Introduction

The multidrug resistant protein MRP2 (or ABCC2/cMOAT) is a member of the ABC superfamily of membrane transporters that mediate the movement of endogenous metabolites and xenobiotics across cellular membranes. MRP1, 2 and 3 (or ABCC1, 2 and 3) have been shown to mediate the transport of a wide variety of organic anions and compounds that are conjugated with sulfate, glucuronate or glutathione (GSH), in addition to a large array of unmodified drugs and natural products (for review, [1, 2]). MRP1, the first to be characterized in drug resistant tumor cells, has been shown to transport similar normal cell metabolites, such as leukotriene C4 (or LTC4) and has been implicated in inflammatory diseases [3]. MRP2, initially identified as the canalicular multi-specific “organic” anion transporter (cMOAT), has been implicated in Dubin-Johnson syndrome a recessive autosomal genetic disorder caused by mutations in MRP2 cytoplasmic domain resulting in truncated and non-functional protein [4, 5]. In rats, MRP2 is thought to mediate hepatobiliary excretion of numerous organic anions and causes hyperbilirubinemia, in addition to its role in the efflux of chemotherapeutic drugs [6, 7]. Moreover, unlike MRP1 which is expressed in most tissues, the tissue distribution of MRP2 is limited to the apical surfaces of the epithelial cells of the hepatocytes, lungs, kidney, and colon [8, 9]; consistent with a role in protecting normal tissues from toxic metabolites and xenobiotics [7]. Hence, MRP2 is believed to play a key role in regulating liver detoxification from metabolites into the bile [10].

MRP2, similar to MRP1 and 3, encodes three hydrophobic transmembrane domains (MSD0, MSD1 and MSD2) with 5+6+6 transmembrane helices and two hydrophilic domains encoding each sequences for an ATP binding cassette (e.g., NBD1 and NBD2) [11, 12]. Moreover, MRP1 and 2 share 48% sequence identity which has been taken to imply functional ho-
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mology and substrate specificity. Indeed, LTC₄ is a normal cell metabolite is one of the highest affinity substrates for MRP1 with Kₘ of 0.1 µM [13, 14]. LTC₄ is also a high-affinity substrate for MRP2 with a Kₘ of 1.0 µM [15], a roughly 10-fold higher Kₘ. However, unlike MRP1, direct binding between LTC₄ and MRP2 has not been demonstrated. In an earlier study, using a photoactive analog of LTC₄ (e.g., IAALTC₄), IAA, Karwatsky et al. [16] demonstrated the direct binding of LTC₄ to MRP1. Moreover, IAALTC₄ was shown to bind several sites in MRP1 that have been previously shown to be important for drug binding and transport of LTC₄ and other drugs [16-18]. In this study, the same photoreactive analog of LTC₄ (IAALTC₄) was used to study LTC₄ binding to MRP2 in plasma membranes and to map its drug binding site. The results of this study are the first demonstration of direct binding between LTC₄ and MRP2 and the localization of LTC₄ binding to a 15 kDa polypeptide in MRP2 which includes several helices in MSD₂, likely TM16 and TM17.

Materials and methods

Materials

Anti-MRP2 (M₂III-6) mAb was purchased from Kamiya Biomedicals (Seattle, Washington). MRP1 pAb was generated against a C-terminal peptide and characterized earlier [19]. The antibody to the α-subunit of Na+/K+ ATPase (Clone M7-PB-E9) was purchased from Sigma (St. Louis, MO). All chemicals used were of the highest grade available.

Cell culture, plasma membrane

HeLa and MDCKII (Madine Darby Canine Kidney cells) cells and their MRP1 and MRP2 transfectant clones were grown in α-MEM media containing 10% fetal calf serum (Bio Media, Canada). Plasma membranes from these cells were prepared as described previously [16]. Membrane pellets were re-suspended in labeling buffer (5 mM Tris-HCl, pH 7.4, 250 mM sucrose) and quantified by Lowry [20], stored at -80 °C if not immediately used.

IAA-LTC₄ synthesis and photoaffinity labeling

The synthesis of radio-photoreactive analog of LTC₄, Iodoarylazido-LTC₄ (IAALTC₄) was done as previously described [16], with some modifications. Plasma membrane aliquots expressing MRP1 or 2 proteins were photoaffinity labeled with IAALTC₄ in the absence or presence of increasing concentrations of various anticancer drugs as previously described [16].

