our proposal of the first MRP model (17). In this second model, MRP is predicted to contain 12 transmembrane segments arranged in a conventional six-plus-six configuration, plus an extremely hydrophobic NH2-terminal region which contains five to six additional transmembrane segments that are not present in the P-glycoproteins. This hydrophobic NH2-terminal extension appears to be a unique feature characteristic of a subfamily of MRP-related ABC transporters which, in addition to human and murine MRP, currently includes the rat sulfonfurylurea receptor (SUR) and yeast cadmium resistance conferring protein, YCF1 (17—19).

To obtain evidence for or against the above topological model of MRP, we are mapping the epitopes for a panel of MRP-specific MAbs we have described previously (20). Herein, we report the identification of the epitope of one of these antibodies, MAB QCRL-1, as a linear human MRP-specific heptapeptide sequence found in the cytoplasmic region joining the two halves of MRP.

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MATERIALS AND METHODS

Cell Lines. MRP-overexpressing, doxorubicin-selected, multidrug-resistant H69AR and revertant H69PR small cell lung cancer cell lines have been described previously (21, 22). TS and C1 are HeLa cell populations transfected with a pRedCMV expression vector containing the entire coding region of human MRP or with the parental vector, respectively (23). mBS is a HeLa cell population which has been transfected with the pCEBV7 expression vector containing the entire coding region of murine MRP (17, 24). The multidrug-resistant 8226/Doxo4 melanoma cell line overexpresses MDR1 and was kindly provided by Dr. W. S. Dalton (Arizona Cancer Center, Tucson, AZ, Ref. 25). FVB and FVB/H mice are SV40-immortalized mouse ear fibroblasts obtained from normal FVB/H mice and FVB mice transgenic for human MDR3, respectively, and were kindly provided by Drs. A. Schinkel and P. Borst (Netherlands Cancer Institute; Amsterdam, the Netherlands; Ref. 26).

Antibodies. MAb QCR1-1 and QCR1-3 are MRP-specific murine MAbS generated from splenocytes of mice immunized with H69AR crude membrane protein (20), and are available from Centocor Diagnostics (Malvern, PA). MABs QCR-1 and QCR-3 are of the IgG2a and IgG2b isotypes, respectively. Ascites was produced by i.p. injection of hybridoma cells into pristane-pretrained nude mice as described previously (20). MRP-1, MRP-2, and MRP-L are polyclonal antisera raised against MRP-specific synthetic peptides (15, 23). MRP-1 and MRP-2 were raised against homologous 15- to 16-amino acid peptides from the first and second NBDs of MRP (amino acids 765–779 and 1427–1441, respectively). The MRP-L antisera was raised against a 12-amino acid peptide (amino acids 932–943) from the proposed cytoplasmic connector region in MRP which joins the two halves of the molecule. The MRP-1 and MRP-L antisera were purified by affinity chromatography as described previously (15). The P-glycoprotein-specific MAB C219 was obtained from Centocor.

Formic Acid Digestion of MRP. Cell membrane-enriched fractions were prepared from H69AR cells, and MRP was immunoprecipitated from 100 µg membrane protein using MAbQCR1-1 and QCR1-3 as described previously (20). Precipitated proteins were separated using SDS-PAGE on 7% gels and transferred as described below. The region of the blot containing MRP was excised and incubated in 70% formic acid for 48 h at 37°C. The supernatant was lyophilized, and the pellet was washed twice by resuspension in distilled water followed by lyophilization. The final pellet was resuspended in Tricine sample buffer (BioRad, Hercules, CA), and polypeptides were separated on a 16.5% Tris-Tricine gel, transferred, and immunoblotted as described below.

Limited Trypsin Digestion of MRP. H69AR membrane proteins in Tris-sucrose buffer (250 mM sucrose/10 mM Tris, pH 7.5) were incubated with trypsin (ICN Biomedicals, St. Laurent, Quebec, Canada) at a trypsin:protein ratio of 1:100 for 10 min at 37°C. To stop digestion, phenylmethylsulfonyl fluoride and leupeptin were added to final concentrations of 10 mM and 16.7 µg/ml, respectively. Samples were solubilized in Laemmli buffer, separated on 7% polyacrylamide gels, and blotted as described below.

