Multidrug Resistance Protein
IDENTIFICATION OF REGIONS REQUIRED FOR ACTIVE TRANSPORT OF LEUKOTRIENE C₄

(Received for publication, December 1, 1997, and in revised form, February 18, 1998)

Mian Gao‡§§, Masayom Yamazaki‡, Douglas W. Loe‡, Christopher J. Westlake‡‡‡, Caroline E. Grant‡, Susan P. C. Cole¶¶¶, and Roger G. Deeley¶¶¶

From the Cancer Research Laboratories and the Departments of Pathology and Biochemistry, Queen’s University, Kingston, Ontario K7L 3N6, Canada

The multidrug resistance protein (MRP) is a broad specificity, primary active transporter of organic anion conjugates that confers a multidrug resistance phenotype when transfected into drug-sensitive cells. The protein was the first example of a subgroup of the ATP-binding cassette superfamily whose members have three membrane-spanning domains (MSDs) and two nucleotide binding domains. The role(s) of the third MSD of MRP and its related transporters is not known. To begin to address this question, we examined the ability of various MRP fragments, expressed individually and in combination, to transport the MRP substrate, leukotriene C₄ (LTC₄). We found that elimination of the entire NH₂-terminal MSD or just the first putative transmembrane helix, or substitution of the MSD with the comparable region of the functionally and structurally related transporter, the canalicular multispecific organic anion transporter (cMOAT/MRP2), had little effect on protein accumulation in the membrane. However, all three modifications decreased LTC₄ transport activity by at least 90%. Transport activity could be reconstituted by co-expression of the NH₂-terminal MSD with a fragment corresponding to the remainder of the MRP molecule, but this required both the region encoding the transmembrane helices of the NH₂-terminal MSD and the cytoplasmic region linking it to the next MSD. In contrast, a major part of the cytoplasmic region linking the NH₂-proximal nucleotide binding domain of the protein to the COOH-proximal MSD was not required for active transport of LTC₄.

*This work was supported by grants from the National Cancer Institute of Canada (No. 4570) with funds from the Canadian Cancer Society and the Medical Research Council of Canada (MT-10519). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported in part by a Queen’s University graduate award.
¶‡ Present address: Dept. of Pharmacokinetics and Biopharmaceutics, Toho University, School of Pharmaceutical Sciences, 2-2-1, Miyama, Funabashi, Chiba, 274, Japan.
¶¶ Senior Career Scientist of Cancer Care Ontario.
¶¶¶ Stauffer Research Professor of Queen’s University. To whom correspondence and reprint requests should be addressed: Cancer Research Laboratories, Rm. A315, Bette Hall, Queen’s University, Kingston, Ontario, Canada, K7L 3N6. Tel: 613-545-2981; Fax: 613-545-6830.

†† The abbreviations used are: MRP, multidrug resistance protein; ABC, ATP-binding cassette; P-gp, P-glycoprotein; LTC₄, leukotriene C₄; CFTR, cystic fibrosis transmembrane conductance regulator; NBD, nucleotide binding domain; MSD, membrane spanning domain; TM, transmembrane; cMOAT/MRP2, canalicular multispecific organic anion transporter; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

transporters (1, 2). When transfected into drug-sensitive recipient cells, MRP increases resistance to natural product chemotherapeutic agents including epipodophyllotoxins, Vinca alkaloids, and certain anthracyclines (3–6). The resistance of MRP-transfected cells is associated with an energy-dependent decrease in drug accumulation and an increase in drug efflux (4, 6). Thus MRP confers a form of multidrug resistance that shares several characteristics with that caused by overexpression of P-glycoprotein (P-gp).

MRP can also act as a primary active transporter of a wide range of organic, anionic conjugates, some of which are potential physiological substrates (reviewed in Loe et al. (7)). These include a structurally diverse array of glutathione, glucuronide, and sulfate conjugates, with the cysteinyl leukotriene, LTC₄, being one of the highest affinity MRP substrates characterized to date (8–11). However, it has not been possible to detect binding or measure primary active transport of unmodified xenobiotics (8, 9, 12). We have been able to demonstrate MRP-mediated, ATP-dependent transport of vincristine, and more recently, aflatoxin B₁, but only in the presence of physiological concentrations of GSH (8, 13).

P-gp (encoded by the MDR1 gene) and MRP share only approximately 18% overall amino acid identity, but despite the lack of primary structure conservation, the hydropathy profiles of the two proteins are quite similar when they are aligned from their COOH termini (14). Like other members of the ABC-superfamily such as the cystic fibrosis transmembrane conductance regulator (CFTR), the nucleotide binding domains (NBDs) of MRP are preceded by a polytopic membrane spanning domain (MSD) that may contain as many as six transmembrane (TM) helices. However, MRP also contains a third, NH₂-proximal MSD. Topology predictions derived from various protein structure algorithms indicate that this region, MSD1, could span the membrane four to six times (15). Mutagenesis of potential N-glycosylation sites has recently shown that the NH₂ terminus of MRP is extracellular (15). Thus it appears most likely that MSD1 spans the membrane five times. A number of ABC transporters relatively closely related to MRP have now been described that also contain a third NH₂-proximal MSD (16–20). Analyses of protein primary structure and gene organization suggest that members of the MRP-related branch of the superfamily may have evolved from a common ancestor that they shared with CFTR (21, 22). Thus the additional NH₂-terminal MSDs of the MRP-related proteins may have been acquired by fusion of a common ancestral gene with one or more genes encoding other membrane proteins. Whether the NH₂-terminal MSDs of these proteins are now essential for function or play some type of regulatory role is not known.