Proteolytic digestion and immunoprecipitation

To determine the trypsin digestion sites in the MRP2, plasma membranes (100 µg) with or without photoaffinity labeling with IAALTC₄ were incubated with increasing concentrations of sequencing grade trypsin at 37 °C for 40 min. Samples were placed on ice to terminate digestion and protease inhibitor cocktail was added. Photolabeled plasma membrane aliquots were trypsin digested and immunoprecipitated using the antibodies to MRP1, 2 or α-subunit of Na⁺/K⁺ ATPase as previously described [16]. The immunoprecipitated proteins were then resolved on SDS-PAGE using the Fairbanks gel system [21]. Gels with radiolabeled proteins were fixed, dried and exposed to Kodak BIO-MAX MS films at -80 °C.

SDS PAGE and immunoblotting

Photoaffinity labeled plasma membrane proteins (20 µg) were resolved on SDS-PAGE using the Fairbanks gel system [21] and gels were either stained with Coomassie blue or transferred to nitrocellulose membrane for Western blotting according to the method of Towbin et al. [22]. Immuno-detection of MRP1, 2 or α-subunit of Na⁺/K⁺ ATPase was achieved by probing the nitrocellulose membranes with specific antibodies to MRP1 [19] (which recognized the last 15 amino acids in the C-terminal of the protein), and MRP2 (clone M₂III-6 which recognizes an epitope in the 202-C-terminal amino acid sequence of MRP2; [23]) at recommended dilutions of 1:5000 (v/v) for MRP1 pAb, and 1:125 (v/v) for M₂III-6. The nitrocellulose membranes were incubated with goat anti-mouse or anti-rabbit antibody conjugated to horseradish peroxidase and immuno-reactive proteins were visualized by chemiluminescence using Pico substrate from Pierce (Rockford, IL).

Results and discussion

To demonstrate the direct binding of LTC₄ to MRP2, a previously characterized radio-photoreactive analog of LTC₄ (e.g., IAALTC₄) was used [16]. Earlier studies [13-15] had shown that MRP2 mediates the transport of LTC₄ however direct evidence for MRP2-LTC₄ interaction...
is lacking. The results in Figure 1 show the photoaffinity labeling of plasma membranes from un-transfected control and previously characterized MRP2 transfected MDCKII cells (or MDCKII\textsubscript{MRP2}) [24]. Figure 1A shows that IAALTC4 bound directly to a 190-kDa protein (lane 2) in MDCKII\textsubscript{MRP2} plasma membrane that was immunoprecipitated with MRP2-specific monoclonal antibody (M2III-6 mAb) [23]. Photoaffinity labeling of plasma membrane from MDCKII cells with IAALTC4, and subsequent immunoprecipitation with MRP2-specific mAb did not reveal a 190-kDa photolabeled protein (lane 1, Figure 1A). The absence of 190-kDa photoaffinity labeled protein in membranes from MDCKII cells (lane 1, Figure 1A), together with the apparent molecular mass of the photoaffinity labeled protein from MDCKII\textsubscript{MRP2} cells are consistent with the identity of the 190-kDa as MRP2 (lane 2, Figure 1A). To rule out the possibility that plasma membranes from MDCKII\textsubscript{MRP2} cells expressed large amounts of a 190-kDa protein that was non-specifically photoaffinity labeled with IAALTC4, Figure 1B shows a Coomassie Blue staining of proteins from MDCKII and MDCKII\textsubscript{MRP2} cells (lanes 3 and 4, respectively). The results in Figure 1B show similar levels of protein expression in MDCKII and MDCKII\textsubscript{MRP2} cells consistent with a specific photoaffinity labeling of MRP2 by IAALTC4. The presence of a photoaffinity labeled protein migrating higher than the 190-kDa protein has been previously observed and is due to differential conformation of MRP2 [25]. Earlier work by Zhang et al. [26] has demonstrated that MRP2 glycosylation modulates its trafficking and function in sandwich-culture Rat hepatocytes. In the latter system, higher level glycosylation of MRP2 was associated with its trafficking to the canalicular membrane together with an increase in its apparent molecular mass on SDS PAGE by roughly 10 kDa [26]. Thus, differential glycosylation of MRP2 and possibly other ABC proteins is likely to modulate their differential targeting to cellular membranes and consequently their physiological functions. It is interesting in this respect that the photoaffinity labeling of differentially glycosylated MRP2 by IAALTC4 does not affect its binding to LTC4 (Figure 1A). Figure 1C shows the photoaffinity labeling of fixed amounts of plasma membranes (20 µg) from MDCKII\textsubscript{MRP2} cells with increasing concentrations of IAALTC4 (1 to 8 µM) which demonstrates saturable photoaffinity labeling signal with increasing concentrations of IAALTC4.