Production of Fusion Proteins. Using a full-length human MRP expression vector as template, portions of the cytoplasmic connecting region of MRP were amplified by PCR. A series of nested 5′ primers, each containing an EcoRI site, was used in reactions with a 3′ primer containing a BamHI site to amplify a series of 5′ end deletion products. These products were predicted to encode MRP amino acids 847–956 (L-847), 865–956 (L-865), 886–956 (L-886), 903–956 (L-903), and 919–956 (L-919) (see Fig. 3). Human MRP primers were also used to amplify a segment encoding amino acids 887–952 of the murine protein (mL-887) from a murine MRP full-length expression construct (17). The PCR products were digested with BamHI and EcoRI and subcloned into the BamHI/EcoRI site of a PGEX2 expression vector (Pharmacia Biotech, Uppsala, Sweden), which had been modified to encode six histidine residues at the COOH-terminus of the fusion protein (a kind gift from Dr. P. Greer, Queen’s University; Kingston, Ontario, Canada). The fidelity of the MRP sequence in each construct was confirmed by DNA sequence analysis. The E. coli strain XL1-BLUE. Cells were harvested, boiled for 10 min in 3% SDS/3% 2-mercaptoethanol/0.3% bromophenol blue/10% glycerol, and lysates were separated using SDS-PAGE on 12.5% polyacrylamide gels and immunoblotted as described below.

SDS-PAGE and Immunoblotting. SDS-PAGE was performed essentially as described (15, 23). Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) using 25 mM Tris base, 192 mM glycine, and 20% methanol buffer. Blots were blocked in TBS-T (10 mM Tris-0.15 M NaCl-0.05% Tween 20, pH 7.5) with 1% BSA and 5% bovine calf serum for 1 h. Antibodies were diluted appropriately in blocking buffer and then incubated with the blots for an additional 1 to 2 h. After washing three times for 5 min in TBS-T, horseradish peroxidase-conjugated goat F(ab')2 fragments [anti-mouse IgG + IgM (H+L); Pierce, Protein Diagnostics Inc., Edmonton, Alberta, Canada, or anti-rabbit IgG (H+L); Jackson ImmunoResearch, West Grove, PA], diluted 1:10,000 in blocking solution, were added, and blots were incubated for 1 h. After washing five times for 5 min in TBS-T, antibody binding was determined by enhanced chemiluminescence detection (Dupont NEN, Boston, MA) and exposure on Kodak X-OMAT film.

Synthesis and Immunoblotting of Peptides. The MRP-L peptide was synthesized by the Biotechnology Service Centre at the Hospital for Sick Children (Toronto, Ontario, Canada). All other peptides were synthesized by Research Genetics (Huntsville, AL). The resin-cleaved peptide SSYSGDI was synthesized by standard Fmoc chemistry. For epitope mapping, peptides were produced using the peptides on paper technique. Briefly, peptides were synthesized in nanomolar quantities in a 96-well format, using standard Fmoc chemistry, directly on the surface of a membrane. The peptides were covalently linked to the membrane by their COOH-terminal ends via a 6-aminohexanoic acid spacer. Overlapping hexapeptides/heptapeptides were synthesized such that each successive peptide contained the last five/six residues of the preceding peptide plus the next amino acid in the sequence. Hexapeptides were synthesized to span MRP amino acids 896–947 (896–901, 897–902, . . . , 942–947), whereas heptapeptides spanned amino acids 895–947. The 96-well format membrane was blocked for 2 h in 1% BSA/1% skim milk powder in TBS-T on an orbital shaker. MAB QCR1-1 asacites (diluted 1:50,000 in blocking solution) was added and incubated overnight. After four 10–15-min washes in TBS-T, a horseradish peroxidase-conjugated F(ab')2 fragment of goat anti-mouse IgG + IgM (H+L; diluted 1:10,000 in blocking solution; Pierce) was added and incubated for 4 h. The blot was washed five times for 10 min in TBS-T, and antibody binding was determined by chemiluminescence detection as described above.