We demonstrated previously that a MRP fragment containing the two NH₂-proximal MSDs (MSD1 and MSD2), their associated NBD (NBD1), and most of the region linking this.
Structure and Function of Multidrug Resistance Protein

segment of the protein to the COOH-proximal MSD (MSD2) had no demonstrable LTC₄ transport activity. However, a functional transporter could be reconstituted by co-expressing this fragment with another containing MSD3 and NBD2 (23). Here, we have used a similar approach to determine whether molecules lacking all or part of MSD1 and the predicted cytoplasmic loop linking it to the rest of the protein can function as transporters, and whether this domain can reassociate with a COOH-proximal MRP fragment when the two are co-expressed. We have also investigated the possibility of exchanging MSD1 of MRP for that of the related canalicular multispecific organic anion transporter, cMOAT/MRP2 (16). In our initial study, the co-expressed fragments corresponded to those that would be generated by cleaving the molecule at Ala₉₃₂, in the cytoplasmic region linking NBD1 to MSD3. Thus although not physically contiguous, the entire linker region between the two halves of the molecule was present in the reconstituted protein (23). We have now determined whether most of this region, which in CFTR and certain other ABC transporters has been reported to have a regulatory function, is required for the LTC₄ transport activity of MRP.

MATERIALS AND METHODS

Generation of Constructs—The expression cassette pBSMRP-fc-ATG and recombinant donor plasmid pFB-MRP, containing the MRP coding sequence, have been described (23). To generate constructs expressing either MSD1 or the remainder of the protein, pBSMRP-fc-ATG was linearized with BamHI, made blunt-ended using a Klenow fragment, and then digested with SacI and KpnI. This digestion generates one fragment (SacI-BamHI) containing the sequence encoding amino acids 1–281 of MRP and another (BamHI-KpnI) encoding the remainder of the protein. The SacI-BamHI* fragment was ligated to the donor plasmid pFASTBAC1 (Life Technologies Inc.) which had been linearized with BamHI and then digested with SacI and KpnI to produce the MRP₂₃₀–₆₅₂ vector. Insertion of the inserted fragment terminated at a stop codon in the vector resulting in the addition of six amino acids, QLVEKY.

To generate a construct lacking the NH₂-proximal 280 amino acids, pFB-MRP was linearized with NcoI, made blunt-ended using a Klenow fragment, and then digested with KpnI. The vector backbone that contains the Kozak sequence and initiator methionine originally introduced into pFB-MRP was ligated to the BamHI*-KpnI fragment. The only amino acid introduced during construction of the MRP₂₃₀–₁₅₃₁ vector was the initiator methionine. To create a similar construct encoding MRP₂₂₉–₁₃₁₅₁, a fragment containing nucleotides 685–1120 was amplified by PCR. The forward primer used introduced a SacI site preceding an in-frame start codon. The PCR fragment was digested with SacI and BamHI, and the SacI-BamHI fragment was isolated from pBSMRP-fc-ATG and then digested with SacI and BamHI, leaving the 3'-proximal sequence beginning at nucleotide 840 attached to the vector backbone. The purified digested vector was ligated to the SacI-BamHI fragment of the MRP₂₃₀–₆₅₂ vector to produce the MRP₂₂₉–₁₃₁₅₁ construct.

To create a construct eliminating amino acids 228–280 of the protein (MRP₂₂₈–₂₈₅), a fragment containing nucleotides 1–681 along with the 5' UTR of pBSMRP-fc-ATG was amplified by PCR. The primer used was 5'-CACGAGCTCAGACCAGAAGTTCTGCAAC-3' (forward primer) which included a SacI site and an in-frame start codon. The 681-base pair fragment was ligated to the 3'-proximal sequence beginning at nucleotide 840 attached to the vector backbone. The purified digested vector was ligated to the SacI-BamHI fragment of the MRP₂₂₉–₁₃₁₅₁ vector. Fidelity of the product was confirmed by dideoxy sequencing. Digestion of the modified vector with SacI and KpnI yielded a fragment of 4.3 kilobase pairs encoding amino acids 67–1531, which was cloned into pFASTBAC1.

A similar strategy was used to create a hybrid protein containing the NH₂-terminal 281 amino acids of cMOAT/MRP2 fused to amino acids 281–1531 of MRP. The cMOAT/MRP2 fragment was created by reverse transcriptase-PCR of poly(A) RNA isolated from the human hepatoma cell line BEL-7402 using an upstream primer corresponding to a perfect 29-nucleotide sequence preceded by a HindIII site and a downstream primer that included a BamHI site. The sequences of the primers were: (forward) 5'-CACAGGATGCTGATTTGATGCCGACG-3', (reverse) 5'-CAGGGATCCTGATTCTTGACAGACAACGACGACACCCTCTACACAAAACC-3' (forward primer which includes a SacI site (underlined) and a conserved Kozak sequence (bold), and 5'-CTCTCCTTGGTCATCCACACCGTGG-3' (reverse primer). The 705-base pair product was digested at the SacI site in the primer and at the BamHI site in the MRP coding sequence to yield a 652-base pair fragment. This fragment was ligated into a MRP vector from which the SacI/BamHI fragment had been removed. Fidelity of the product was confirmed by dideoxy sequencing. Digestion of the modified vector with SacI and KpnI yielded a fragment of 4.3 kilobase pairs encoding amino acids 67–1531, which was cloned into pFASTBAC1.

