Structural Determinants of Substrate Specificity Differences between Human Multidrug Resistance Protein (MRP) 1 (ABCC1) and MRP3 (ABCC3)

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ABSTRACT:

Multidrug resistance proteins (MRPs) are members of the “C” branch of the ATP-binding cassette transporter superfamily. Human MRP1 transports a wide range of natural product drugs and structurally diverse conjugated and unconjugated organic anions. Its closest relative is MRP3. Despite their structural similarity, the homologs differ substantially in their substrate specificity. It is noteworthy that MRP1 transports glutathione (GSH) and GSH conjugates and displays GSH-stimulated transport of a number of unconjugated and conjugated compounds. In contrast, MRP3 does not transport GSH and is a poor transporter of GSH conjugates. However, both proteins transport glucuronide conjugates, such as 17β-estradiol 17-(β-D-glucuronide). We have constructed a series of MRP1/MRP3 hybrids and used them to identify a region of MRP1 that is critical for binding and transport of GSH conjugates such as leukotriene C₄ (LTC₄). Substitution of this region encompassing transmembrane helices 8 and 9 and portions of cytoplasmic loops 4 and 5 of MRP1 with the equivalent region of MRP3 eliminated LTC₄ transport. Transport of other substrates was either unaffected or enhanced. We identified three residues in this region: Tyr440, Ile441, and Met445, mutation of which differentially affected transport. It is noteworthy that substitution of Tyr440 with Phe, as found in MRP3, reduced LTC₄ and GSH-stimulated estrone-3-sulfate transport without affecting transport of other substrates tested. The mutation increased the $K_m$ for LTC₄ 5-fold and substantially reduced photolabeling of MRP1 by both [³H]LTC₄ and the GSH derivative, azidophenacyl-[³⁵S]GSH. These results suggest that Tyr440 makes a major contribution to recognition of GSH and the GSH moiety of conjugates such as LTC₄.

The ATP-binding cassette (ABC) transporter, multidrug resistance protein (MRP) 1 was identified in a human small cell lung cancer cell line, H69AR, that exhibited cross-resistance to a broad range of natural product-type drugs (Cole et al., 1992). MRP1 was highly overexpressed in H69AR cells and after gene transfer studies was shown to cause a multidrug resistance phenotype (Cole et al., 1994; Grant et al., 1994). MRP1 has since been found to be expressed in many solid tumors and hematological malignancies, and MRP1 is a negative prognostic indicator of treatment outcome in some (Deeley et al., 2006). In vitro, MRP1 confers resistance to many structurally and functionally diverse natural product chemotherapeutic agents, including anthracyclines, Vinca alkaloids, and epipodophyllotoxins. MRP1 also actively transports a range of glutathione (GSH)-, glucuronide- and sulfate-conjugated organic anions (Deeley et al., 2006). The GSH-conjugated, cysteinyi leukotriene C₄ (LTC₄) is the best characterized physiological substrate of MRP1 (Leier et al., 1994; Loe et al., 1996b). Other potential physiological substrates include the peptides, GSH and oxidized GSH, the glucuronide conjugate 17β-estradiol 17-(β-D-glucuronide) (E₂17βG), and the steroid sulfate estrone 3-sulfate (E₃SO₄) (Leier et al., 1994, 1996; Loe et al., 1996a; Qian et al., 2001b). MRP1 also transports chemotherapeutic agents to which it confers resistance, such as methotrexate (MTX), vincristine, daunorubicin, and etoposide (VP-16) (Loe et al., 1996b; Rappa et al., 1997; Hooiberg et al., 1999; Renes et al., 1999). Transport of certain substrates, both unconjugated (e.g., vincristine and VP-16) and conjugated (e.g., E₃SO₄), is stimulated by GSH (Loe et al., 1996b; Sakamoto et al., 1999; Qian et al., 2001b; Zelcer et al., 2003). In some cases (e.g., vincristine), the GSH-dependent substrate may reciprocally stimulate GSH transport, whereas in others (e.g., E₃SO₄) no stimulation is observed (Loe et al., 1998; Qian et al., 2001b).

MRP1 is a member of the “C” branch of the ABC superfamily,

ABBREVIATIONS: ABC, ATP-binding cassette; MRP, multidrug resistance protein; GSH, glutathione; LTC₄, cysteinyi leukotriene C₄; E₂17βG, estradiol-17β-D-glucuronide; E₃SO₄, estrone 3-sulfate; MTX, methotrexate; VP-16, etoposide; MSD, membrane-spanning domain; TM, transmembrane; NBD, nucleotide-binding domain; SUR, sulfonylurea receptor; HEK, human embryonic kidney; β-gus, β-glucuronidase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.
which includes the cystic fibrosis transmembrane conductance regulator (ABC7), the two sulfonlurea receptors (SUR1/ABCC8, SUR2/ABCC9), and eight other MRP1-related proteins (ABCC2–6 and 10–12/MRP2–6 and 7–9). Most ABC transporters are made up of two membrane spanning domains (MSDs), each composed of six transmembrane (TM) helices and two cytosolic nucleotide binding domains (NBDDs). However, MRP1, MRP2, MRP3, MRP6, and MRP7 and the two sulfonlurea receptors (SURs) have an additional relatively poorly conserved MSD, MSD0, which contains five TM helices and a glycosylated extracellular NH₂-terminal region (Deeley et al., 2006).

MRP1 is most closely related to MRP3 (58% identity), whereas the protein’s substrate specificity is most similar to that of MRP2 (49% identity). MRP3 differs from MRP1 and MRP2 in both its drug resistance and conjugated anion transport profiles (Deeley et al., 2006). Like MRP1 and MRP2, MRP3 confers resistance to epipodophyllotoxins, such as VP-16, but not to vincristine alkaloids or anthracyclines (Kool et al., 1999; Zelcer et al., 2001; Zhang et al., 2003). MRP3 also transports certain glucuronides conjugates, including E₂₁βG, that are MRP1 and MRP2 substrates, as well as other compounds that are not transported by the other two proteins (Hiromachi et al., 2000; Zeng et al., 2000; Zelcer et al., 2001; Zhang et al., 2003). Unlike MRP1 and MRP2, MRP3 does not transport GSH, and the efficiency with which it transports GSH conjugates, such as LTC₄, is low (Zeng et al., 2000; Zelcer et al., 2001).