Competitive Immunoprecipitation. Membrane-enriched fractions were prepared from H69AR cells that had been metabolically labeled with [14C]methionine as described previously (20), resuspended in Tris-sucrose buffer, aliquoted, and frozen at −70°C. For immunoprecipitation, 20 µg radiolabeled membrane protein per sample was pelleted by centrifugation at 100,000 × g for 20 min, and then solubilized for 1 h at 4°C in PBS with 1% CHAPS. Insoluble material was removed by recentrifugation at 100,000 × g for 20 min. Ascites fluid containing approximately 2–2.5 µg MAb was incubated for 1 h at room temperature with 0.4–2 nmol free peptide in PBS. The mixture was then adjusted to 1% CHAPS and added to the solubilized membranes. After incubation for 4 h at 4°C, 30 µl of 25% GammaBind Plus protein G Sepharose (Life Technologies Inc., Gaithersburg, MD) in 1% CHAPS/PBS was added to each sample and incubated for 1 additional h at room temperature. After brief microcentrifugation, the Sepharose beads were washed three times with 1% CHAPS/PBS, and precipitated proteins were eluted from the beads with Laemmli buffer and analyzed using SDS-PAGE and fluorography. Densitometric analysis using a Molecular Dynamics computing densitometer (Sunnyvale, CA) was used to estimate the amount of MRP immunoprecipitated. Results were expressed as a percentage of MRP immunoprecipitated in the absence of competing peptide.

RESULTS

Cross-Reactivity of MAB QCR1-1 with Other ABC Transport Proteins. Previous studies suggested that MAB QCR1-1 reacts with a linear cytoplasmic epitope in MRP (20). Some cytoplasmic regions of members of the ABC transporter superfamily (most notably the NBDs) are highly conserved, raising the possibility that antibodies raised against MRP might cross-react with other members of the superfamily. Therefore, we tested the cross-reactivity of MAB QCR1-1 with some additional mammalian ABC transporters using immunoblot analysis. Membranes from MDR1-overexpressing 8226/
Mab QCRL-1 also did not detect the presence of the human CFTR expressed at high levels in membranes from T84 colon cells (Ref. 27; results not shown). Mab C219 reacted with the $M_r 170,000$ proteins encoded by the $MDR_1$ and $MDR_3$ genes in $8226/Dox40$ and V01V01 cells, respectively, as expected, but not with MRP.

Cross-reactivity of Mab QCRL-1 with murine MRP was also assessed. MRP-1 is an anti-peptide polyclonal antiserum raised against a highly conserved motif in the first NBD of human MRP, which is 100% conserved in the murine MRP sequence. This antiserum detected MRP in membrane protein preparations from human MRP-transfected HeLa cells (75), but not (under the conditions used) in the control transfected cells (C1; Fig. 1B, left). The MRP-1 antiserum also reacted with the slightly faster migrating murine MRP protein in murine MRP-transfected mB cells (17). When a duplicate blot was incubated with Mab QCRL-1, however, only human MRP was detected (Fig. 1B, right).

Localization of the QCRL-1 Epitope to the Cytoplasmic Connecting Region. To more precisely localize the epitope of Mab QCRL-1, MRP was fragmented by complete digestion with formic acid or by limited digestion with trypsin, followed by immunoblotting with anti-peptide antisera. A schematic representation of MRP is shown in Fig. 2A, and the locations of the peptides used to produce antisera MRP-1, MRP-2, and MRP-L are indicated. The MRP-1 and MRP-2 antisera were raised against peptides within the highly conserved ATP-binding signature motifs in NBD1 and NBD2 (3), respectively, and both are known to cross-react with P-glycoprotein* (15). Although 10 of 15 amino acids in the MRP-1 and MRP-2 peptides are identical, there are no continuous stretches of identity longer than three amino acids. Moreover, neither antisera cross-reacted with the peptide from the other half of MRP as determined by the ELISA (data not shown). Consequently, the MRP-1 and MRP-2 antisera can distinguish between the NH$_2$- and COOH-proximal NBDs of MRP, respectively.