Viral Infection and Membrane Vesicle Preparation—Recombinant baculoviruses were generated as described elsewhere (23). Conditions used for viral infection were similar to those described previously, except that the multiplicity of infection used for each co-expressed construct was adjusted to achieve comparable levels of the two MRP fragments (23). Cells were disrupted by nitrogen cavitation to generate membrane vesicles that were purified by sucrose gradient centrifugation, as described previously (8, 27).

Immunoblotting and Quantification of MRP Fragments—SDS-polyacrylamide gel electrophoresis (PAGE) of membrane vesicles proteins was as described using 5–15% gradient gels (3, 24). Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) in 0.1% SDS and MRP fragments were detected using an enhanced chemiluminescence kit (ECL) and murine mAbs QCRL-1 and MRPm6, as described previously (23, 25, 26).

The MR specific rat anti-mAb MRPr1 was used to detect some MRP fragments, in which case, horseradish peroxidase-conjugated goat antirabbit IgG and IgM (H + L), F(ab')₂ fragment (Pierce, Professional Diagnostics Inc., Edmonton, Alberta, Canada) was used instead of horseradish peroxidase-conjugated goat anti-mouse IgG and IgM (H + L), F(ab')₂ (26). The relative amount of various MRP fragments was estimated by comparison to full-length MRP expressed in Sf21 insect cells (23).

Vesicle Transport of LTC₄—Uptake of [³H]LTC₄ (50 nM, 132.0 Ci mmol⁻¹, NEN Life Science Products) into membrane vesicles was measured at 23 °C in the presence of ATP or AMP (4 mM) using a rapid filtration technique, as described elsewhere, with modifications (8, 27).

To determine kinetic parameters such as Kₘ and Vₘₐₓ, initial rates of LTC₄ uptake were measured for 60 s at various substrate concentrations. Data from initial rate measurements were fitted to the Michaelis-Menten type equation by an iterative nonlinear least-squares method using a MULTI program with a weight of 1/ν² (28).

RESULTS

Generation of Recombinant Baculoviruses—A possible topology of MRP derived from computer-assisted hydropathy analyses (7, 14, 15) and locations of known N-glycosylation sites (15) is shown in Fig. 1. Sf21 cells were transfected with baculoviruses encoding the regions of MRP indicated (Fig. 1). Whole cell lysates of the transfected cells were then analyzed by SDS-PAGE and immunoblotting using various MRP-specific antibodies (data not shown). Viral stocks were then prepared and their titers determined, as described previously (23).

MSD1 Is Required for Efficient LTC₄ Transport—The cytoplasmic loop connecting MSD1 to MSD2 is predicted to extend some distance from its putative transmembrane regions, as described elsewhere (27). Our results indicate that the cytoplasmic loop connecting MSD1 and MSD2 is predicted to extend some distance from its putative transmembrane regions, as described elsewhere (27). This was confirmed by the finding that MSD1 is required for LTC₄ transport, MRP fragments...
extending from either Val229 or Asp281 to the COOH terminus of the protein were expressed in Sf21 insect cells. SDS-PAGE and immunoblotting of membrane vesicle proteins revealed that MRP229–1531 and MRP281–1531 were expressed efficiently (Fig. 2A), at levels slightly higher than full-length MRP in comparable vesicle preparations. However, ATP-dependent LTC4 uptake was approximately 4.0 pmol min⁻¹ mg⁻¹ vesicle protein (at 23 °C and 50 nM LTC4) (Fig. 2B). This is only 2–3-fold higher than observed with vesicles from cells infected with a control virus encoding β-glucuronidase and less than 10% of the rates of uptake typically obtained with vesicles containing comparable levels of full length MRP (data not shown).

Elimination of the First Putative TM Helix of MSD1 Has Little Effect on Accumulation of MRP in the Membrane but Markedly Decreases LTC4 Transport—To determine whether elimination of just the first putative TM helix of MSD1 influenced accumulation of the protein in the membrane and/or transport activity, we constructed a vector encoding a polypeptide lacking amino acids 1–66 of MRP (MRP67–1531). Immunoblotting of membrane vesicle proteins indicated that MRP67–1531 accumulated to levels approaching those of full length MRP (Fig. 2A). However, initial rates of ATP-dependent LTC4 uptake were very similar to those observed following deletion of the entire MSD1 (Fig. 2B).

Co-expression of MRP1–281 with either MRP229–1531 or MRP281–1531 Restores ATP-dependent Transport of LTC4—To investigate the possibility that co-expression of MSD1 with the remainder of the protein could reconstitute a functional transporter, Sf21 cells were co-infected with constructs encoding MRP1–281 and MRP229–1531 or MRP281–1531. The levels of MRP1–281 were determined by immunoblotting using the MRP-specific mAb, MRPr1 (26). The epitope for this antibody has recently been located to amino acids 238–247 and thus is known to be present in both MRP1–281 and MRP229–1531 (Fig. 3, left).2 The levels of MRP1–281 and MRP229–1531 were estimated with mAb QCRL-1, the epitope for which includes amino acids 918–924 (29) (Fig. 3, right). The levels of each fragment were normalized by comparison with vesicle proteins from Sf21 cells expressing full-length MRP that were included on immunoblots probed with either mAb MRPr1 or mAb QCRL-1. The multiplicity of infection used for each vector was varied to ensure that similar levels of each of the encoded MRP fragments were obtained. This was achieved by using a 2-fold

