Monoclonal Antibodies That Inhibit the Transport Function of the 190-kDa Multidrug Resistance Protein, MRP

LOCALIZATION OF THEIR EPITOPEs TO THE NUCLEOTIDE-BINDING DOMAINS OF THE PROTEIN*

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Multidrug resistance in tumor cells is often accompanied by overexpression of multidrug resistance protein (MRP), a 190-kDa transmembrane protein that belongs to the ATP-binding cassette superfamily of transport proteins. MRP mediates ATP-dependent transport of a variety of conjugated organic anions and can also transport several unmodified xenobiotics in a glutathione-dependent manner. To facilitate structure-function studies of MRP, we have generated a panel of MRP-specific monoclonal antibodies (mAbs). Four of these mAbs, QCRL-2, -3, -4, and -6, bind intracellular conformation-dependent epitopes, and we have shown that they can inhibit the transport of several MRP substrates. Binding competition and immunoprecipitation assays indicated that mAbs QCRL-4 and -6 probably recognize the same detergent-sensitive epitope in MRP, whereas mAbs QCRL-2, -3, and -4 each bind distinct, non-overlapping epitopes. Fab fragments inhibit transport as effectively as the intact mAbs, suggesting that inhibition results from direct interactions of the mAbs with MRP. Immunodot blot and immunoprecipitation analyses revealed that the minimal regions of MRP sufficient for full reactivity of mAbs QCRL-2 and -3 are amino acids 617–858 and 617–932, respectively, which encompass the NH₂-proximal nucleotide-binding domain (NBD). In contrast, the epitope bound by mAb QCRL-4 localized to amino acids 1294–1531, a region that contains the COOH-proximal NBD. However, none of the mAbs inhibited photo-labeling of intact MRP with 8-azido-[α-³²P]ATP. This suggests that rather than preventing nucleotide binding, the mAbs inhibit transport by interfering with substrate binding or by trapping MRP in a conformation that does not allow transport to occur. Our results also demonstrate for the first time that the NBDs of MRP can be expressed as soluble polypeptides that retain a native conformation.

The development of resistance to multiple natural product and semi-synthetic drugs commonly used in cancer chemotherapy, termed multidrug resistance, is a serious obstacle to successful treatment. This phenomenon has been extensively studied in vitro using tumor cell lines selected for resistance by chronic or intermittent exposure to drugs. The multidrug resistance observed in these cell lines is most commonly accompanied by overexpression of one or both of the integral membrane transporters, MRP and P-glycoprotein (1–3). The 190-kDa MRP and 170-kDa P-glycoprotein are members of the ATP-binding cassette superfamily of transport proteins, which in eukaryotes are typically composed of one or two highly conserved nucleotide-binding domains (NBDs) and one to three membrane-spanning domains (MSDs) (4). The NBDs of ATP-binding cassette transporters are characterized by the presence of three short sequences, the so-called Walker A and B motifs and the transport family signature motif (3–6). Outside of their two NBDs, however, MRP and P-glycoprotein show relatively little sequence homology, and it is now clear that there are significant structural and functional differences between these two proteins (7–11).

Transfection studies have confirmed that overexpression of either MRP or P-glycoprotein is sufficient to confer resistance to a similar, but not identical, spectrum of drugs (1, 12–15). A substantial body of experimental evidence indicates that P-glycoprotein confers multidrug resistance by binding and transporting unmodified chemotherapeutic drugs and other compounds out of cells (1, 2). In contrast, direct binding of drugs to which it confers resistance has not been demonstrated for MRP (3, 16–19). However, MRP has been shown to be a primary active transporter of amphiphilic conjugated organic anions. Established substrates of MRP include a number of GSH-, glucuronide-, and sulfate-conjugated compounds, with the cysteinyl leukotriene LTC₄ and the GSH conjugate of the potent carcinogen aflatoxin B₁ being the highest affinity substrates identified to date (17, 18, 20, 21). In addition to conjugated anions, MRP has been shown to mediate ATP-dependent transport of some unmodified compounds, such as aflatoxin B₁ and the anticancer drugs vincristine and daunorubicin, but only in a GSH-stimulatable manner (16, 19, 21–23). Recently, we have shown that the GSH-stimulated transport of unmodified vincristine by MRP-enriched membrane vesicles involves cotransport of GSH (19). It may be that MRP confers resistance...
to at least some classes of xenobiotics in intact cells by such a cotransport mechanism.