Formic acid digestion of MRP is predicted to yield nine fragments ranging in size from 13 to 376 amino acids (Fig. 2A). Immunoblots of formic acid digests of immunoprecipitated MRP showed that Mab QCRL-1 reacted with a fragment of approximately $M_r 23,000$ (Fig. 2A).
Attenuation of Telomerase Activity by a Hammerhead Ribozyme Targeting the Cancer Therapy. We designed hammerhead ribozymes against human Department of Obstetrics and Gynecology, Gifu University School of Medicine, Gifu, Gifu 500. Japan Teruhiko Tamaya template region is a good tool to repress telomerase activity in cancer cells. We could diminish the telomerase activity. Next we subcloned the nbozyme trial carcinoma cells, only a ribozyine targeting the RNA template region telomerase RNA were designed. In a cell-free system, these three ham merhead ribozymes efficiently cleaved the RNA substrate. When these being increasingly considered and used as human gene therapeutic the costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with INTRODUCTION Telomerase is a ribonucleoprotein believed to play a role in cellular immortalize cells and also to be a significant step in the carcinogen and the restoration of telomerase activity is currently considered to Senescence and immortalization (1—3). It synthesizes telomeric DNA shortening (4). Telomerase activity has been determined in various tissues and cells during the past years, and it has been shown that most somatic cells lose telomerase activity in the early stage of embryogenesis (6), cancer cells, germ cell lines, and some somatic cells express telomerase to exert its action (1 1). Telomerase is composed of a RNA molecule and the associated proteins (10). Telomerase RNA functions as a template for the extension of cells. The specificity of telomerase in cancer cells suggests that telomerase is an excellent target for therapy. Three ribozymes targeting the 3' end of the GUC sequence at the telomerase RNA and which GUC sequence could be eligible. There are 14 GUC sequences may carry out additional action by the ribozymes in targeting sequences. This observation suggested that Mab QCRL-1 epitope was likely to be asymmetrically located toward the COOH-terminal end of the connecting region.

Localization of the Epitope with Fusion Proteins. A fusion protein consisting of a portion of the MRP connecting region fused to the COOH-terminal end of bacterial GST was produced and used for immunoblotting analyses. This fusion protein, L-847, contains amino acids 847—956 of MRP and is illustrated in Fig. 3A. L-847 migrated as a band of approximately $M_r$ 40,000 in SDS-polyacrylamide gels as detected by Coomassie staining (data not shown). Since the MRP-L peptide sequence is contained within this fragment, the MRP-L antiserum served as a positive control for immunodetection and reacted with the $M_r$ 40,000 L-847 fusion protein but not the $M_r$ 27,000 bacterial GST itself (Fig. 3B, upper panel), as expected. Mab QCRL-1 also reacted with L-847 but not with GST (Fig. 3B, lower panel). To further localize the Mab QCRL-1 epitope, PCR was performed on a human MRP cDNA template with the downstream primer used to generate L-847 and a series of nested upstream primers to produce progressively shorter DNA fragments truncated at their 5’ ends. A schematic of the fusion proteins produced with these truncated PCR products, L-865, L-886, L-903, and L-919, is shown in Fig. 3A. The Coomassie-stained proteins were of the predicted sizes, ranging from approximately $M_r$ 31,000—38,000 (results not shown). As expected, the MRP-L antiserum reacted with all four products (Fig. 3B, upper panel), whereas Mab QCRL-1 reacted with L-865, L-886, and L-903, but not L-919 (Fig. 3B, lower panel). These results indicated that the QCRL-1 epitope resided between MRP amino acids 903 and 956. To confirm the absence of cross-reactivity of Mab QCRL-1 with murine MRP (Fig. 1), a portion of murine MRP cDNA encoding amino acids 887—952 of the connecting region was amplified by PCR. The murine MRP-GST fusion protein (mL-887) generated from this product was tested for immunoreactivity with MRP-L antiserum and Mab QCRL-1 (Fig. 4). The human MRP L-903 and L-919 fusion proteins were included as controls. Coomassie staining revealed that
Although expression of mL-887 was somewhat higher than that of the human fusion proteins (Fig. 4A), the MRPL-L antisense was much less reactive with the murine fusion protein mL-887 than with the human proteins (Fig. 4B, left). MAb QCRL-1 reacted with human L-903 but did not hit with human L-919, as expected, and did not react with mL-887 (Fig. 4B, right), confirming the inability of this MAb to detect murine MRP.

Because MAb QCRL-1 reacted with human but not mouse MRP, it was reasoned that its epitope would not reside in any regions of extended identity between the two proteins. In Fig. 4C, amino acids 903–956 of human MRP are shown aligned with the corresponding mouse sequence. Of the 54 amino acids in the human sequence, the COOH-proximal 16 are identical to the mouse protein, thus excluding this region as a potential epitope. The remaining 38 amino acids are less well conserved, consistent with the MAb QCRL-1 epitope residing in this region. Lys-940 in human MRP is absent from the mouse protein, and the two proteins differ at 12 other residues. Ten of the 12 amino acids corresponding to the MRPL-L peptide sequence (indicated in Fig. 4C) are identical between the two proteins, including all of the first six, which probably accounts for the (weak) reactivity of the MRPL-L antisense with the mL-887 fusion protein.