---

FIG. 1. Topology of MRP and illustration of various MRP constructs. The schematic shown is derived from computer predictions using several algorithms and identification of utilized glycosylation sites (Y) by site directed mutagenesis (15). Approximate locations of predicted individual transmembrane helices are indicated (TM 1–17) as is their proposed organization into three MSDs. Positions of nucleotide binding domains (NBD1 and NBD2), as well as the relatively poorly conserved linker region connecting NBD1 to MSD3 are also shown. Linear representations of the various MRP fragments expressed in Sf21 cells are aligned below the schematic. The locations in full-length MRP of NH2-terminal and COOH-terminal amino acid residues of the fragments are indicated as are any amino acids introduced during construction of the respective expression vectors.
To further characterize the LTC₄ uptake by vesicles from cells co-expressing MRP₁–281 and MRP₂₂₉–₁₅₃₁, rates of uptake were measured at several LTC₄ concentrations (10–1000 nM). The apparent $K_m$ and $V_{max}$ values for LTC₄ were $136 \pm 42$ nM and $102 \pm 20$ pmol min⁻¹ mg⁻¹, respectively (Fig. 4B). This $K_m$ value is similar to those reported previously for vesicles from MRP-transfected HeLa T14 cells (105 nM) (8) and HeLa T5 cells (97 nM) (10), but slightly higher than those obtained with vesicles from Sf21 cells expressing either intact MRP (67 nM) or co-expressing both half-molecules (56 nM) (23). Comparable analyses were also performed to determine an apparent $K_m$ for ATP of 136 ± 24 μM (Fig. 4C), a value similar to that obtained previously with intact protein (100 μM) (23).

**MSD1 from cMOAT/MRP2 Is Not Exchangeable with MSD1 of MRP—** MRP and cMOAT/MRP2 share 49% overall amino acid identity and transport a similar range of substrates. Comparison of hydropathy profiles suggests that the topology of MSD1 in each protein may be similar and like MRP, cMOAT/MRP2 has a potential N-glycosylation sequon very near the NH₂-terminus. To determine whether the NH₂-proximal MSDs of the two proteins might be exchangeable, a vector was constructed that encoded a fusion protein comprised of amino acids 1–280 from cMOAT/MRP2 and amino acids 281–1531 of MRP. Immunoblotting of membrane vesicles from cells infected with the vector confirmed that encoded a fusion protein comprised of amino acids 1–280 from cMOAT/MRP2 and amino acids 281–1531 of MRP. Immunoblotting of membrane vesicles from cells infected with the vector confirmed that encoded a fusion protein comprised of amino acids 1–280 from cMOAT/MRP2 and amino acids 281–1531 of MRP. Immunoblotting of membrane vesicles from cells infected with the vector confirmed that encoded a fusion protein comprised of amino acids 1–280 from cMOAT/MRP2 and amino acids 281–1531 of MRP. Immunoblotting of membrane vesicles from cells infected with the vector confirmed that encoded a fusion protein comprised of amino acids 1–280 from cMOAT/MRP2 and amino acids 281–1531 of MRP. Immunoblotting of membrane vesicles from cells infected with the vector confirmed that encoded a fusion protein comprised of amino acids 1–280 from cMOAT/MRP2 and amino acids 281–1531 of MRP. Immunoblotting of membrane vesicles from cells infected with the vector confirmed that encoded a fusion protein comprised of amino acids 1–280 from cMOAT/MRP2 and amino acids 281–1531 of MRP. Immunoblotting of membrane vesicles from cells infected with the vector confirmed that encoded a fusion protein comprised of amino acids 1–280 from cMOAT/MRP2 and amino acids 281–1531 of MRP. Immunoblotting of membrane vesicles from cells infected with the vector confirmed that encoded a fusion protein comprised of amino acids 1–280 from cMOAT/MRP2 and amino acids 281–1531 of MRP.

---

**FIG. 2. Expression and LTC₄ transport activity of MRP fragments lacking MSD1 or the NH₂-terminal TM helix.** A, membrane proteins (1 μg) from Sf21 cells expressing MRP or NH₂-terminal truncations of the protein were resolved by SDS-PAGE through a 5–15% gradient gel (MRP, MRP₁–281, MRP₂₂₉–₁₅₃₁, MRP₂₂₉–₁₅₃₁), or a standard 7.5% gel (MRP₂₂₉–₁₅₃₁ transferred to Immobilon-P membrane and detected with MRP-specific mAb QCRL-1. The sizes of protein standards are indicated in kilodaltons. B, membrane vesicles containing either MRP₂₂₉–₁₅₃₁ or MRP₂₂₉–₁₅₃₁ were assayed for ATP-dependent LTC₄ transport activity at 23 °C for up to 2 min in transport buffer containing [³H]LTC₄ (50 nM), as described under “Materials and Methods.” Time dependence of uptake is shown for MRP₁–281 in the presence of AMP (○) or ATP (□) and for MRP₂₂₉–₁₅₃₁ in the presence of AMP (●) or ATP (▲). LTC₄ uptake by vesicles prepared from cells infected with a control vector was detectable when AMP was used in place of ATP. We also examined membrane vesicles from cells co-infected with MRP₁–281 and MRP₂₂₉–₁₅₃₁ (Fig. 4A). The initial rates of uptake (approximately 25 pmol min⁻¹ mg⁻¹) were 20–30% of those obtained with membrane vesicles from cells producing similar levels of intact MRP that were prepared contemporaneously (data not shown). No change in the level of LTC₄ uptake relative to vesicles from control cells was detectable when AMP was used in place of ATP. We also examined membrane vesicles from cells co-infected with MRP₁–281 and MRP₂₂₉–₁₅₃₁. Despite the fact that these fragments overlap by 52 amino acids in the region predicted to connect MSD1 to MSD2, vesicles containing them displayed initial rates of ATP-dependent uptake of LTC₄ similar to the rate obtained with vesicles containing MRP₁–281 and MRP₂₂₉–₁₅₃₁ (25–30 pmol mg⁻¹ min⁻¹).