We previously reported the isolation and initial characterization of a panel of five MRP-specific mAbs (QCRL-1, -2, -3, -4, and -6) (24). These mAbs were raised against crude membranes prepared from multidrug-resistant H69AR small cell lung cancer cells, which express very high levels of MRP. All five mAbs reacted only with permeabilized cells in immunocytochemical analyses, indicating that they recognize intracellular epitopes (24). The linear epitope bound by one of the mAbs, QCRL-1, was subsequently mapped to amino acids 918–924 in the cytoplasmic region connecting the two halves of MRP (25). This knowledge has facilitated the use of mAb QCRL-1 for a number of purposes, including analysis of MRP expression in clinical tumor specimens (26–31), structure-function studies (10, 25, 32, 33), and immunofluorescence purification of MRP.2

In contrast to mAb QCRL-1, the remaining four mAbs recognize conformation-dependent epitopes in the MRP molecule (24). mAbs QCRL-2,-3, and -4 also have the ability to inhibit the transport of several conjugated substrates (including LTC4, 17β-estradiol 17(β-glucuronide), and aflatoxin B1-glutathionyl aflatoxin B1) into inside-out MRP-enriched plasma membrane vesicles (16, 21, 23, 34). Similarly, GSH-stimulatable transport of unmodified vincristine and aflatoxin B1 is inhibited by these mAbs (19, 21, 23). These results suggest that the conformation-dependent epitopes recognized by mAbs QCRL-2,-3, and -4 are localized in or near functionally important regions of MRP. In this study, we show that mAbs QCRL-2,-3, and -4 recognize distinct, non-overlapping epitopes of MRP and that their ability to inhibit MRP-dependent transport results from direct interactions with the NBDs of the protein that do not prevent the binding of ATP.

MATERIALS AND METHODS

Cell Lines and Antibodies—The MRP-overexpressing multidrug-resistant H69AR small cell lung cancer cell line has been described previously (7, 35). T5 cells are HeLa cells that have been transfected with a full-length MRP cDNA expression vector, pRo/CMV-MRP1, and C1 cells are HeLa cells transfected with empty vector (13, 14). mAbs QCRL-1, -2, -3, -4, and -6 are murine mAbs raised against cell membranes from MRP-overexpressing H69AR cells (24). The mAbs were used in the form of crude or DEAE-blue-purified ascites fluid (26), were obtained as purified preparations from Centocor (Malvern, PA), or were purified using GammaBind Plus Sepharose resin (Amersham Pharmacia Biotech). Fab fragments of mAbs QCRL-1, -2, -3, and -4 were prepared using the ImmunoPure Fab kit (Pierce). MRP-1 and -2 are polyclonal antisera raised against homologous 15-amino acid peptides from the first and second NBDs of MRP (amino acids 765–779 and 1427–1441, respectively) (24, 25, 36). The rat mAb MRP1 and the mouse mAbs MRP5n and MRP6m were kindly provided by Dr. R. J. Scheper (Free University Hospital, Amsterdam) (19, 37, 38). mAbs MRP1 and MRP6m recognize linear epitopes between MRP amino acids 238–247 and 1511–1520, respectively (39), whereas the epitope for mAb MRP5n is located between MRP amino acids 986–1096.2