Fine Mapping of the QCRL-1 Epitope Overlapping Peptides. An array of overlapping hexapeptides and heptapeptides spanning human MRP amino acids 895–947 was synthesized directly on the surface of an inert membrane and immunoblotted with MAb QCRL-1. A summary of the reactivity of the MAb QCRL-1 with individual hexapeptides and heptapeptides is depicted in Fig. 5. Peptide 71, a heptapeptide with the sequence SSYSGDI corresponding to human MRP amino acids 918–924, was the most reactive. The strength of MAb QCRL-1 binding to this peptide could not be compared quantitatively with that of the next most reactive peptide, a hexamer of sequence SSYSGD (peptide 23), because the difference in intensity of the corresponding spots on the fluorogram was too great. However, analysis of the reactivity of neighboring peptides allowed the specific residues involved in the MAb QCRL-1/MPR peptide interaction to be identified. Reactivity with both the hexapeptide and heptapeptide series decreased greatly when Ser-918 was removed. Marked increases in reactivity were observed when Ile-924 was included in the heptapeptide series, and when Asp-923 was included in the hexapeptide series. The presence of both Ser-918 and Ile-924, the first and last residues in the epitope respectively, is critical for maximum binding. Tyr-920 is also important for binding because reactivity was first detected when this residue was added to the COOH termini of both the hexapeptide and heptapeptide series.

Confirmation of the Specificity of Peptide Binding by Competitive Immunoprecipitation. To provide corroborating evidence for the specificity of MAb QCRL-1 for peptide 71, the SSYSGDI peptide was synthesized and used in a competitive immunoassay. MRP-
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3' erase activity in transfectants was simply due to the clonal divergence, we included pooled clones of the vector transfectants and the ribozyme transfectants. As shown in Fig. 5, most clones showed an apparently reduced telomerase activity compared with that of the vector transfectant control and parental AN3CA cells. In some clones (clones 5, 9, and 10), telomerase activity was almost undetectable. Five of 10 clones (clones 5, 7, 8, 9, and 10) in which the telomerase activity was diminished to a variable extent were further studied for expression of the ribozyme and telomerase RNA. To study the expression of the ribozyme, RT-PCR and Southern blot analysis were performed. Ribozyme expression was found in all of the transfectants with pH(3APr-l-neo-36RZ) and its pooled clone (Fig. 6), implying that ribozyme RNA was successfully expressed in these clones, although the expression level differed in the clones.

The expression of telomerase RNA in the transfectants was analyzed by Northern blotting. The telomerase RNA expression of the vector transfectants was unchanged when compared with that of the parental AN3CA cells. Transfectants with pH(3APr-l-neo-36RZ) and its pooled clone clearly reduced the level of telomerase RNA (Fig. 6). The reduction level roughly inversely paralleled the expression level of the ribozyme. This suggested that the reduced telomerase activity was associated with the reduction of the telomerase RNA expression.

Fig. 2. Structure of the 36-ribozyme. The 20-mer antisense sequences against the target region were placed upstream and downstream of the catalytic core of the ribozyme. The cleavage site is localized in the RNA template region, which is underlined.

Fig. 3. In vitro cleavage reaction. The ribozymes and substrate RNA were mixed and incubated for 3 h. All three ribozymes cleaved the RNA substrate, which was 601 bases long. RZ, ribozyme.

Fig. 4. Ribozyme RNA was introduced into Ishikawa cells. Note that the 36-ribozyme diminished telomerase activity most efficiently in 48 h. C, control; DOTAP, DOTAP (liposome) only; RZ, ribozyme; IS, internal standard.

Fig. 5. Telomerase activity in transfectants and parental AN3CA cells. Vector Pool, a pooled clone of vector transfectant; Ribozyme Pool, a pooled clone of 36-ribozyme transfectant; Lanes 1–10, clones 1–10; IS, internal standard. Note that the telomerase activity was reduced in all of the ribozyme transfectants including the pooled clone.