To further characterize the LTC₄ uptake by vesicles from cells co-expressing MRP₁–281 and MRP₂₂₉–₁₅₃₁, rates of uptake were measured at several LTC₄ concentrations (10–1000 nM). The apparent $K_m$ and $V_{max}$ values for LTC₄ were $136 \pm 42$ nM and $102 \pm 20$ pmol min⁻¹ mg⁻¹, respectively (Fig. 4B). This $K_m$ value is similar to those reported previously for vesicles from MRP-transfected HeLa T14 cells (105 nM) (8) and HeLa T5 cells (97 nM) (10), but slightly higher than those obtained with vesicles from Sf21 cells expressing either intact MRP (67 nM) or co-expressing both half-molecules (56 nM) (23). Comparable analyses were also performed to determine an apparent $K_m$ for ATP of 136 ± 24 μM (Fig. 4C), a value similar to that obtained previously with intact protein (100 μM) (23).

**FIG. 3. Co-expression of MRP₁–281 with either MRP₂₂₉–₁₅₃₁ or MRP₂₉₉–₁₅₃₁.** Membrane proteins (1 μg) from cells expressing full length MRP or co-expressing MRP₁–281 with either MRP₂₂₉–₁₅₃₁ or MRP₂₉₉–₁₅₃₁ were separated by SDS-PAGE through a 5–15% gradient gel and immunoblotted, as described under “Materials and Methods.” Left, expression of MRP₁–281 was detected with mAb MRPr1. Right, expression of MRP₂₂₉–₁₅₃₁ and MRP₂₉₉–₁₅₃₁ was detected with mAb QCRL-1. The sizes of protein standards are indicated in kilodaltons. The minor species of approximately 67 kDa visible in some lanes is a COOH-proximal proteolysis fragment generated by cleavage at a hypersensitive site in the vicinity of amino acids 920–930 (29).
The presence of AMP or ATP is indicated by (\( f \)). Uptake by vesicles from cells infected with control vector in the presence of AMP or ATP was assessed using a MULTI program. The broken line represents the ATP dependence of LTC4 uptake by vesicles containing co-expressed MRP1–281 and MRP281–1531 in the presence of AMP or ATP (Fig. 6A). The initial rate of LTC4 uptake was measured at various concentrations of ATP (10–1000 \( \mu \)M). Kinetic parameters (\( V_{\text{max}} \) and \( K_m \)) were determined by iterative nonlinear least-square methods using a MULTI program. The line represents a computer best fit to the data. The initial rate of ATP-dependent uptake of \(^{3}H\)LTC4 by membrane vesicles containing MRP1–281 and MRP281–1531 was measured at various LTC4 concentrations (10–1000 nM). Kinetic parameters (\( V_{\text{max}} \) and \( K_m \)) were determined by iterative nonlinear least-square methods using a MULTI program. The line represents a computer best fit to the data. The initial rate of ATP-dependent uptake of \(^{3}H\)LTC4 was measured at various concentrations of ATP (10 \( \mu \)M to 4 \( \mu \)M) in the presence of 50 nM \(^{3}H\)LTC4. Kinetic parameters were determined as described in Fig. 6. Data points are means of triplicate determinations in a typical experiment. Where not visible, standard error bars are within the limits of the symbol.

Fig. 4. Kinetics of ATP-dependent LTC4 uptake by membrane vesicles from Sf21 cells co-expressing MRP1–281 and MRP281–1531. A. LTC4 uptake was assayed as described under “Materials and Methods.” Uptake by vesicles from cells infected with control vector in the presence of AMP or ATP is indicated by (■) and (○), respectively. The broken line represents the ATP dependence of LTC4 uptake by vesicles containing co-expressed MRP1–281 and MRP281–1531 in the presence of AMP or ATP from that obtained in the presence of ATP. B. The initial rate of ATP-dependent \(^{3}H\)LTC4 uptake by membrane vesicles containing MRP1–281 and MRP281–1531 was measured at various LTC4 concentrations (10–1000 nM). Kinetic parameters (\( V_{\text{max}} \) and \( K_m \)) were determined by iterative nonlinear least-square methods using a MULTI program. The line represents a computer best fit to the data. C. The initial rate of ATP-dependent uptake of \(^{3}H\)LTC4 was measured at various concentrations of ATP (10 \( \mu \)M to 4 \( \mu \)M) in the presence of 50 nM \(^{3}H\)LTC4. Kinetic parameters were determined as in B. Data points are means of triplicate determinations in a typical experiment. Where not visible, standard error bars are within the limits of the symbol.

The mutant protein was expressed efficiently in Sf21 cells and accumulated to levels which are approximately one-half of those obtained with full-length MRP (Fig. 6A). However, ATP dependent LTC4 transport in membrane vesicles containing the mutant protein did not differ significantly from the basal activity detected in membranes from control cells (data not shown).