Site-directed mutagenesis studies have identified a number of residues that are either critical for overall activity or influence the substrate specificity of MRP1, but few have been identified that selectively affect transport of GSH conjugates, such as LTC₄ (reviewed in Deeley et al., 2006). Two notable exceptions are Lys³³² and His³³³ in TM6. Certain mutations of both residues reduce LTC₄ and apigenin-stimulated GSH transport without affecting transport of other MRP1 substrates, such as E₂₁βG or MTX (Haimeur et al., 2002, 2004). However, these studies have also shown that it is not possible to predict structure/substrate specificity relationships based on amino acid sequence conservation among MRP homologs, and that mutation of even exceptionally conserved amino acids can differentially and selectively affect the transport of shared substrates such as E₂₁βG (reviewed in Deeley et al., 2006).

In the present study, we have used MRP1/MRP3 hybrid proteins to screen for regions, rather than individual amino acids, of MRP1 that are important for the transport of LTC₄ and other GSH conjugates as a first step toward locating the critical residues involved. Using MRP1/MRP3 hybrids that covered all the MSD1 and MSD2, a region of MRP1 that includes TMs 8 and 9 (amino acids 425–516) was located, which, when replaced with amino acids 411 to 502 of MRP3, completely eliminated LTC₄ transport while modestly enhancing transport of E₂₁βG. Within this region there are 26 amino acids that differ between the two proteins; among them we were able to identify a cluster of three residues in TM8, Tyr⁴⁴⁰, Ile⁴⁴¹, and Met⁴⁴² in MRP1 and Phe⁴₂⁶, Leu⁴²⁷, and Leu⁴²⁹ in MRP3, which contributes significantly to this major difference in substrate specificity between the two proteins.

Materials and Methods

Materials. [³H]LTC₄ (165.7 Ci/mmol), [³H]E₂₁βG (45 Ci/mmol), [³H]E₂₃SO₄ (57.3 Ci/mmol), and [³⁵S]GSH (1498 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Waltham, MA), and [³H]MTX (28Ci/mmol) was from Moravek Biochemicals, Inc. (Brea, CA). Hygromycin, doxorubicin hydrochloride, VP-16, vincristine sulfate, S-methyl GSH, the unlabeled equivalents of the triitated compounds, and the reagents for synthesis of azidoephanyl-³⁵S[GSH were purchased from Sigma-Aldrich (St. Louis, MO). Methotrexate was obtained from Faulding (Canada) Ltd. (Montreal, QC, Canada).

Cell Lines and Tissue Culture. Human embryonic kidney (HEK) 293 cells were grown in Dulbecco’s minimal essential medium plus 7.5% fetal bovine serum (Sigma-Aldrich) and SiF21 cells in Grace’s medium supplemented with 0.2% tryptose broth and 10% fetal bovine serum (Sigma-Aldrich). The HEK293 cell line transfected with the pCEBV7 vector, with and without the MRP1 coding sequence (HEK-MRP1 and PC7, respectively), has been described previously (Cole et al., 1994). The mutant MRP1 cDNAs were also cloned into the pCEBV7 vector, and HEK293 cells were transfected using Fugene (Roche Applied Science, Laval, QC, Canada) according to the manufacturer’s directions. After at least 2 weeks of selection in 100 µg/ml hygromycin, cloned cell lines were isolated by limiting dilution and tested for MRP1 expression.

Wild-type MRP1 was expressed in SiF21 insect cells as two half-molecules, amino acids 1 to 932 and 932 to 1531, using the baculovirus expression vector pFASTBAC dual (Invitrogen, Carlsbad, CA) as described previously (Gao et al., 1996). Likewise, β-glucuronidase (β-gus) was expressed in SiF21 cells using the expression vector pFASTBAC. Both generation of recombinant bacmids and baculoviruses and the conditions for viral infection have also been described previously (Gao et al., 1996).

Hybrid cDNA Construction and Site-Directed Mutagenesis. To generate the vectors expressing the hybrid MRP1/MRP3 proteins, the relevant portions of MRP3 were amplified by polymerase chain reaction (PCR) using oligonucleotide primers with the appropriate MRP1 restriction enzyme cleavage sites added to the 5’ ends. If the MRP3 fragment contained the cleavage site of choice, then a compatible cleavage site was used instead. After cleavage of both the PCR fragments (and the appropriate vector containing a portion of the MRP1 cDNA, the DNA fragments (two or three) were ligated together. The identity of each construct was confirmed by restriction enzyme digestion, and the fidelity of the PCR was checked by sequencing. Finally, the MRP1/MRP3 hybrid portion of the construct was moved into the pFASTBAC dual-MRP1 expression vector.

To generate the vectors expressing the mutant MRP1 proteins, site-directed mutagenesis was performed using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The target sequence for the PCR was an approximately 2.7-kilobase 5’ end of MRP1 cDNA cloned into the pGEM3Zf+ vector (Fisher Scientific, Waltham, MA). After sequencing to ensure the fidelity of the PCR, the MRP1 fragment was cloned into pFASTBAC dual-containing wild-type MRP1, replacing the equivalent unmutated fragment. Likewise, a BglIII fragment from the pFASTBAC dual clone containing the mutant MRP1 cDNAs was moved into the mammalian expression vector, pCEBV7-MRP1. Each construct was verified to be correct by both sequencing and restriction enzyme analysis.