Immunoprecipitation—For immunoprecipitation of MRP, membrane-enriched fractions were prepared from H69AR cells that had been metabolically labeled with [35S]methionine (24). Membrane proteins were solubilized in PBS containing 1% CHAPS at detergent/protein ratios ranging from 1:1 to 10:1 (w/w) for 3 hr at 4°C. After centrifugation at 100,000 × g, 1 µl of crude ascites fluid was mixed with 22.5 µg of solubilized membrane protein and incubated overnight at 4°C. For immunoprecipitation of polypeptides MRP-(617–932) and MRP-(1294–1531) (prepared as described below), ~4 µg of total cytosolic protein from infected SF21 cells was diluted to 100 µl with PBS, and 1 µl of ascites (mAb QCRL-1, -2, -3, or -4) or 5 µl of MRP-2 antiserum was added. After incubation for 2 h at 4°C, immune complexes were recovered by the addition of 30 µl of 25% GammaBind Plus Protein G-Sepharose (Amersham Pharmacia Biotech) in PBS. After incubation for 1 h at room temperature, the beads were washed four times with PBS, and precipitated proteins were eluted with Laemmli buffer and analyzed by SDS-PAGE and fluorography.

Labeling of mAbs—Sixty µg each of DEAE Blue-purified mAbs QCRL-1, -2, -3, and -4 were passed over an Econo-Pac 10DG column (Bio-Rad) and eluted in PBS. Protein-containing fractions were pooled, and the volume was brought up to 850 µl with PBS. IODO-BEADS (Fierce) were washed with PBS and dried on filter paper. For each mAb, one IODO-BEADS was added to 150 µl of PBS containing 400 µCi of 125I (17 Ci/mg of iodine; ICN, Montreal, Quebec, Canada) and incubated for 5 min. The purified mAb was then added, and labeling was allowed to proceed for 15 min. To separate 125I-labeled mAb from free 125I, the reaction mixture was loaded onto an Econo-Pac 10DG column, the eluates were pooled with PBS, and radioactive fractions were collected.

Dot Blot Assay of Direct Binding Competition between Unlabeled and 125I-Labeled mAbs—To quantitate the relative amount of mAb in DEAE blue-purified mAb samples and crude ascites fluid, the purified samples were separated by SDS-PAGE together with serial dilutions of the corresponding ascites fluid. The amount of IgG heavy chain protein in each was assessed by densitometric analysis of the Coomassie-stained gels on a Molecular Dynamics computing densitometer using ImageQuant software. Dot blots were prepared on Immobilon P membrane in a 96-well manifold by blotting 4 µg of H69AR crude membranes in 50 µl of TBS/well and draining slowly by gravity (see below). After washing with TBS containing Tween 20 (TBS-T), 100 µl of blocking solution (0.1% gelatin and 1% bovine serum albumin in TBS-T) per well was added and allowed to drain slowly by gravity. Approximately 30 ng of each 125I-labeled mAb was mixed with QCRL-1, -2, -3, -4, and -6 crude ascites or with mouse IgG (Fierce) in 200 µl of blocking solution/well at ratios of unlabeled competing antibody to 125I-labeled antibody ranging from 1:125 to 125:1. The labeled/unlabeled antibody mixtures were added to the blot and drained slowly by gravity. Finally, the wells were rinsed four times with 200 µl of TBS-T, the blot was removed from the manifold and dried, and bound antibody was detected by exposure to Kodak X-Omat AR film at ~70°C.

Generation of Expression Constructs, Production of Reconstituent Bacterial Cells, and Viral Infection—The preparation of constructs encoding wild-type and truncated (MRP-(1–858), MRP-(1–932), MRP-(229–1531), MRP-(281–1531), and MRP-(932–1295)) MRP molecules has been described previously (32, 33). Additional constructs (MRPpen5n, MRP-(281–932), MRP-(1–616), MRP-(281–1295), MRP-(932–1295), MRP-(617–1295), and MRP-(1294–1531)) were also prepared using similar methods. All constructs were transfected into the recombinant donor plasmid pFASTBAC1 (Life Technologies, Inc., Burlington, Ontario, Canada), and recombinant bacmids and baculovirus were produced as described previously and used to infect Spodoptera frugiperda SF21 cells as described previously (32).