**DISCUSSION**

Most ABC transporters are comprised of two polytopic MSDs and two NBDs. In proteins such as the P-gps, CFTR, and STE6, all four domains are contained within a single polypeptide, but this is frequently not the case (2). For example, bacterial transporters such as the maltose and histidine permeases consist of four polypeptides each encoding a single domain (31, 32), while mammalian TAP1 and TAP2, which are involved in antigen presentation, each contain one MSD and one NBD, and heterodimerize to form a functional complex (33, 34). ABC transporters, such as STE6, CFTR, and P-gp, are thought to have evolved by duplication or fusion of genes encoding half-translators with domain organizations similar to those of TAP1 and TAP2. This suggestion is supported by studies in which co-expression of half molecules of transporters such as STE6, CFTR, and P-gp has been shown to reconstitute some level of function (35–37).

MRP was the first example of an ABC transporter with a predicted topology inconsistent with the typical four-domain structure. More recently identified ABC transporters with a NH2-terminal MSD include MCOAT/MPR2, the yeast cadmium resistance factor, and the sulfonylurea receptors, as well as several less well characterized MRP-related proteins (16, 18, 20). Comparisons of protein primary structure and gene organization suggest that the MRP-related proteins shared a common, four-domain ancestor with CFTR (21). It is also apparent from such comparisons that the NH2-terminal MSDs of the MRP-related proteins are poorly conserved relative to the other four domains in these proteins (15, 22). These observations raise the question of whether or not the additional MSD is essential for transport activity, or for some other function of the protein. Our results demonstrate that two truncated molecules (MRP1–858 and MRP281–1531) lacking MSD1 can be expressed in Sf21 cells as efficiently as the full-length protein. However, ATP-dependent transport of LTC4 by the mutant was less than 10% of that obtained with vesicles containing comparable amounts of intact protein.

If transporters such as MRP evolved by fusion of a common ancestral gene with one or more genes encoding other integral membrane proteins, the additional MSDs might be expected to be capable of folding independently and of being appropriately inserted in cell membranes. In support of this suggestion, we found that a polypeptide corresponding to amino acids 1–281 is efficiently produced and incorporated into membranes. In addition, when this fragment was co-expressed with four-domain residues, but the protein kinases involved have not been identified (24, 30). To investigate the functional requirement for the linker, we co-expressed two MRP fragments, comprising amino acids 1–858 (MRP1–858) and 932–1531 (MRP932–1531). This eliminated the most serine-rich segment of the linker between amino acids 859 and 931. Immunoblotting of membrane proteins from cells co-infected with MRP1–858 and MRP932–1531 confirmed that both fragments were efficiently expressed and accumulated to comparable levels (Fig. 6B). The initial rates of LTC4 accumulation by vesicles from the co-infected cells were approximately 35% of those obtained with vesicles from Sf21 insect cells producing full-length MRP (Fig. 7A). This initial rate is somewhat higher than we have achieved previously by co-infection with both half-molecules that contained the entire linker region. \( K_m \) and \( V_{\text{max}} \) values for LTC4 transport by MRP1–858/MPR932–1531 vesicles were determined to be 134 ± 18 nM and 218 ± 18 pmol min\(^{-1}\) mg\(^{-1}\), respectively (Fig. 7B). The \( K_m \) for ATP was estimated to be 27 ± 3 \( \mu \)M (Fig. 7C). This value was lower than obtained previously with vesicles from cells producing the full-length protein (100 \( \mu \)M) or co-infected with both half-molecules (44 \( \mu \)M) (23).

**Comparison of the primary structures of**

**Structure and Function of Multidrug Resistance Protein**

10737

**Comparison of the primary structures of**

**Structure and Function of Multidrug Resistance Protein**

10737
fragments lacking MSD1, it increased the ability to transport LTC₄ 5–6-fold. The kinetic parameters of the reconstituted transporter were also similar to those of the intact protein. These observations confirm the importance of MSD1 for transport activity. They also strongly suggest that MSD1 is not simply tethered to the rest of the molecule by the polypeptide backbone, but must be capable of forming stable interactions with other protein domains.

The ability to reconstitute transport activity by co-expressing the 1–281 fragment with a partially overlapping fragment extending from amino acid 229 to the COOH terminus, raised the possibility that the region of overlap in the predicted cytoplasmic linker between MSD1 and MSD2 may not be critical for function. However, elimination of the overlap by creating an internal deletion in MRP (MRP₃₋₂₂₈₋₂₈₀) also eliminated LTC₄ transport activity. This suggests either that the region between amino acid 228 and 280 may be involved in LTC₄ binding and/or transport, or that shortening of the loop by 53 amino acids may have altered the topological relationships between MSD1 and other, essential regions of the protein. To try to distinguish between these two possibilities, we co-expressed fragments extending from amino acid 1–227 and 281–1531. This combination of fragments failed to reconstitute LTC₄ transport (data not shown). However, suitable antibodies for determining the level of expression of the 1–227 fragment are currently not available and we cannot exclude the possibility that this fragment is not expressed efficiently.

In addition to their very similar substrate specificities, MRP and cMOAT/MRP2 also share considerable amino acid identity (16, 38). The corresponding NBDs of MRP and cMOAT/MRP2 are approximately 70% identical while both MSD2 and MSD3 share approximately 50% identity between the two proteins. In contrast, although the predicted topologies of MSD1 of MRP and cMOAT/MRP2 are similar, their amino acid identity is only approximately 25%. This could reflect independent acquisition of the additional MSD of each protein or a relative lack of functional constraints on the primary structure of the domain. Consequently, we determined if it was possible to exchange MSD1 of MRP for the comparable region of cMOAT/MRP2 without loss of function. Although the hybrid protein could be expressed at high levels in Sf21 cells, it had no detectable LTC₄ transport activity. Interestingly, the presence of MSD1 from cMOAT/MRP2 in the fusion protein, did not interfere with the restoration of transport activity when MSD1 from MRP was co-expressed with the cMOAT₁₋₂₈₀–MRP₂₈₁₋₁₅₃₁ hybrid. Given the very similar substrate specificities of the two proteins, these observations suggest that there may be a requirement for specific interactions between MSD1 and other domains of MRP, that cannot be fulfilled by MSD1 of cMOAT/MRP2.