Determination of Relative MRP1 Protein Levels. Membrane vesicles were prepared from either SiF21 or HEK293 cells by nitrogen cavitation followed by layering on a 35% sucrose cushion as described previously (Loe et al., 1996b). Total membrane protein concentration was determined by Bradford assay (Bio-Rad, Mississauga, ON, Canada), and then serially diluted protein samples from each membrane preparation were analyzed either in SDS-polyacrylamide gel electrophoresis (PAGE), followed by electroblotting to Immobilon-P membranes (Millipore Corporation, Billerica, MA), or transferred to the same membrane using a Bio-dot SF microfiltration apparatus (Bio-Rad). In each case, the appropriate wild-type MRP1, either the full-length protein or protein expressed as two half-molecules, was included on each blot. The membranes were probed using anti-MRP1 monoclonal antibodies (mAbs) MRP6, MRP1 (Alexis Biochemicals, San Diego, CA), and QCRL-1 (Hipfner et al., 1996), and antibody binding was detected using horseradish peroxidase-conjugated goat anti-mouse or anti-rat IgG (Pierce Biotechnology, Rockford, IL), enhanced chemiluminescence detection, and X-OMat Blue XB-1 film (PerkinElmer Life and Analytical Sciences). Densitometry of the film images was performed using a ChemiImager 4000 (Alpha Innotech Corp., San Leandro, CA) or a ScanMaker i900 scanner (Microtek, Carson, CA) and Photoshop 5.5 software (Adobe Systems, Ottawa, ON, Canada). Either multiple blots or multiple exposures of the same blot were used to analyze the relative MRP1 protein levels. Expression of each protein was determined relative to wild-type MRP1, and the transport levels normalized accordingly. For proteins expressed as half-molecules, the levels of expression of each...
half-molecule compared with the equivalent wild-type fragment were determined. In some cases, the half-molecules of the mutant protein were expressed within ±10% of the wild-type fragments. In this case, no normalization was done. Where expression of the mutant protein differed more substantially, normalization was done using the expression levels of the least abundant half-molecule compared with its wild-type counterpart.

Transport of Trans-Labeled Substrates into Membrane Vesicles. Plasma membrane vesicles were prepared as described previously, and ATP-dependent transport into inside-out membrane vesicles was measured using a rapid filtration technique (Loe et al., 1996b). LTC₄ transport assays were performed at 23°C in a 20-μl reaction volume containing 2 to 4 μg of membrane vesicle protein, 50 nM [³H]LTC₄ (20 nCi/reaction), 10 mM MgCl₂, 4 mM ATP or AMP in transport buffer (250 mM sucrose, 50 mM Tris HCl, pH 7.5). ATP-dependent uptake was calculated by subtracting the uptake in the presence of AMP from the uptake measured in the presence of ATP. The results are expressed as mean ± S.D. of triplicate determinations in each assay.

Uptake of [³H]E₂₁₇βG was measured in a comparable fashion except that transport was measured at 37°C in a reaction containing 400 nM [³H]E₂₁₇βG (40 nCi/reaction). [³H]MTX uptake was also measured at 37°C for 20 min in a reaction mixture containing 100 μM [³H]MTX (200 nCi/reaction). [³H]E₂₁₇βG uptake was measured at 37°C at a final concentration of 6 μM [³H]E₂₁₇βG, 80 nCi/reaction) with the addition of 2 mM S-methyl GSH. Kₘ and Vₘₘₐₓ values for ATP-dependent [³H]LTC₄ uptake in membrane vesicles (4 μg) were measured at six or seven LTC₄ concentrations (25 nM to 1 μM) for 1 min at 23°C in transport buffer containing 4 mM AMP or ATP and 10 mM MgCl₂. Data were analyzed using GraphPad Software Inc. (San Diego, CA) Prism software, and kinetic parameters were determined by linear regression analysis of a Hanes-Wolff plot. Likewise, initial rates of [³H]E₁₃SO₄ uptake were measured as described for LTC₄ except that the assay was done at 37°C using a 30-s time point and concentrations of E₁₃SO₄ ranging from 3 to 20 μM as described above.

Photoaffinity Labeling of Wild-Type and Mutant MRP1 Proteins with [³H]LTC₄₆. Samples containing approximately equivalent levels of MRP1 protein were mixed in a 30-μl reaction volume, and an aliquot (2 μl) was removed. The remaining 28 μl was then mixed with [³H]LTC₄ (200 nM, 0.13 μCi) and incubated for 30 min at room temperature. After freezing in liquid N₂, samples were cross-linked at 302 nm for 1 min as described previously (Loe et al., 1996b). The freezing and cross-linking cycle was repeated nine more times. Both the cross-linked and native proteins (the 2-μl sample) were resolved by electrophoresis through 5 to 15% gradient SDS-polyacrylamide gels. The gel containing the cross-linked proteins was fixed, processed for fluorography by soaking in Amplify (GE Healthcare, Baie d’Urfe, QC, Canada), dried, and then exposed to film for approximately 2 weeks. The nonradioactive gel was processed as described for immunoblots above.

Preparation of Azidophenacyl-[³⁵S]GSH and Photolabeling of Wild-Type and Mutant MRP1 Proteins. Azidophenacyl-[³⁵S]GSH was prepared essentially as described previously except that 0.1 μCi of [³⁵S]GSH (1498 Ci/mmol) was diluted to approximately 500 Ci/mmol with cold GSH (Qian et al., 2002). For photolabeling, HEK 293 membrane vesicles containing approximately equivalent amounts of wild-type or mutant MRP1 protein were mixed with membrane vesicles from the control cell line, PC7, such that each sample contained 120 μg of total membrane protein in 60 μl of transport buffer. A sample (4 μg) was taken before photolabeling for immunoblot analysis, as described for [³H]LTC₄ photolabeling. Each sample was then incubated with 0.25 μCi of azidophenacyl-[³⁵S]GSH at room temperature for 10 min and irradiated at 312 nm on ice for 5 min. The membrane vesicles were collected by centrifugation at 14,000 rpm for 15 min before solubilization in 50 mM Tris, pH 7.5, and Laemmli buffer. After resolving the proteins in 6% SDS-PAGE, the gel was treated with Amplify (GE Healthcare) for 20 min, dried, and exposed to film with an intensifying screen for up to 3 days.

Chemosensitivity Testing. Drug resistance was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay as described previously (Cole et al., 1994). Mean values ± S.D. of quadruplicate determinations were plotted using GraphPad software. IC₅₀ values were obtained from the best fit of the data to a sigmoidal curve. Relative resistance is expressed as the ratio of the IC₅₀ values of cells expressing mutant or wild-type MRP1 compared with cells containing the empty expression vector. Resistance values were the average of three or more independent experiments.