Direct binding of LTC4 to MRP1 was previously demonstrated by Karwatsky et. al. [16] using the same photoreactive analog, IAALTC4. In the present study it was of interest to compare the photoaffinity labeling efficiencies of MRP1 and 2 with IAALTC4. Figure 2A shows Western blot results demonstrating the expression of MRP1 and 2 in plasma membranes from untransfected and MRP1 and 2 transfected cells (e.g., HeLa\textsubscript{MRP1} and MDCKII\textsubscript{MRP2} transfectants) as revealed with MRP1- and 2-specific antibodies.
The results in Figure 2A show similar levels of MRP1 and 2 in membrane fractions from HeLaMRP1 and MDCKII MRP2 transfectants. Differences in the apparent expression of MRP1 and 2 in HeLaMRP1 and MDCKII MRP2 cells, as determined by Western blotting with their respective antibodies is not readily explainable as differences in the apparent signal of these two proteins are likely due to differences in the affinities of antibodies to each protein rather than quantitative differences in protein expression. Consequently, the results in Figure 2A show qualitative differences in MRP1 and 2 expressions in membrane fractions from HeLaMRP1 and MDCKII MRP2 transfectants. Indeed, a quantitative comparison of MRP1 and 2 in different membranes may be possible with the use of an anti-tag specific antibody directed towards an identical epitope tag in both proteins. However, such comparison can suffer from other factors such as differences in the transfer of MRP1 and 2 to nitrocellulose membranes due to differences in their amino acid sequences.

Photoaffinity labeling of equal aliquots of the above plasma membranes under identical conditions were immunoprecipitated with specific antibodies to MRP1 and 2 (e.g. MRP1 peptide pAb and M2III-6 mAb). The results of Figure 2B (lanes 1 and 6) show the photoaffinity labeling of plasma membranes from HeLaMRP1 and MDCKII MRP2, showing a direct and specific photolabeling of MRP1 and 2 with IAALTC4, respectively. Interestingly, IAALTC4 photoaffinity labeled the α-subunit of Na+/K+ ATPase in both membrane preparations (see Figure 2A). The differential affinity of MRP1 and 2 towards LTC4, it was important to show that photoaffinity labeling of MRP2 was due to specific protein-drug interaction and not due to its mere presence in the lipid bilayer. To rule out the latter possibility, it was of interest to determine if IAALTC4 photoaffinity labeled a ubiquitously expressed transmembrane protein, (e.g., α subunit of Na+/K+ ATPase) in both membrane preparations (see Figure 2A). The
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results in Figure 2B (lanes 3-4 and 7-8) show the lack of photoaffinity labeling of the α-subunit of Na⁺/K⁺ ATPase with IAALTC₄.

To determine if IAAATC₄ photoaffinity labels MRP2 at a physiologically relevant site, plasma membranes from MDCKII-MRP2 cells were photoaffinity labeled with IAALTC₄ (1 µM) in the presence of increasing concentrations of normal cell metabolites and drugs. The results of Figure 3A show photoaffinity labeling of MRP2 in the presence of increasing molar excess of unmodified LTC₄ (2.5-fold and 25-fold molar excess). Interestingly, increasing concentrations of LTC₄ caused a significant increase in the photolabeling of MRP2 with IAALTC₄. Although not entirely clear, it is likely that the observed increase in MRP2 photoaffinity labeling in the presence of molar excess of LTC₄ increases the affinity of the protein to IAALTC₄ and consequently this translates into higher photoaffinity labeling (~200% relative increase; Figure 3B). These results are in contrast with those obtained with MRP1 using the same photoreactive analog of LTC₄, whereby increasing concentrations of LTC₂ inhibited or caused a marked reduction in the photoaffinity labeling of the protein [16]. By contrast, the presence of molar excess of MK571 (25-fold and 50-fold) shows a significant decrease in MRP2 photoaffinity labeling with IAALTC₄ (~75% relative decrease, Figure 3B). Of considerable interest are the findings that 25-50 fold molar excess of GSH and Quercetin, a dietary flavonoid, have no significant effect on MRP2 photoaffinity labeling by IAALTC₄ (Figure 3). However, it is possible that neither compound was used at sufficiently high molar excess to reveal a change in IAALTC₄ labeling of MRP2. Together, these results demonstrate that IAALTC₄ binds to MRP2 at a physiologically relevant site(s); while demonstrating differences in IAALTC₄ binding to MRP2 and MRP1. For example, in the case of MRP1, molar excess of LTC₄, MK571 and GSH caused a significant decrease in IAALTC₄ binding to MRP1 [16]. Although these differences are not entirely clear, they may be due to differences in the amino acid sequence of the two proteins.