MRP has the characteristics of a type IIIb polytopic protein (reviewed in Singer (39)). These proteins are typified by a short NH₂-terminal extracellular sequence without a cleavable signal peptide and an NH₂-terminal TM segment that is often followed by a region of relatively positive charge. In MRP, both Asn¹⁹ and Asn²³ are glycosylated and amino acids 1–37 have a net negative charge (15). Thus they are predicted to be on the
outside of the membrane while all five charged residues in the first predicted cytoplasmic loop of 21 amino acids are positively charged. In some type IIIb proteins, the NH2-terminal TM of the first predicted cytoplasmic loop of 21 amino acids are positively charged. In some type IIIb proteins, the NH2-terminal TM and the COOH-proximal MSD of ABC-proteins such as P-gp and CFTR is highly variable among superfamilies and is relatively poorly conserved among subfamily isoforms (41–43). It is also frequently post-translationally modified by phosphorylation of serine residues (24, 30, 44–46). In CFTR, this region forms the “R” domain which mediates CAMP dependent regulation of the chloride channel (45, 46). In contrast, phosphorylation status has no apparent effect on the drug transport activity of P-gp (47), although it may influence other functions of the protein (48). Before examining the possible functional implications of phosphorylation of the comparable region of MRP, we investigated whether the region is actually required for ATP-dependent transport. Because creation of a large internal deletion in the linker region could potentially alter interactions between the two NBDs and possibly other domains of the protein, we examined the possibility of reconstituting LTC4 transport by co-expressing fragments, 1–858 and 932–1531. This eliminated 74 amino acids that included the most serine rich and divergent portion of the linker. The co-expressed fragments actively transported LTC4 with a \( V_{\text{max}} \) that was not significantly different from that obtained previously for co-expressed fragments 1–932 and 932–1531 and a \( K_{\text{m}} \) that was approximately two fold greater. Thus this part of the linker is clearly not essential for transport and presumably is not necessary for interaction between the two halves of the protein. The only difference observed between the transport activity reconstituted from fragments 1–858 and 932–1531 and that of intact MRP was an approximate four fold decrease in the \( K_{\text{m}} \) for ATP. Whether this indicates that the linker region and/or modifications to it might influence the affinity or catalytic activity of the NBDS remains to be determined.

The results of the present study demonstrate the importance of MSD1 and the first TM segment of MRP for active transport of LTC4. The co-expression experiments also suggest that this domain can form stable, functional interactions with other regions of the protein. Studies are in progress to localize the sites of interaction and to determine how this additional domain is integrated into the evolutionarily conserved four domain structure typical of the majority of ABC transporters.

Acknowledgment—We thank Dr. R. Schepers (Free University Hospital, Amsterdam, The Netherlands) for providing MRP-specific monoclonal antibodies MRPr1 and MRPm6, David Hipfner for helpful discussion and advice and Dr. J. Gerlach and Leah Young for the gift of dsDNA from HepG2 cells. The excellent technical assistance from Monika Vasa is appreciated.