Cytosolic and Membrane Protein Isolation—H69AR, empty vector-transfected HeLa C1, MRP-transfected HeLa T5, or SF21 cells were harvested, and crude membrane fractions were prepared (24, 32). For some experiments, plasma membrane vesicles and cytosolic fractions were prepared. Briefly, cells were homogenized in buffer containing 50 mM Tris-HCl, 250 mM sucrose, 0.25 mM CaCl2, pH 7.5, and protease inhibitors (CompleteTM, Roche Molecular Biochemicals). Cell pellets were frozen at ~70°C for at least 1 h, thawed, and then disrupted by N2 cavitation. EDTA was added to 1 µl, and after centrifugation at 500 × g for 15 min, the supernatant was layered over 35% (w/w) sucrose, 50 mM Tris-HCl, 250 mM sucrose, pH 7.5, and protease inhibitors and used to infect Spodoptera frugiperda SF21 cells as described previously (32).

MRP-mediated LTC4 Transport in Inside-out Membrane Vesicles—MRP-mediated LTC4 uptake into membrane vesicles was measured by a rapid filtration technique (16, 40). Plasma membrane vesicles from MRP-transfected T5 cells (2 µg of protein in a 60-µl reaction volume) were preincubated alone or with mAb QCRL-1, -2, -3, or -4 (10 µg/ml intact mAb or Fab fragment) for 1 h at room temperature. Uptake of [3H]LTC4 (50 nM, 40 Ci/reaction, 146 Ci/mmol; Life Science Products, Markham, Ontario) was then determined in the presence of 10 mM MgCl2 and 4 mM ATP for 1 min at 37°C. Uptake was stopped by

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Inhibitory mAbs Localize to MRP Nucleotide-binding Domains

RESULTS

Immunoprecipitation of Full-length MRP—We demonstrated previously that mAbs QCRL-1, -2, and -3 could immunoprecipitate MRP from 1% CHAPS-solubilized membranes, confirming that they are MRP-specific (24). In contrast, mAbs QCRL-4 and -6, which bound strongly and specifically to crude membranes from MRP-overexpressing cell lines in immunodot blots, did not immunoprecipitate MRP solubilized under similar conditions or with different detergents, including 0.1% SDS, 1% Nonidet P-40, or 0.5% deoxycholate (data not shown). Consequently, it could not be concluded with absolute certainty that they reacted exclusively with MRP. To test the possibility that mAbs QCRL-4 and -6 recognized conformation-dependent epitopes that were particularly sensitive to denaturation, milder conditions were used for extracting MRP from cell membranes prior to immunoprecipitation. At a detergent/protein ratio of 1:1, mAb QCRL-3 immunoprecipitated the 190-kDa MRP, as expected (Fig. 1). mAbs QCRL-4 and -6 also immunoprecipitated a protein of the same size under these conditions, indicating that they are indeed MRP-specific. Higher detergent/protein ratios (e.g. 10:1) had little effect on the interaction of mAb QCRL-3 with MRP. However, binding and precipitation of MRP by both mAbs QCRL-4 and -6 were substantially reduced, confirming the detergent sensitivity of the epitopes recognized by these mAbs.

Assay of Binding Competition between MRP-specific mAbs—Binding competition assays were carried out to determine whether mAbs QCRL-1, -2, -3, -4, and -6 recognize overlapping epitopes using H69AR membrane dot blots. A constant amount of 125I-labeled mAb QCRL-1, -2, -3, -4, or -6 either alone or premixed with increasing amounts of unlabeled competing antibody (mouse IgG or mAb QCRL-1, -2, -3, -4, or -6; indicated to the left of each blot) that had been preincubated with unlabeled competing antibodies (mAb QCRL-1, -2, -3, -4, or -6 crude ascites or mouse IgG; indicated to the right of each blot). The unlabeled/125I-labeled mAb ratios are indicated at the top of the blots. After allowing the antibody mixtures to drain slowly by gravity, the wells were rinsed. The blots were dried, and 125I-labeled mAb binding was detected by autoradiography.