Fig. 6. Competitive immunoprecipitation of MRP. MRP metabolically labeled with [35S]methionine was immunoprecipitated from detergent-solubilized membranes of H69AR cells using MRP-specific MAb preincubated with increasing amounts, (0–42 nmol) of competing peptides. Immunoprecipitates were analyzed using SDS-PAGE followed by fluorography. A, MRP immunoprecipitated with MAb QCRL-1 or MAb QCRL-3 in the presence of increasing amounts of peptide 71 (SSYSGD) or MRP-L peptide (MRP amino acids 932–943). B, amounts of immunoprecipitated MRP were estimated by densitometry, expressed as a percentage of the amount of MRP immunoprecipitated in the absence of competing peptide, and plotted as a function of the amount of competing peptide. The results shown are the average of two experiments. ■, MAb QCRL-1 versus peptide 71; ○, MAb QCRL-3 versus peptide 71; Δ, MAb QCRL-1 versus MRP-L peptide.

specific MAbs were preincubated with increasing concentrations of peptide and then used to immunoprecipitate [35S]-labeled MRP from solubilized H69AR cell membranes. As the amount of peptide 71 (MRP amino acids 918–924) increased from 0 to 42 nmol, immunoprecipitation of MRP by MAB QCRL-1 was inhibited (Fig. 6A, upper panel). With 42 nmol of competing peptide, the amount of MRP immunoprecipitated was decreased by nearly 90% (Fig. 6B). This inhibition was shown to be specific for MAB QCRL-1, since the same peptide failed to inhibit immunoprecipitation of MRP by MAB QCRL-3 which recognizes a conformation-dependent epitope of MRP (Ref. 20; Fig. 6A, middle panel). Furthermore, equimolar amounts of the MRP-L peptide which resides in an adjacent region of the protein (MRP amino acids 932–943) did not inhibit immunoprecipitation of MRP by MAB QCRL-1 (Fig. 6A, lower panel).

DISCUSSION

We have previously raised and characterized a panel of MRP-specific MAbs and found that one of these MAbs (QCRL-1) recognizes a linear epitope, whereas the remaining MAbs (QCRL-2, -3, -4, and -6) recognize conformation-dependent epitopes (20). These MAbs have since been tested for their ability to affect MRP function, and several have been shown to inhibit the ATP-dependent uptake of LTC₄ and 17β-estradiol 17-(β-D-glucuronide) into membrane vesicles derived from MRP-transfected cells (12, 13). We are now mapping the epitopes of these MAbs to aid in determining the topology of MRP, and to locate regions of potential functional importance identified by the MAb-specific inhibition of MRP-mediated transport and/or ATP binding.

Using a series of overlapping hexapeptides and heptapeptides, we have identified the linear epitope of MAB QCRL-1 as SSYSGD, corresponding to amino acids 918–924 in the region connecting the two halves of MRP. This result was confirmed by competitive immunoprecipitation experiments which showed that this peptide could specifically inhibit the interaction between MAb QCRL-1 and MRP. The first residue of this peptide sequence, Ser-918, is absolutely critical for high-affinity binding of MAB QCRL-1 since the MAB bound very poorly to peptides 24 (SSYSGD) and 72 (SSYSGD), in which the entire epitope sequence except for the first serine is present. Similarly, binding of MAB QCRL-1 to the fusion protein L-919, which lacks only Ser-918, was not detectable. Ile-924 and, to a lesser extent, Asp-923 and Tyr-920 were also found to be important for MAB QCRL-1 binding. Analysis of MAB QCRL-1 reactivity with peptides of different lengths, as well as with peptides in which critical amino acids have been substituted, will be required to identify precisely the residues involved in binding.

We have previously described polyclonal antisera raised against pentadecapeptides containing the highly conserved active transport family signature regions of NBD1 and NBD2 of MRP, and, although the peptides are only 47 and 67% identical to the corresponding sequences in P-glycoprotein, both antisera cross-reacted with P-glycoprotein (15). Similarly, a polyclonal antisera raised against a peptide from the same NBD region in P-glycoprotein which differed at more than half the residues from the corresponding sequence of MRP cross-reacted with both proteins (28). It therefore appears that, at least in the NBDs, there is more potential for antibody cross-reactivity among ABC transporters than might be suspected from their amino acid sequences. For this reason, we tested MAB QCRL-1 for cross-reactivity with several other mammalian ABC superfamliy transporters (human MDR1, MDR3, CFTR, and murine MRP). All of these transport proteins have a cytoplasmic connecting region analogous to that of human MRP to which the MAB QCRL-1 epitope was mapped. In P-glycoprotein, this region includes the so-called linker (2), whereas in CFTR the connecting region makes up the regulatory R-domain (29). This connecting region is one of the most highly divergent regions of the ABC transporters, showing significant sequence divergence even among highly conserved cross-species homologues (2, 17, 30). Of the proteins examined, MRP shares less than 20% overall identity with the human P-glycoproteins and with human CFTR, and this is confined mostly to the generally conserved NBDs (3). Consequently, the lack of cross-reactivity of MAB QCRL-1 with these proteins is not surprising. In contrast, human and murine MRPs are highly conserved with an overall amino acid identity of 88%. However, identity in the connecting region of MRP decreases to less than 78% (17), and examination of the murine sequence corresponding to the QCRL-1 epitope (amino acids 918–924 of human MRP) reveals differences at two positions, Tyr-920 and Ile-924 (substituted in murine MRP by His and Thr, respectively; Fig. 4). Both of these differences occur at residues identified as important for MAB QCRL-1 binding by peptide mapping. Thus, the observed absence of cross-reactivity of MAB QCRL-1 with murine MRP is consistent with the location of its epitope.