Results

Identification of Regions in MRP1 and MRP3 Responsible for Differences in LTC₄ Transport. Based on the relatively high amino acid sequence identity and similar predicted topology of MRP1 and MRP3 (Fig. 1A), 12 hybrid expression vectors were constructed such that limited regions of MSD1 and MSD2 of MRP1 were replaced with the predicted equivalent from MRP3 (Fig. 1B). Because we and others have shown previously that the MSD0 region of MRP1, defined here as amino acids 1 through 203, is not required for transport of a number of substrates, including LTC₄, this region was not included in our analysis (Bakos et al., 1998; Westlake et al., 2003). Each hybrid protein was expressed in SF21 cells as an NL₄-terminal fragment of amino acids 1 through 932 and a COOH-terminal fragment of amino acids 932 through 1531 (Fig. 1B). When coexpressed, these two fragments of wild-type MRP1 associate correctly and support ATP-dependent LTC₄ transport with an efficiency of approximately 90% compared with the intact protein (Gao et al., 2000). After preparation of membrane vesicles, the ability of each of the hybrid proteins to transport LTC₄ and E₂₁₇βG was compared (data not shown). None of the constructs supported LTC₄ transport, with the notable exception of constructs C and J, which displayed transport activity similar to, or approximately 50% of, wild-type levels, respectively. Construct C contained four of the six TM of MSD2 from MRP3 (TMs 12–15), whereas construct J contained only TMs 12 and 13 of MRP3. Neither of these constructs transported E₂₁₇βG, nor did constructs A, E, F, H, K, and L, although a low level of transport was detected with constructs B, D, and I. The only construct that retained E₂₁₇βG transport activity while losing the ability to transport LTC₄ was construct G, in which amino acids 425 through 516 in MSD1 of MRP1 (TMs 8 and 9) were replaced with amino acids 411 through 502 of MRP3 (Fig. 1B). The G construct showed enhanced E₂₁₇βG (Fig. 1D) transport compared with wild-type MRP1, whereas LTC₄ transport was reduced to approximately 15% of wild-type levels (Fig. 1C). This observation, coupled with the ability to exchange four TMs of MSD2 of MRP1 for those of MRP3 without loss of LTC₄ transport (construct C), strongly suggested that the region encompassing TMs 8 and 9 of MRP1 contained nonidentical residues that were critical for LTC₄ binding and/or transport.

To determine whether a smaller region of MRP1 could be exchanged with MRP3 and produce a similar effect on transport, hybrid constructs G1 (substitution of amino acids 425–477 of MRP1 with amino acids 411–463 of MRP3) and G2 (substitution of amino acids 478–516 of MRP1 with amino acids 464–502 of MRP3) were made (Fig. 2A), and the level of expression in SF21 cells (Fig. 2B) and transport of LTC₄ and E₂₁₇βG were determined (Fig. 2, C and D). LTC₄ transport by the G1 construct was approximately 40% of wild-type levels (Fig. 2C), whereas E₂₁₇βG transport was 2 times the level obtained with wild-type MRP1 (Fig. 2D). For the G2 construct, LTC₄ transport was reduced by 80% (Fig. 2C), but E₂₁₇βG transport was similar to wild-type MRP1 (Fig. 2D). Thus, both halves of the G region contain residues that alone or in concert affect LTC₄ transport by MRP1, whereas E₂₁₇βG transport was either unchanged or enhanced by introduction of corresponding nonidentical amino acids from MRP3.

Analysis of the Contribution of Nonidentical Amino Acids in the G Region of MRP1 and MRP3 to LTC₄ and E₂₁₇βG Transport. The results obtained with the hybrid proteins indicated that multiple residues throughout this region could contribute to interactions with LTC₄. Within the G region, there are 26 amino acid differences between MRP1 and MRP3 (Fig. 2A). Using site-directed mutagenesis, we attempted to identify those nonidentical amino acids...
that had a major and selective effect on LTC₄ transport. Each of the 26 nonidentical amino acids in MRP3 was introduced into MRP1, either as single residue substitutions or as double substitutions where adjacent residues were involved. Constructs were expressed in Sf21 cells, and after determining their expression levels (Fig. 3A), LTC₄ and E₂₁₇/H₉₂₅₂ transport was assessed for each mutant protein. In a preliminary screen based on single time point assays, the majority of individual, single mutations had negligible effect on LTC₄ transport (data not shown). The most dramatic reduction in transport was found for one double mutation located in the G1 region, Y₄₄₀F/I₄₄₁L, which reduced LTC₄ transport to less than 20% of wild-type levels (Fig. 3B). However, this double mutation also decreased E₂₁₇/H₉₂₅₂ transport to approximately 25% of wild-type MRP1 (Fig. 3C). A second double mutation, A₄₈₁G/V₄₈₂A, located in the G2 region also reduced LTC₄ transport by 6-fold, and in this case, E₂₁₇/H₉₂₅₂ transport was reduced to approximately one third of wild-type MRP1 levels (data not shown).

Because no single- or double-substituted mutant was found that alone explained the dramatic reduction in LTC₄ transport by the G hybrid protein while leaving E₂₁₇/H₂₅₂ transport intact, a series of multiple mutations were made predominantly targeting clusters of nonidentical amino acids (Table 1). With the exception of one triple mutation, in which substitution of M₄₄₃ with L was introduced into the LTC₄ transport-deficient Y₄₄₀F/I₄₄₁L double mutant, no combination of mutations tested reduced LTC₄ transport by more than 60 to 70% (data not shown). The Y₄₄₀F/I₄₄₁L/M₄₄₃L virtually eliminated LTC₄ transport (Fig. 3B) but also reduced E₂₁₇/H₂₅₂ transport by approximately 80% (Fig. 3C).

Transport of LTC₄, E₂₁₇/H₂₅₂, E₁₃SO₄, and MTX by Y₄₄₀F/I₄₄₁L, and M₄₄₃L MRP1 Mutant Proteins. To further characterize the effects of the double and triple mutations on substrate specificity, we investigated the influence of individual contributing amino acid substitutions on transport of LTC₄ and E₂₁₇/H₂₅₂, as well as two additional substrates, E₁₃SO₄ and MTX. These two organic anion substrates were chosen because the former is transported by MRP1 in a GSH- or S-methyl GSH-stimulated manner but is not a substrate for MRP3, whereas MTX is transported by both proteins (Deeley et al., 2006).