Inhibition of MRP-mediated LTC4 Transport by Fab Fragments of mAbs QCRL-2, -3, and -4—mAb QCRL-3 and, in some cases, mAbs QCRL-2 and -4 have been shown to inhibit MRP-mediated transport of several conjugated substrates as well as GSH-stimulatable transport of several unmodified xenobiotics (16, 19, 21, 23, 34). However, intact (bivalent) mAbs were used in these studies, raising the possibility that inhibition resulted from steric effects caused by the Fc regions of these mAbs or by cross-linking of adjacent MRP molecules. To address this pos-
sibility, Fab fragments of mAbs QCRL-2, -3, and -4 were prepared and tested to determine whether these monovalent fragments are also capable of inhibiting MRP-mediated transport activity. As expected, the Fab fragment of the non-inhibitory mAb QCRL-1 had little effect on MRP-mediated LTC₄ transport relative to the control sample with no mAb (Fig. 3). In contrast, Fab fragments of mAbs QCRL-2, -3, and -4 inhibited transport by >85% at 10 μg/ml. Similar results were obtained with the intact mAbs, in agreement with previous observations. These results indicate that inhibition of MRP-mediated transport by mAbs QCRL-2, -3, and -4 is caused by their direct interaction with the protein.

**Binding of mAbs to Sf21-expressed Modified MRP Molecules—**To localize the conformation-dependent epitopes of mAbs QCRL-2, -3, and -4, the reactivity of these mAbs with a panel of truncated or otherwise altered MRP molecules expressed in Sf21 insect cells was examined in a series of immunodot blot analyses. In the first set of experiments, the mAbs were tested against MRP polypeptides with NH₂-proximal truncations (MRP-(229–1531) and MRP-(932–1531)) or COOH-proximal truncations (MRP-(1–858) and MRP-(1–932)). In addition, a mutated protein (MRPins708) that contains 13 additional amino acids (DIRTINVRFLREI) inserted at amino acid 708 between the Walker A and B motifs of NBD1 was examined. These amino acids are present in the comparable location of P-glycoprotein, but are absent from most MRP-related ATP-binding cassette transporters (3, 7, 11). A diagram of MRP showing the approximate locations of its three MSDs and two NBDs is shown below the box model of MRP. The locations of the three MSDs and two NBDs in MRP are indicated, as are the positions of the epitopes recognized by the three mAbs used as positive controls (MRPr1, QCRL-1, and MRPr6) (dotted lines). B, identical dot blots of crude membrane protein isolated from Sf21 cells expressing MRP constructs (5 μg/spot for MRP-(1–858) and 2 μg/spot for all other polypeptides) were prepared and probed with mAb QCRL-2, -3, or -4 or with control mAbs MRPr1, QCRL-1, or MRPr6.

As positive controls, the dot blots were probed in parallel with mAbs MRPr1, MRPr6, and QCRL-1, the epitopes of which have been defined previously (Fig. 4A) (25, 39). These blots provided standards against which the immunoreactivity of mAbs QCRL-2, -3, and -4 with each polypeptide could be compared (25, 39). All three of the positive control mAbs showed patterns of immunoreactivity with the MRP fragments consistent with the locations of their epitopes (Fig. 4B). However, mAb MRPr1 reacted more strongly with the MRP-(229–1531) polypeptide than the other two mAbs, and mAb QCRL-1 reacted more strongly with the MRP-(1–932) polypeptide than mAb MRPr1. In each case, the truncation in the MRP molecule is very near the site of the epitope of the more strongly reacting mAb (amino acids 238–247 for mAb MRPr1 and amino acids 918–924 for mAb QCRL-1) (25, 39). This may increase accessibility of the mAbs to these epitopes and hence account for the stronger signal observed.

mAbs QCRL-2 and -3 showed similar patterns of immunoreactivity with the MRP fragments (Fig. 4B). Both mAbs bound to the NH₂-proximal half-molecule MRP-(1–932) and to the polypeptide lacking MSD1 (MRP-(229–1531)), and neither bound to the COOH-proximal half-molecule MRP-(932–1531). In addition, the binding of both mAbs was markedly diminished by the insertion of 13 amino acids in the NH₂-proximal NBD (MRPins708). The only major difference between the two mAbs was that mAb QCRL-2 did not bind to MRP-(1–858), whereas mAb QCRL-3 reacted strongly with this polypeptide. These results suggest that the sequences bound by mAbs QCRL-2 and -3 are located somewhere in MSD2 and/or NBD1. In addition, at least part of the mAb QCRL-2 epitope is between amino acids 859 and 932 in the region connecting NBD1 to MSD3. In contrast, mAb QCRL-4 reacted only with those polypeptides containing the COOH-proximal half of MRP (MRP-(932–1531) and MRP-(229–1531)) and bound as well to MRPins708, as the control mAbs. These observations suggest that the QCRL-4 epitope is in MSD3 and/or NBD2.