There is increasing evidence for the involvement of MRP in drug resistance in certain tumors. Overexpression of MRP has been shown to account for multidrug resistance in cell lines derived from a number of different tumor types (5). MRP expression has been demonstrated in a variety of tumors, and several recent clinical reports have implicated MRP in drug resistance in neuroblastoma and certain forms of leukemia (5). Although much of the work to date has examined the
levels of MRP mRNA expression, detection of MRP protein in these samples will be necessary to assess accurately the role of MRP in clinical drug resistance. Identification of the MAb QCRL-1 epitope will facilitate the use of this antibody for such studies. We have done a FASTA search of the SWISS-PROT database using the MAb QCRL-1 epitope sequence, SSYSGDI, to determine whether there are proteins with which MAb QCRL-1 is likely to cross-react. Human MRP was the only protein found to contain the entire epitope sequence. While not conclusive, this finding suggests that MAb QCRL-1 will be highly specific for the immunodetection of MRP. We have shown that the membrane-coupled peptide 71, to which MAb QCRL-1 bound most strongly, inhibits the interaction of MAB QCRL-1 with MRP in solution. This peptide should thus be a valuable tool for demonstrating the specificity of MAb QCRL-1 staining in histological sections and in other immunoassays.

Proteins of the ABC superfamily have a common structural organization consisting of four domains: two domains of six transmembrane segments, each associated with a highly conserved NBD. These four domains may be contained in one continuous polypeptide chain, or the domains may be found in separate subunits which associate to form a functional protein (14). According to the Eisenberg hydropathy analyses, P-glycoprotein is predicted to conform to this six-plus-six model of ABC transporters (31, 32). Some other experimental evidence in support of this model has been obtained from epitope mapping studies with P-glycoprotein specific MAbS. Mapping of the epitopes of MAbS MRK-16 (33), MM4.17 (34), and MM6.15 (35) demonstrated that the proposed first, fourth, and sixth extracellular loops of P-glycoprotein were indeed located on the extracellular side of the plasma membrane as predicted. Similar studies with the P-glycoprotein-specific MAbS C219, C494, and C32 confirmed the intracellular localization of the first and second NBDs of this protein (36). We have previously demonstrated that MAb QCRL-1 reacts with intact MRP-overexpressing cells only after permeabilization (20), indicating that it recognizes an intracellular epitope. Combined with the current epitope mapping data, this provides strong evidence that at least the COOH-proximal portion of the region predicted to connect the two halves of MRP is indeed cytoplasmic, as suggested by computer-assisted hydropathy analysis.