![Predicted topology of human MRP1 and location of the MRP3 segments that were exchanged in the MRP1/MRP3 hybrid proteins. A, the predicted topology of human MRP1 with 17 TM helices, organized into three MSDs and two NBDs. Also depicted is the site of division of the protein used for expression as two half-molecules: MRP₁₁–₄₂₅ and MRP₁₄₂₅–₁₅₃₁. B, portions of MSD1 and MSD2 of MRP1 that were exchanged for the equivalent regions of MRP3 (fragments A–L) and expressed as MRP1/MRP3 hybrid proteins in the Sf21 expression system, as described in the text. ATP-dependent LTC₄ (C) and E₂₁₇/H₂₅₂ (D). Transport by membrane vesicles expressing wild-type MRP1 (dh), the MRP₁₁–₄₂₅/₄₁₁–₅₀₂MRP₁₅₁₇–₁₅₃₁ hybrid protein (G), and vesicles expressing a control protein β-gus. Shown are transport levels obtained at 2 min for LTC₄ and at 3 min for E₂₁₇/H₂₅₂. Conditions used for the transport assays were as described under Materials and Methods. The results shown are the averages and S.D. of triplicate assays from a typical experiment. Similar results were obtained with a second set of independently produced membrane vesicle preparations.](image-url)
The Y440F and M443L mutations each independently decreased initial rates of LTC₄ transport by approximately 60 and 50%, respectively, whereas the I441L mutation had little or no effect (Fig. 3B). In contrast, E217/H9252G transport was decreased approximately 50% by both the I441L and M443L mutations but only 20% by the Y440F mutation (Fig. 3C). All three mutations significantly decreased E13SO₄ transport in the presence of 2 mM S-methyl GSH, with the Y440F, I441L, and M443L mutations reducing transport at 1 min by approximately 65, 50, and 90%, respectively (Fig. 3D). However, none of the single mutations or the double mutation had a statistically significant effect on transport of the common MRP1/MRP3 substrate MTX (Fig. 3E). Thus, mutation of each of these three amino acids differentially affects

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substrate specificity as opposed to overall transport activity, strongly suggesting that they are not simply perturbing the conformation of the protein and that they contribute to substrate recognition.

Kinetic Parameters of $[^3H]LTC_4$ Transport by Y440F and I441L Single and Double Mutants. We also determined the effects of the Y440F and I441L single and double mutations on the $K_m$ and $V_{max}$ for LTC$_4$ transport. The $K_m$ and $V_{max}$ values obtained for wild-type MRP1 were 72 nM and 37 pmol/mg/min, respectively, whereas the $K_m$ for the Y440F mutation was 328 nM and the normalized $V_{max}$ was 41 pmol/mg/min (Fig. 4, A and B). The increase in $K_m$ suggested that the relatively conservative substitution of Phe for Tyr results in an almost 5-fold decrease in apparent affinity for LTC$_4$ with no change in the maximal rate of transport. Although LTC$_4$ transport by the I441L mutant was only marginally decreased at a fixed concentration of substrate, linear regression analysis indicated that the $K_m$ for LTC$_4$ was increased approximately 2-fold (149 nM for the I441L mutant protein versus 72 nM for wild-type MRP1), again with no significant change in $V_{max}$ (normalized $V_{max}$ for the I441L mutation 42 versus 37 pmol/mg/min for the wild-type protein) (Fig. 4, A and B). Transport of LTC$_4$ by the double mutant was markedly reduced compared with either of the single mutations alone. As a

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**TABLE 1**

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<th>Mutant No.</th>
<th>Mutations Included</th>
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**FIG. 3.** ATP-dependent uptake of $[^3H]$-labeled LTC$_4$, E$_2$17$, G$,$ E$,$ E$_3$, and MTX by membrane vesicles prepared from SF21 cells infected with virus encoding wild-type or mutant MRP1. A, levels of wild-type and mutant MRP1 proteins were determined by transferring vesicle protein to membranes using a slot blot apparatus, followed by sequential detection with mAbs QCRL1 and MRPm6 and densitometry, as described under Materials and Methods. Shown are scans of 1-µg samples only. Not shown are scans of the 0.5-, 1.5-, and 2-µg protein samples. The numbers below the blots refer to the levels of mutant MRP1 proteins relative to the level of the wild-type protein (dh), as determined by densitometry. B–E, ATP-dependent uptake of $[^3H]$LTC$_4$ measured for 2 min (B); $[^3H]$E$_2$17$, G$ measured for 3 min (C); $[^3H]$E$_3$, S$_3$O$_4$ measured with the addition of 2 mM S-methyl GSH for 1 min (D); and $[^3H]$MTX measured for 20 min (E). The relative levels of protein expression determined by densitometry (A) were used to normalize transport data to the expression level of wild-type MRP1. Values are mean ± S.D. of three determinations in a single experiment. Similar results were obtained with two or more independently produced membrane vesicle preparations.
consequence, it was technically impossible to determine an accurate $K_m$ for LTC$_4$.

**Photolabeling of the NH$_2$- and COOH-Terminal Halves of MRP1 Mutant Proteins by LTC$_4$.** To confirm whether the increase in $K_m$ resulting from the Y440F mutation reflected a decreased affinity for substrate, we examined the effect of the mutation on photolabeling of MRP1 with [3H]LTC$_4$. Based on the expression levels of the three mutant proteins relative to wild-type MRP, the quantity of membrane vesicles used for photolabeling was adjusted to yield equivalent amounts of wild-type or mutant MRP1 in each photolabeled sample. To confirm that this was achieved, an aliquot of each protein sample used for photolabeling was re-examined by SDS-PAGE, immunoblotting, and densitometry (Fig. 5A).

We have previously shown that LTC$_4$ binds to both NH$_2$- and COOH-terminal fragments of MRP1 asymmetrically (Qian et al., 2001a). As shown in Fig. 5B, [3H]LTC$_4$ predominantly labels the NH$_2$-terminal fragment containing amino acids 1 through 932 of wild-type MRP1, although labeling of the COOH-terminal fragment, amino acids 932 through 1531, was still readily detectable. Photolabeling of both fragments of the I441L mutant protein, which displayed only a 2-fold increase in $K_m$, was essentially indistinguishable from that obtained with the wild-type MRP1 protein. However, photolabeling of the NH$_2$-terminal fragments of both the Y440F and double Y440F/I441L mutant proteins was similarly, substantially reduced compared with both the wild-type and the I441L mutant. These data are consistent with the results of kinetic studies and strongly support the suggestion that the Y440F mutation results in a substantial decrease in affinity for LTC$_4$. Furthermore, photolabeling of the COOH-terminal fragment of the Y440F and Y440F/I441L mutant proteins was also reduced when compared with wild-type MRP1 or the I441L mutant, despite the fact that this region is identical in all four proteins. Thus, photolabeling of the NH$_2$- and COOH-proximal halves of the protein does not seem to be the result of interaction of LTC$_4$ with two functionally independent sites.