To further localize the QCRL-2 and -3 epitopes, a second series of immunodot blotting was carried out with these mAbs using MRP polypeptides containing MSD2 or MSD2 + NBD1 (Fig. 5, A and B). The polypeptides used were MRP-(1–932), MRP-(281–1531), and MRP-(1–616). As positive controls, the dot blots were probed with mAb MRPr1 and the MRP-1 polyclonal antiserum. MRP-(229–1531) was included in these analyses to
Inhibitory mAbs Localize to MRP Nucleotide-binding Domains

![Diagram](Image)

**FIG. 5. Immunodot blot analysis of SF21-expressed MRP domains.** A, shown is a schematic diagram depicting the domain structure of MRP and approximate locations of amino acids that mark the five domains of the protein. Locations of the epitopes recognized by mAbs MRPr1 and MRPrm5 and polyclonal antiserum MRP-1 are indicated below the box model of MRP. B, identical dot blots of crude membrane protein prepared from SF21 cells expressing constructs encoding linear combinations of MRP domains (1 μg/well) were probed with mAb QCRL-2 or -3 or with mAb MRPr1 or the MRP-1 antiserum as a control. Membrane protein from cells expressing the NH2-terminally truncated MRP-(1299–1531) construct (3 μg/spot) was included on the dot blots for normalization of signal intensities. C, identical dot blots of crude membrane protein prepared from SF21 cells expressing constructs encoding linear combinations of MRP domains (3 μg/well for MRP-(932–1531) and 1 μg/well for others) were probed with mAb QCRL-4 or with control mAb MRPrm5.

allow normalization of signal intensity since this polypeptide contains the epitopes bound by all four antibodies used. mAb MRPr1 reacted with MRP-(1–616), and the MRP-1 antiserum reacted with MRP-(1281–932) and MRP-(281–1531), consistent with the known locations of their epitopes (Fig. 5B). mAbs QCRL-2 and -3 showed a pattern of reactivity similar to that of the MRP-1 antiserum, reacting only with polypeptides containing MRP amino acids 617–932 (Fig. 5B). This suggests that the conformation-dependent epitopes recognized by these mAbs are wholly or partially contained within this region corresponding to NBD1.

To further localize the QCRL-4 epitope, immunodot blotting was performed using MRP fragments containing MSD3 or MSD3 + NBD2 (Fig. 5, A and C). In these experiments, the polypeptides used were MRP-(932–1531), MRP-(281–1295), and MRP-(932–1295). As a positive control, the blot was also probed with mAb MRPrm5, whose linear epitope lies between amino acids 986 and 1096.3 mAb MRPrm5 bound to all three polypeptides as expected, whereas mAb QCRL-4 reacted only with MRP-(932–1531) (Fig. 5C). These results suggest that the QCRL-4 epitope is contained wholly or in part within the region corresponding to NBD2.

**Immunoprecipitation of Soluble Polypeptides Corresponding to NBD1 (MRP-(617–932)) and NBD2 (MRP-(1294–1531))**—To determine whether the epitopes recognized by the mAbs were entirely contained between amino acids 617 and 932 (QCRL-2 and -3) or amino acids 1296 and 1531 (QCRL-4), the ability of the mAbs to immunoprecipitate the MRP-(617–932) or MRP-(1294–1531) polypeptide was examined. MRP-(617–932) migrated as a polypeptide of ~36 kDa in SDS-PAGE and could be immunoprecipitated from the cytosolic fraction of SF21 cells with mAbs QCRL-2 and -3 as well as with mAb QCRL-1, as expected (Fig. 6A). Immunoprecipitation of MRP-(617–932) with mAbs QCRL-2 and -3 was specific because this polypeptide was not immunoprecipitated with mAb QCRL-4. MRP-(1294–1531) was also present in the cytosolic fraction of SF21 cells and migrated as a doublet between 25 and 30 kDa (Fig. 6B). The exact nature of these doublet MRP NBD-derived bands is not known. However, we speculate that there is a protruding “tail” at the NH2 or COOH terminus of the folded NBD polypeptide that is susceptible to proteolysis. Cleavage of the tail would result in lower levels of the larger, but less stable full-length polypeptide and higher levels of the slightly smaller, but more stable protein. MRP-(1294–1531) could be immunoprecipitated with mAb QCRL-4 as well as with the MRP-2 antiserum, as expected (Fig. 6B). Immunoprecipitation with these antibodies was specific because the only proteins detectable in the mAb QCRL-2 and -3 immunoprecipitates were the immunoglobulin light chains of the precipitating antibodies, which migrated at ~25 kDa.