In addition to identifying the epitope of MAb QCRL-1, the present studies have also revealed the presence of a number of a very protease-sensitive sites in the connecting region of MRP. Limited trypsin digestion of native MRP resulted in the rapid appearance of initial cleavage products of approximately M, 120,000 and 80,000. The M, 120,000 product reacted with polyclonal antisera MRP-1 and weakly with MAb QCRL-1. The M, 80,000 fragment reacted more strongly with MAb QCRL-1 as well as with the MRP-L and MRP-2 antisera. Similar results were obtained when V8 protease was substituted for trypsin (data not shown), suggesting that this protease-hypersensitive region may be readily accessible to cytoplasmic proteins. Both sets of initial cleavage fragments are glycosylated, and when deglycosylated their sizes are consistent with cleavage in the connecting region (data not shown). From these studies, we conclude that these fragments are produced by enzymatic cleavage in the cytoplasmic connecting region at a number of sites on both sides of the MAb QCRL-1 epitope. The relative reactivity of MAb QCRL-1 with the two fragments suggests that proteolytic digestion of MRP occurs much more frequently on the NH2-proximal side of the MAb QCRL-1 epitope, despite the distribution of potential cleavage sites. There are a total of 15 lysine and 5 arginine residues in the connecting region, and of these sites, 40% (7 of the lysine and 1 of the arginine residues) are located on the COOH-proximal side of the MAb QCRL-1 epitope. The relative reactivity of MAb QCRL-1 with the M, 120,000 and 80,000 fragments suggests that these COOH-proximal sites are relatively inaccessible to the protease, possibly as a result of their proximity to the first predicted transmembrane segment of the second half of MRP. Consistent with this suggestion, the MRP-L antiserum, which was raised against a peptide just COOH-proximal to the MAb QCRL-1 epitope, showed no reactivity with the first half of MRP, indicating that little or no cleavage occurs between the MRP-L peptide sequence and the first transmembrane segment of the second half. However, some digestion may occur at three lysine residues in the MRP-L peptide sequence itself, resulting in the destruction of the MRP-L epitope while generating a M, 120,000 fragment immunoreactive with MAb QCRL-1.

Alignment of the hydropathy profiles of human and murine homologues of P-glycoprotein and MRP suggests that the membrane topology of these two proteins may be much more similar than their low amino acid identity might suggest. Thus, they may share a similar organization of putative transmembrane domains (with the exception of the hydrophobic NH2-proximal 230 amino acids of MRP, which have no counterpart in the P-glycoproteins; Ref. 17). The present proteolytic mapping studies provide additional experimental evidence for structural similarities between MRP and P-glycoprotein. Upon limited proteolysis of the native protein products of the human MDR1 gene (37), hamster ppgl gene (38), and murine mdr1b gene (39), the initial cleavage occurs in the cytoplasmic linker region, yielding two major products representing the intact halves of the protein, as was observed for MRP. This similarity suggests that the transmembrane segments and NBDs of both MRP and P-glycoprotein form relatively inaccessible structures while the connecting (or linker) region is relatively exposed in the cytoplasm. The accessibility of this cytoplasmic region to enzymes is consistent with it being a major site of reversible phosphorylation. The linker region has been shown to be the principal site of phosphorylation in P-glycoprotein (40). MRP has been shown to be phosphorylated, primarily on serine residues (15, 41, 42). By analogy, phosphorylation of MRP may be expected to occur in the connecting region, and we are currently investigating the localization of the residues involved to confirm this prediction.

We have tested our panel of MRP-specific MAbS for their ability to inhibit LTC4 and 17β-estradiol 17-(β-d-glucuronide) uptake into inside-out plasma membrane vesicles prepared from MRP-transfected cells (12, 13). MAb QCRL-3, which recognizes a conformation-dependent epitope in MRP, abolished both 3H-labeled LTC4 and 3H-labeled 17β-estradiol 17-(β-d-glucuronide) uptake into vesicles, whereas MAb QCRL-1 at equal concentrations had no effect on transport. Similar results were obtained when 3H-labeled LTC4 was used to photoaffinity label MRP in the presence of MRP-specific MAbS, viz., MAb QCRL-3 inhibited photolabeling whereas MAb QCRL-1 did not. This difference in activity of the two MRP-specific MAbS may be explained by the present epitope-mapping studies. The transmembrane domains of ABC transporters are believed to be the primary regions involved in substrate binding (14). Our results indicate that in the case of MRP, binding of an antibody (i.e., MAb QCRL-1) to the cytoplasmic connecting region does not interfere with substrate access or transport. We have also tested the ability of these MAbS to inhibit photolabeling of MRP by 8-azido-[32P]ATP, and have found that some of them appear capable of inhibiting ATP binding. By mapping the conformation-dependent epitopes of the remaining MAbS, we will gather further evidence for the membrane topology of MRP and should be able to precisely identify functionally important regions of this molecule. Moreover, definition of the epitopes recognized by MAb QCRL-1 and the other MAbS will facilitate the use of these reagents for immunohistological studies of MRP expression in drug-resistant tumors.
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REFERENCES


Location of a Protease-hypersensitive Region in the Multidrug Resistance Protein (MRP) by Mapping of the Epitope of MRP-specific Monoclonal Antibody QCRL-1

David R. Hipfner, Kurt C. Almquist, Brenda D. Stride, et al.


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