**Mutational Analysis of Residue Tyr$^{440}$.** We next examined the functional consequences of less conservative substitutions of Tyr$^{440}$. These included nonaromatic neutral (Ala), polar (Ser), polar neutral (Gln), and acidic (Glu) residues, as well as a polar aromatic substitution with Trp. Vesicles containing the mutant proteins were then tested for their ability to transport LTC$_4$. All of these mutations had a greater effect on transport than the more conservative Y440F mutation. Transport by the two polar mutations Y440Q and Y440S was decreased by 75 to 80%, whereas the charged and neutral mutations decreased transport by more than 90% (Fig. 6A). To determine whether the size of the aromatic side chain was critical, as well as its polarity, we created a Tyr to Trp mutation. This mutation decreased LTC$_4$ transport by 75% compared with the wild-type protein (Fig. 6A). In addition, unlike the Y440F mutation, which had little effect on E$_{17}$BG transport, the Y440W mutation essentially eliminated transport of the conjugated estrogen (Fig. 6B).

**Kinetic Parameters of S-Methyl GSH-Stimulated [3H]Estrone 3-Sulfate Transport and Azidophenacyl[35S]-GSH Photolabeling of Wild-Type and Mutant MRP1 Proteins.** The Y440F mutation has a major deleterious effect on the transport of LTC$_4$ and the S-methyl GSH-stimulated transport of E$_3$S$_4$, (Fig. 3, B and D) but not on the other estrogen conjugate tested (E$_2$17BG or MTX). Because we have shown that the Y440F mutant protein has reduced affinity for LTC$_4$ compared with wild-type MRP1 (Fig. 4B), it is possible that the Y440F mutation also affects the affinity for E$_3$S$_4$ and/or S-methyl GSH. To test the former hypothesis, we attempted to determine a $K_m$ for the transport of E$_3$S$_4$ by wild-type MRP1 and the Y440F mutant. It is unfortunate that the levels of transport by the mutant precluded determination of a reliable $K_m$. However, the transport deficiency showed no indication of being overcome by using concentrations of E$_3$S$_4$ as much as 10-fold
higher than the $K_m$ of wild-type MRP1 in transport assays (Fig. 7A). Thus, although we were unable to determine whether $K_m$ was affected, it seems likely that the mutation decreases the $V_{\text{max}}$ for E13SO$_4$ transport possibly as a result of decreasing the affinity for S-methyl GSH.

To test whether the Y440F mutation alters the binding characteristics of S-methyl GSH and thus E13SO$_4$ transport, we examined the binding of a GSH analog, azidophenacyl-GSH, to wild-type and mutant MRP1 expressed in stably transfected HEK cells. We have previously shown that this analog can substitute for GSH or S-methyl GSH in stimulating E13SO$_4$ transport and, when radiolabeled with $^{35}$S, binds to MRP1 in a fashion similar to that found for LTC4 (Qian et al., 2002). The M443L mutation reduced S-methyl GSH-stimulated E13SO$_4$ transport by 90% in Sf21 cells (Fig. 3D). As shown in Fig. 7, B and C, binding of azidophenacyl-$^{35}$S[GSH by this mutant protein was barely detectable when compared with wild-type MRP1, despite the use of almost 2-fold more mutant than wild-type protein in the samples shown. Thus, the affinity for azidophenacyl-GSH is severely affected by the M443L mutation. In contrast, the I441L mutant protein, which decreased S-methyl GSH-stimulated transport of E13SO$_4$ by 55% compared with wild-type MRP1 (Fig. 3D), bound approximately equivalent levels of azidophenacyl-$^{35}$S[GSH (Fig. 7C), suggesting that, as was determined for LTC$_4$, the affinity for azidophenacyl-GSH is relatively unaffected by the I441L mutation. In contrast, the Y440F mutation, which reduced S-methyl GSH-stimulated transport of E13SO$_4$ by approximately 65% (Fig. 3D), markedly decreased photolabeling with azidophenacyl-$^{35}$S[GSH (Fig. 7C). This suggests that the affinity for this GSH derivative is dramatically affected by the Y440F mutation, as was found for LTC$_4$. The reduced affinity for both azidophenacyl GSH and LTC$_4$ suggests that the Y440F mutation may alter the interaction of MRP1 with the GSH moiety of both compounds and thus may decrease E13SO$_4$ transport by reducing the affinity for GSH and S-methyl GSH.

Effect of the Y440F, I441L, M443L, and Y440F/I441L Mutations on Resistance to Vincristine, Doxorubicin, and VP-16. Lastly, the drug-resistance profiles of Y440F, I441L, M443L, and Y440F/I441L mutant proteins were examined because unlike MRP1, the profile of resistance to natural product drugs conferred by MRP3 is restricted primarily to epipodophyllotoxins (Deeley et al., 2006). Each single and the double-mutant proteins were stably expressed in cloned populations of HEK293 cells, and the subcellular localization of wild-type and mutant MRP1s was compared by immunostaining with the MRP1-specific mAb MRPM6 and confocal microscopy to ensure that none of the mutations adversely affected protein trafficking (see supplemental data).
Cells expressing MRP3 were significantly resistant to VP-16 (6.4-fold) but not to either vincristine (1.1-fold) or doxorubicin (0.87-fold), whereas cells expressing wild-type MPR1 were resistant to all three classes of drugs (15.6-, 16.2-, and 4.5-fold for vincristine, VP-16, and doxorubicin, respectively), consistent with previous results (Table 2) (Cole et al., 1994; Grant et al., 1994; Kool et al., 1999; Zelcer et al., 2001; Zhang et al., 2003). The three single mutations each decreased resistance to vincristine and VP-16, 2- to 3-fold, whereas only the Y440F mutation resulted in a major decrease in resistance to doxorubicin. The effect of the double Y440F/I441L mutation seemed to be additive with respect to both VP-16 and doxorubicin resistance but resulted in no greater decrease in resistance to vincristine than either mutation alone. Thus, as observed during organic anion transport studies, each mutation had different effects on the drug-resistance profile of MPR1, suggesting that all three of these residues contribute to recognition of natural product drugs, as well as the organic anion substrates tested. However, none of the mutations resulted in a profile more closely resembling that of MRP3.