REFERENCES

1. Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. V., and Deeleuy, R. G. (1992) Science 258, 1650–1654
2. Higgins, C. F. (1992) Annu. Rev. Cell Biol., 8, 67–113
3. Grant, C. E., Valdimarsson, G., Hipfner, D. R., Almquist, K. C., Cole, S. P. C., and Deeleuy, R. G. (1993) Cancer Res. 53, 357–361
4. Zaman, G. J. R., Flens, M. J., van Leusden, M. R., De Haas, M., Mulder, H. S., Lankelevie, J., Pineso, H. M., Scheper, R. J., Baas, F., Broxterman, H. J., and Borst, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1649–1652
5. Mulder, M., Meijer, C., Zaman, G. J. R., Borst, P., Scheper, R. J., Mulder, N. H., de Vries, E. G. E., and Janssen, P. L. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 13033–13037
6. Leier, I., Jedlitschky, G., Buchholz, U., Cole, S. P. C., Deeleuy, R. G., and Keppler, D. (1996) Cancer Res. 56, 9902–9910
7. Lowe, D. W., Almquist, K. C., Deeleuy, R. G., and Cole, S. P. C. (1996) Eur. J. Cancer 32A, 945–957
8. Lowe, D. W., Almquist, K. C., Deeleuy, R. G., and Cole, S. P. C. (1996) J. Biol. Chem. 271, 9675–9682
9. Muller, M., Meier, C., Zaman, G. J. R., Borst, P., Scheper, R. J., Mulder, N. H., de Vries, E. G. E., and Janssen, P. L. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 13033–13037
10. Leier, I., Jedlitschky, G., Buchholz, U., Cole, S. P. C., Deeleuy, R. G., and Keppler, D. (1996) Cancer Res. 56, 988–999
and Deeley, R. G. (1996) Mol. Pharmacol. 49, 962–971
15. Hipfner, D. R., Almquist, K. C., Leslie, E. M., Gerlach, J. H., Grant, C. E., Deeley, R. G., and Cole, S. P. C. (1997) J. Biol. Chem. 272, 23625–23630
16. Paulusma, C. C., Bosma, P. J., Zaman, G. J. R., Bakker, C. T. M., Otter, M., Scheffer, G. L., Scheper, R. J., Borst, P., and Oude Elferink, R. P. J. (1996) Science 271, 1126–1128
17. van Kuijck, M. A., van Aubel, R. A. M. H., Busch, A. E., Lang, F., Russel, F. G. M., Bindels, R. J. M., van Os, C. H., and Deen, P. M. T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5401–5406
18. Szczypka, M. S., Wemmie, J. A., Moye-Rowley, W. S., and Thiele, D. J. (1994) J. Biol. Chem. 269, 22853–22857
19. Katzmann, E. J., Hallstrom, T. C., Voet, M., Wysock, W., Golin, J., Volckaert, G., and Moye-Rowley, W. S. (1995) Mol. Cell. Biol. 15, 6875–6883
20. Aguilar-Bryan, L., Nichols, C. G., Wechsler, S. W., Clement, J. P., Boyd, A. E., Gonzalez, G., Herrera-Sosa, H., Nguy, K., Bryan, J., and Nelson, D. A. (1995) Science 268, 423–426
21. Grant, C. E., Kurz, E. U., Cole, S. P. C., and Deeley, R. G. (1997) Genomics 45, 368–378
22. Bakos, V., Hegedus, T., Hollis, Z., Welker, E., Tuszynsky, G. E., Zaman, G. J. R., Flens, M. J., Várádi, A., and Sarkadi, B. (1996) J. Biol. Chem. 271, 23623–23630
23. Gao, M., Loe, D. W., Grant, C. E., Cole, S. P. C., and Deeley, R. G. (1996) J. Biol. Chem. 271, 27782–27787
24. Almquist, K. C., Loe, D. W., Hipfner, D. R., Mackie, J. E., Cole, S. P. C., and Deeley, R. G. (1995) Cancer Res. 55, 102–110
25. Hipfner, D. R., Gauldie, S. D., Deeley, R. G., and Cole, S. P. C. (1994) Cancer Res. 54, 5795–5799
26. Flens, M. J., Izquierdo, M. A., Scheffer, G. L., Fritz, J. M., Meijer, C. J. L. M., Scheper, R. J., and Zaman, G. J. R. (1994) Cancer Res. 54, 4557–4563
27. Leier, I., Jedlitschky, G., Buchholz, U., and Keppler, D. (1994) Eur. J. Biochem. 220, 599–606
28. Yamaoka, K., Tanigawara, Y., Nakagawa, T., and Uno, T. (1981) J. Pharmacobio-Dyn. 4, 879–885
29. Hipfner, D. R., Almquist, K. C., Stride, B. D., Deeley, R. G., and Cole, S. P. C. (1996) Cancer Res. 56, 3307–3314
30. Ma, L., Krishnamachary, N., and Center, M. S. (1995) Biochemistry 34, 3338–3343
31. Bishop, L., Aqbayani, R. J., Ambudkar, S. V., Maloney, P. C., and Ames, G. F. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6953–6957
32. Panagiotidis, C. H., Reyes, M., Sievertsen, A., Boos, W., and Shuman, H. A. (1993) J. Biol. Chem. 268, 23685–23696
33. Androlewicz, M. J., Ortmann, B., van Endert, P. M., Spies, T., and Cresswell, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12716–12720
34. Russ, G., Esquivel, F., Yewdell, J. W., Cresswell, P., Spies, T., and Bennink, J. R. (1995) J. Biol. Chem. 270, 21312–21318
35. Berkower, G., and Michaelis, S. (1991) EMBO J. 10, 3777–3785
36. Ostedgaard, L. S., Rich, D. P., DeLerg, L. G., and Welsh, M. J. (1997) Biochemistry 36, 1287–1294
37. Loo, T. W., and Clarke, D. M. (1994) J. Biol. Chem. 269, 7750–7755
38. Mayer, R., Kartenbeck, J., Buchler, M., Jedlitschky, G., Leier, I., and Keppler, D. (1995) J. Cell Biol. 131, 137–150
39. Singers, S. J. (1990) Annu. Rev. Cell Biol. 6, 247–296
40. Dalbey, R. E., and Wickner, W. (1987) Science 235, 783–787
41. Ueda, K., Cardarelli, C., Gottesman, M. M., and Pastan, I. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3004–3008
42. Chin, J. E., Soffer, R., Noonan, K. E., Choi, K. Y., and Roninson, I. B. (1989) Mol. Cell. Biol. 9, 3868–3880
43. Van der Bliek, A. M., Baas, F., Ten Houte de Lange, T., Konimam, P. M., Van der Velde-Koerte, T., and Borst, P. (1987) EMBO J. 6, 3325–3331
44. Chambers, T. C., Pohl, J., Glass, D. B., and Kuo, J. F. (1994) Biochem. J. 299, 309–315
45. Cheng, S. H., Rich, D. P., Marshall, J., Gregory, R. J., Welsh, M. J., and Smith, A. E. (1991) Cell 66, 1027–1036
46. Chang, X.-B., Tabcharani, J. A., Hou, Y.-X., Jensen, T. J., Kartner, N., Alon, N., Haraunen, J. W., and Riordan, J. R. (1995) J. Biol. Chem. 270, 11304–11311
47. Germann, U. A., Chambers, T. C., Ambudkar, S. V., Pastan, I., and Gottesman, M. M. (1995) J. Bioenerg. Biomembr. 27, 53–61
48. Szabo, K., Bakos, E., Welker, E., Muller, M., Goodfellow, H. R., Higgins, C. F., Varadi, A., and Sarkadi, B. (1997) J. Biol. Chem. 272, 23165–23171