The observed increase in IAALTC₄ binding to MRP2 in the presence of molar excess of LTC₄ may be due to the presence of two LTC₄ binding sites. Zelcer et al. [27] suggested the presence of two sites in MRP2, where the “S” site transports the substrate, and the “M” site modulates substrate transport. Hence, it is possible that the binding of unmodified LTC₄ to the allosteric binding site “M” on MRP2 increases the binding of IAALTC₄ to the “S” site, where IAALTC₄ would have lower affinity to “M” site than the “S” site. By contrast, the binding of MK571 to the “S” or “M” site could negatively modulate the binding of IAALTC₄ to MRP2 as demonstrated by the reduction in MRP2 photoaffinity labeling. Alternatively, MK571 and LTC₄ may bind to different sites on MRP2. A similar two-site mechanistic model was previously suggested for the transport of E₂₁₇βG by MRP1 [28].

Several studies using photoreactive drugs and mutational analysis have lead to the identification of specific domains in MRP proteins as sites for protein-drug interactions [29-33]. In an attempt to elucidate IAALTC₄ binding domain
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MRP2 was photoaffinity labeled with IAALTC₄ and subjected to limited trypsin digestion prior to immunoprecipitation of the digested and photoaffinity labeled polypeptides with MRP2 mAb (M₂III-6) which recognizes the C-terminal 202 amino acids [23]. Figure 4A shows a Western blot of MRP2 digested with increasing amounts of trypsin and probed with M₂III-6 mAb. The results in Figure 4A show three major (72 kDa, 50 kDa and 25 kDa polypeptides) and four minor (115 kDa, 45 kDa, 40 kDa and 37 kDa) tryptic polypeptides of MRP2 that contain M₂III-6 epitope (see, Figure 4C). Figure 5B shows the results of immunoprecipitation of MRP2 photoaffinity labeled tryptic peptides with M₂III-6 mAb.

Figure 4. Mapping of IAALTC₄ photoaffinity labeled domains of MRP2. Native (Panel A) and IAALTC₄ photoaffinity labeled plasma membranes from MDCKII and MDCKIIMRP² were resolved on SDS-PAGE without or with trypsin (1:800 and 1:100 enzyme:protein). Panel A shows Western blot of the above plasma membranes without and with trypsin probed with MRP2-specific mAb. Panel B shows an immunoprecipitation of IAALTC₄ photoaffinity labeled proteins or tryptic peptides from samples without or following trypsin digestion using MRP2-specific mAb. The apparent molecular sizes of native and tryptic peptides of MRP2 are indicated to the right of each figure in panels A and B. Panel C shows a schematic representation of MRP2-domains (MSD₀-L₀-MSD₁-NBD₁-L₁-MSD₂-NBD₂) with relative position of M₂III-6 epitope sequence in NBD₂. The lines below the schematic representation show the relative sizes of possible tryptic peptides of MRP2 that correspond to M₂III-6 reactive tryptic peptides in panels A.
sensitivity in L1 linker domain is thought to be due to the presence of positively charges amino acids (e.g., lysine and arginine), in addition to being accessible or exposed. The second photolabeled peptide (e.g., 40kDa) encodes the C-terminal ATP binding domain and at least the last two transmembrane helices (e.g., TM16-17) of MSD2 (Figure 4C; [17, 18]). A major tryptic peptide of MRP2 is a 25kDa peptide which encodes the C-terminal ATP-binding domain, minus TM16-17 was not photoaffinity labeled and consequently localizes IAALTC4 photolabeled domain to a 15 kDa polypeptide that sequences from MSD2, likely TM16 and 17. Together, these results are consistent with earlier findings which have implicated the MSD regions in MRP2 substrate specificity [32, 33]. In one study, mutation of the tryptophan1254 in TM 17 of MRP2 to alanine disrupted the MSD regions in MRP2 substrate specificity [32, 33]. In one study, mutation of the tryptophan1254 in TM 17 of MRP2 to alanine disrupted the MSD regions in MRP2 substrate specificity [32, 33].

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Abbreviations: MDR, multidrug resistance; MRP2, multidrug resistance protein 2; ABC, ATP-binding cassette; IAALTC4, iodoaryl azido-leukotriene C4; MK571, leukotriene D4 antagonist; GSH, glutathione.

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