**Fab Fragments of mAbs QCRL-2, -3, and -4 Do Not Inhibit Azido-ATP Labeling of MRP**—To determine whether mAbs QCRL-2, -3, and -4 interfere with the ability of MRP to bind nucleotide, membrane vesicle proteins from MRP-transfected T5 cells and control C1 cells were incubated with Fab fragments of the mAbs individually or together and then photolabeled with 8-azido-[32P]ATP. As shown in Fig. 7, labeling of MRP in T5 membranes was unaffected by the Fab fragments, suggesting that the mAbs do not prevent nucleotide binding by the protein.

**DISCUSSION**

To provide molecular probes for studying both the structure and transport mechanism of MRP, we isolated a panel of mAbs...
that reacted preferentially with cell membranes from MRP-overexpressing cell lines (24). Four of these mAbs (QCRL-2, -3, -4, and -6) recognize intracellular conformation-dependent epitopes and are able to inhibit the transport activity of MRP. At the time of their initial characterization, immunoblot analyses strongly suggested that mAbs QCRL-4 and -6 recognize MRP, but we were unable to confirm this directly by immunoprecipitation (24). By using very low detergent/protein ratios, we have now shown that mAbs QCRL-4 and -6 immunoprecipitate a single 190-kDa protein from solubilized membranes (Fig. 1). These observations establish the MRP specificity of mAbs QCRL-4 and -6 and indicate that both mAbs recognize detergent-sensitive epitopes. Binding competition assays revealed that mAbs QCRL-4 and -6 recognize identical or overlapping epitopes (Fig. 2). None of the remaining mAbs competed reciprocally for binding, demonstrating that mAbs QCRL-2, -3, and -4 recognize distinct, non-overlapping epitopes of MRP.

mAbs QCRL-2, -3, and -4 have been shown to be effective inhibitors of MRP-mediated ATP-dependent transport of several conjugated organic anion substrates, and mAb QCRL-3 has been shown to inhibit GSH-stimulatable transport of unmodified vincristine, daunorubicin, and aflatoxin B1 (16, 19, 21, 23, 34). This inhibitory property is not shared by mAb QCRL-1, which may be explained by the fact that its linear heptapeptide epitope is within a region of MRP that is not required for LTC4 transport activity in Sf21 cell membranes (25, 32). The Fab fragments of mAbs QCRL-2, -3, and -4 inhibited MRP-mediated transport as effectively as the intact mAbs (Fig. 3). This finding indicates that inhibition is caused by direct interaction of the antigen-binding sites of these mAbs with MRP, rather than being the result of a steric effect caused by the Fc regions of the immunoglobulins or by cross-linking of adjacent MRP molecules.