Discussion

In studies described here, we sought to identify additional residues involved in the binding and transport of GSH and GSH conjugates by MRP1. We did so by constructing a series of MRP1/MRP3 hybrids and searching for regions that, when exchanged, decreased transport of the high-affinity MRP1 substrate, LTC₄, without affecting transport of the shared substrate, E₂₁₇BG. The approach was based on the premise that such regions would contain amino acids that differ between MRP1 and MRP3, which are determinants of LTC₄ specificity. We found that it was possible to exchange most of MSD2, encompassing TM12 through TM15, with little effect on LTC₄ transport. We observed, together with previous studies showing that point mutations in TMs 16 and 17 have little effect on LTC₄ transport by MRP1, suggested that nonidentical residues crucial for LTC₄ transport were localized in MSD1 and possibly the cytoplasmic linker region (CL3) (Deelely et al., 2006). The only hybrid that displayed a major, selective decrease in LTC₄ transport contained amino acids 411 through 502 of MRP3 in place of amino acids 425 through 516 of MRP1. Exchange of this region, spanning TMs 8 and 9, completely eliminated LTC₄ transport with little effect on transport of E₂₁₇BG. Four amino acids in this region of MRP1 (Arg⁴³³, Asp⁴³⁶, Trp⁴⁵⁹, and Pro⁴⁷⁸) have previously been shown to be determinants of the substrate specificity of MRP1 (Conrad et al., 2002; Koike et al., 2002, 2004; Haimeur et al., 2004). Mutation of three of them (Arg⁴³³, Asp⁴³⁶, and Pro⁴⁷⁸) affects transport of LTC₄. Arg⁴³³ and Asp⁴³⁶ are predicted to be located in a cytoplasmic helical region that is an extension of TM8, whereas Pro⁴⁷⁸ is located within TM9. However, all of these residues are identical between MRP1 and MRP3 and thus would not be revealed by the approach we have taken.

The G region contains 26 amino acids that differ between MRP1 and MRP3. Analysis of hybrids containing subfragments spanning either TM 8 or TM 9 suggested that multiple nonidentical residues were likely to contribute to LTC₄-specific transport. Consequently, we were unable to completely replicate the results of exchanging the entire region by mutating the 26 nonidentical amino acids singly or as clusters of up to eight residues. Furthermore, individual mutation of many of these residues had negligible effects on transport of any of the substrates tested. This result is consistent with current models of the interaction of multidrug transporters with their structurally diverse substrates, which is believed to involve multiple, often overlapping, weak interactions between the ligand and a relatively large and flexible binding pocket or surface (reviewed in Deelely and Cole, 2006). However, we did locate a trio of nonidentical amino acids predicted to be in the inner leaflet region of TM8, each of which selectively affected the substrate specificity of MRP1. It is noteworthy that conservative substitution of Tyr⁴⁴⁰ with Phe as present in MRP3 reduced LTC₄ transport by ~60% with little effect on transport of E₂₁₇BG. This mutation also reduced transport of the GSH-dependent MRP1 substrate E₃₅₄O₃ but not the unconjugated organic anion MTX, which is transported by both MRP1 and MRP3 (Deelely et al., 2006).

The Y440F mutation resulted in a significant decrease in the apparent affinity for LTC₄ (4.5-fold increase in $K_{ass}$), whereas $V_{max}$ was not affected. In addition, photolabeling of the Y440F protein by [³H]LTC₄ was markedly decreased. Taken together, the data strongly suggested that the primary defect in the Y440F protein was at the level of LTC₄ binding. Sequence comparison with the human MRP family (Fig. 2A) revealed that seven of nine members have Tyr or Phe at the position corresponding to MRP1 Tyr⁴⁴⁰. Thus, the presence of an aromatic amino acid at this position is relatively highly conserved across family members and may be of broad functional importance for substrate recognition. In addition to MRP1, MRP6 and the more distantly related MRP4 have been shown to transport LTC₄ with relatively high affinity (Iliaś et al., 2002; Rius et al., 2008). However, whereas Tyr⁴⁴⁰ is conserved in MRP6, the corresponding residue in MRP4 is Phe, as it is in MRP3. The lack of complete conservation among the LTC₄ transporters is not unexpected. Extensive studies have shown that structure/substrate specificity relationships cannot be predicted based on amino acid sequence conservation among MRP homologs, and that even exceptionally conserved amino acids do not necessarily make the same contributions to recognition of common substrates (reviewed in Deelely et al., 2006).

Other conservative (W) and nonconservative (A, E, Q, and S) substitutions of Tyr⁴⁴⁰ caused significant reductions in LTC₄ transport ranging from 75% (Y440Q) to 90% (Y440A and Y440E). In particular, the relatively conservative substitution with Trp not only decreased LTC₄ transport by ~75%, but unlike the Y440F mutation, also essentially eliminated transport of E₂₁₇BG. Thus, it seems that both the size and the polarity of the aromatic side chain at this location are important for the interaction of MRP1 with LTC₄, whereas it is primarily the size that is critical for interaction with E₂₁₇BG. Whether the mutations exert their effects by altering direct contacts between substrate and the mutated residue or by localized perturbations in regions of the binding pocket important for interaction with certain substrates and not others is presently not known.

In contrast to the Y440F mutation, the conservatively substituted I441L mutation had no effect on LTC₄ or MTX transport but decreased transport of both E₂₁₇BG and E₃₅₄O₃, whereas the M443L mutation decreased transport of all three conjugated substrates but not

### TABLE 2

**Relative Drug Resistance of HEK293 Cells Transfected with Wild-Type and Mutant MRP1**

| Transfectant | Drug (Relative Resistance Factor$^*$) |  |  
|--------------|-------------------------------------|---|---
|              | Vincristine | VP-16 | Doxorubicin |
| HEKMRP1      | 15.6 ± 2.5 | 16.2 ± 4.9 | 4.5 ± 0.3 |
| HEKMRP1-Y440F | 2.4 ± 0.5 (5.1) | 2.7 ± 0.8 (6.0) | 1.3 ± 0.2 (1.9) |
| HEKMRP1-I441L | 8.9 ± 2.5 (9.7) | 4.1 ± 1.2 (4.4) | 3.7 ± 1.4 (4.1) |
| HEKMRP1-M443L | 6.5 ± 1.2 (6.0) | 5.8 ± 0.5 (5.4) | 3.8 ± 0.7 (3.6) |
| HEKMRP1-Y440F/I441L | 3.9 ± 1.0 (7.5) | 1.3 ± 0.3 (1.7) | 1.2 ± 0.2 (1.5) |
| HEKMRP3      | 1.1 ± 0.1 | 6.4 ± 1.9 | 0.87 ± 0.2 |

$^*$ The relative resistance factor was obtained by dividing the IC₅₀ values for the wild-type or mutant MRP1-transfected cells by the IC₅₀ value for cells transfected with the expression vector alone. Each value represents the mean ± S.D. of three or more independent experiments.

Resistance factors normalized for differences in MRP1 protein expression are indicated in parenthesis.
MTX. Likewise, mutation of each of these three residues caused differential effects on the drug-resistance profile of MRP1, consistent with their importance in determining recognition and transport of a number of substrates in addition to LTC₄. As suggested previously, these observations are compatible with the existence of a common binding pocket with each substrate establishing multiple overlapping but not identical interactions with the protein (Deeley et al., 2006).

The clustering of Tyr⁴⁴⁰, Ile⁴⁴¹, and Met⁴⁴³ in a single turn of TM8 is similar to that of three previously identified mutation-sensitive residues in TM6: Lys³³², His³³⁵, and Asp³³⁶. Mutations of Lys³³² and His³³⁵ affect substrate specificity in a similar and selective fashion, whereas mutation of Asp³³⁶ affects overall transport activity (Haimeur et al., 2002, 2004). Most significantly, the conservative substitution of Lys³³² by Arg increased the $K_m$ for LTC₄ ~5-fold without affecting the $V_{max}$, as is the case with the Y440F mutation. Other nonconservative mutations of Lys³³² abrogated LTC₄ transport, as was observed with certain nonconservative substitutions of Tyr⁴⁴⁰. In addition, photolabeling with $[^3H]$LTC₄ of K332D and K332L mutant proteins was severely reduced compared with wild-type MRP1. Thus, mutations of both TM6-Lys³³² and TM8-Tyr⁴⁴⁰ decrease LTC₄ binding, and both the charge (or polarity) and volume of the residue at either location are critical for interaction with LTC₄.

The Y440F mutation had little effect on either E₁βG or MTX transport but markedly decreased $S$-methyl GSH-stimulated $E_₂S$₄ transport, and photolabeling with the GSH analog azidophenacyl-$[³⁵S]$GSH was severely reduced. Because of the magnitude of the effect, we were unable to determine the kinetic parameters of $E₂S₄$ transport. However, it is clear that the Y440F mutation almost entirely eliminates binding of azidophenacyl-GSH, as well as LTC₄. Because both $S$-methyl GSH and azidophenacyl-GSH can substitute for GSH in transport of $E₂S₄$ (Qian et al., 2002; Leslie et al., 2003), Tyr⁴⁴⁰ may interact with the GSH moiety of LTC₄, $S$-methyl GSH, and azidophenacyl-GSH and thus reduce transport of both LTC₄ and $E₂S₄$. Consistent with this suggestion, the Y440F mutation resulted in a major decrease in resistance to all three classes of drugs, transport of, or resistance to which, has been shown to be GSH-dependent (Loe et al., 1996b, 1998; Rappa et al., 1997; Renes et al., 1999).

We have previously used molecular modeling to examine possible spatial relationships among residues that affect the substrate specificity of MRP1, as well as the disposition of specific amino acid side chains with respect to the putative translocation pathway of the protein (Campbell et al., 2004; Deeley et al., 2006). The models of MRP1 and several other ABC transporters were based on the crystal structure of MsbA published by Chang (2003). This structure differs significantly from that determined more recently for the Staphylococcus aureus multidrug ABC transporter, Sav1866, and was retracted because of major errors in the packing and tilt of a number of TMs (Chang et al., 2006; Dawson and Locher, 2006; Dawson et al., 2007). Consequently, we developed a revised model based on the more recent Sav1866 structure (DeGorter et al., 2008). However, this structure corresponds to the ADP-trapped form of Sav1866, in which the protein is thought to be in its low-affinity substrate binding state, with the putative translocation pathway open to the extracellular side of the membrane. In such a configuration, it is presumed that the high-affinity site is occluded and inaccessible to substrate in the cytoplasm or membrane. Three views of this model are shown in Fig. 8, illustrating the predicted locations of residues in TM8, which when mutated selectively affect substrate specificity, in relation to those previously identified in TM6, which also differentially influence transport of LTC₄.

The TMs in the Sav1866-based structure are tilted relative to the likely translocation pathway, rather than being parallel to it, and several of the helices display significant curvature (Fig. 8B) (DeGorter et al., 2008). For example, TM6 and TM8 are adjacent to each other and closely aligned in the inner leaflet region of the membrane but tilt away from each other in the outer leaflet. The cluster of residues we have identified is located in the inner leaflet of the membrane with the side chains of Tyr⁴⁴⁰ and Ile⁴⁴¹ aligned tangentially to the translocation pathway, whereas Met⁴⁴³ projects into it. In TM6, the previously identified residues are predicted to be located in the outer leaflet, with Lys³³² and Asp³³⁶ projecting into the “open” end of the pathway. It has been proposed that changes in the conformation of the NBDs on binding and release of nucleotide are transmitted to the MSDs via two coupling helices in each MSD, one of...
which (helix 1) interacts with both NBDs in the closed configuration, whereas the other (helix 2) interacts with the apposing NBD. In MSD1 of MRP1, coupling helix 1 is predicted to connect TM7 and TM8. Thus, one or both of these helices seem likely candidates for transmitting conformational changes induced by movement of coupling helix 1. It remains to be determined to what extent such a conformational change affects the accessibility of the residues in TM8, such as Tyr440 and Ile441, to hydrophilic substrates such as LTC4 and GSH.

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References