As an initial step toward understanding the mechanism by which mAbs QCRL-2, -3, and -4 inhibit the transport activity of MRP, we have localized the conformation-dependent epitopes bound by these mAbs. With respect to mAbs QCRL-2 and -3, we have determined that the minimal regions of MRP sufficient for full reactivity are amino acids 617–932 and 617–858, respectively. These regions include NBD1 and different amounts of the segment that connects NBD1 to MSD3. Thus, a portion of the epitope recognized by mAb QCRL-2 is located between amino acids 859 and 932 in the connector region of the protein. Insertion of 13 amino acids at position 708 in NBD1 of MRP resulted in a markedly decreased reactivity of mAbs QCRL-2 and -3 and also reduced the transport activity of the protein.4 Taken together, these observations indicate that the determinants of the epitopes bound by both of these mAbs are contained within NBD1 and that the relative positioning of these determinants is perturbed when the distance between the Walker A and B motifs of this domain is altered. In contrast to mAbs QCRL-2 and -3, the epitope bound by mAb QCRL-4 appears to reside wholly within the second NBD of MRP, between amino acids 1294 and 1531. Since mAbs QCRL-2, -3, and -4 do not react with MRP in its denatured form, the ability of these mAbs to immunoprecipitate MRP-(617–932) (NBD1) and MRP-(1296–1531) (NBD2) indicates that both of these soluble truncated polypeptides have folded correctly and assumed a configuration similar or identical to the NBDs when they are present in the context of the full-length protein in its native state. Consistent with this conclusion is our observation that the individual NBDs have the ability to bind and hydrolyze ATP.4 This knowledge is important with respect to ongoing structural and functional studies of these MRP domains and their interactions with other regions of the protein as well as in comparisons with the NBDs of other ATP-binding cassette transporters such as P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (42–46).

The location of the QCRL-2, -3, and -4 epitopes in the NBDs of MRP raised the possibility that one or more of the mAbs might inhibit transport by interfering directly with ATP binding. However, the inability of both of the intact mAbs (data not shown) and their Fab fragments to inhibit labeling of MRP by 8-azido-ATP suggests that this is not the case (Fig. 7). We have shown previously that intact mAb QCRL-3 can inhibit photolabeling of MRP by [3H]LTC4 (16), indicating that this mAb may inhibit transport by directly limiting access of LTC4 to its binding site. Further experiments examining the effects of the Fab fragments of mAbs QCRL-2, -3, and -4 on [3H]LTC4 photolabeling of MRP and MRP-derived polypeptides will be required to determine conclusively whether these mAbs inhibit transport of this substrate by preventing its binding to the protein. In turn, these studies may provide important information about the location of the LTC4 and other substrate binding site(s) on MRP.

Structure-function studies of P-glycoprotein have been aided by several P-glycoprotein-specific mAbs that have been reported to interfere with substrate interactions and/or the transport activity of this protein. The first P-glycoprotein-reactive mAb to be characterized was mAb C219, which recognizes a linear cytoplasmic epitope located six amino acids downstream from the Walker B motif in both the NH2- and COOH-proximal NBDs of this protein (47, 48). mAb C219 inhibited photolabeling of P-glycoprotein with the calcium channel blocker photoaffinity analog [3H]azidopine (49), but unlike mAbs QCRL-2, -3, and -4 in the present study, also inhibited photolabeling with 8-azido-ATP (49, 50). The inhibition of ATP binding by mAb C219 was associated with a decrease in ATPase activity present in membranes from P-glycoprotein-overexpressing cells (50), whereas we have noted no such effect of the MRP-specific mAbs on the ATPase activity of MRP.2 Despite these effects on drug binding and ATPase activity, inhibition of P-glycoprotein transport activity by mAb C219 has not been reported. On the other hand, several other mAbs that bind extracellular epitopes of P-glycoprotein specifically reverse or reduce the drug resistance and/or drug accumulation deficit of viable, P-glycoprotein-overexpressing tumor cells (51–54). The mechanism by which one of these mAbs, UC12, inhibits P-glycoprotein function appears to involve trapping of the protein in an inactive conformation (55). Similarly, mAbs QCRL-2, -3, and -4 may stabilize or trap MRP in a given conformational state and, in doing so, prevent the conformational changes that are presumed to occur during the transport cycle of the protein. To explore this possibility, it will be necessary to determine if the mAbs are able to modulate MRP conformational transitions. mAbs QCRL-2, -3, and -4 cannot be used to detect MRP in intact, non-permeabilized cells because their epitopes are cytoplasmic. Nevertheless, it should be possible to test their reactivity with functional MRP in cell membranes, in the presence
and absence of ATP and transport substrates, using filter bind-
ing assays. Such studies will provide important insight into the
 mechanism by which MRP transports its conjugated organic
 anion and drug substrates.

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