APPLICATION OF CYSTEINE SCANNING MUTAGENESIS TO THE MULTIDRUG RESISTANCE PROTEIN (MRP)1

by

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Abstract

Multidrug resistance protein (MRP)1, a member of the ABCC branch of the ATP-binding cassette (ABC) superfamily of transporters, can confer resistance to a broad spectrum of chemotherapeutic agents. In addition to the core functional unit of ABC transporters that consists of two membrane spanning domains (MSD) and two nucleotide binding domains (NBD), MRP1 contains a third MSD (MSD0) resulting in the following arrangement: NH$_2$-MSD-(MSD-NBD)$_2$. In lieu of high-resolution structural information for MRP1, cysteine scanning mutagenesis (CSM) was applied to MRP1 and involves the development of a functional template devoid of cysteines into which paired cysteines can be introduced. Previous attempts to create a functional, cys-less template of MRP1 demonstrated that cysteines in MSD0 were structurally and functionally important (1;2). However, given that MRP1 lacking MSD0 remains functional, a partially functional, cys-less MRP1 lacking this domain has been expressed in yeast (3-5). Given these results, with the ultimate goal of applying CSM to MRP1 in its entirety, we investigated the endogenous cysteines within MSD0 and co-expressed MRP1 half-molecules and validated these potential CSM templates by transport and ATP binding/hydrolysis assays. Mutation of cysteines within the core of MRP1 had detrimental effects on MRP1 transport activity and further mutation of cysteines by domain revealed that wild-type activity was retained in an MSD0-less MRP1 dual lacking cysteines in both NBDs. This construct was used for introduction of cysteines on juxtaposed faces of the NBD1:NBD2 heterodimer at positions 775 and 1329; comparable residues in the related Cystic Fibrosis Transmembrane Regulator (CFTR/ABCC7) have been suggested to be evolutionarily coupled and joined by a hydrogen bond, maintained in structures of related proteins (6). Unfortunately, functional assays revealed that introduction of cysteines at these positions greatly reduced transport activity of MRP1 and diminished trapping of nucleotide at both NBDs. Finally, alanine substitution of the seven cysteines in MSD0 was not without effect.
and cellular trafficking assays, co-expression studies and SDS-PAGE analysis suggested an altered conformation of this domain. In addition, a disulfide pair of Cys7 and Cys32 was suggested by these experiments in MSD0 and further supported by examination of these mutants in full-length MRP1.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ABCC</td>
<td>subfamily C of the ABC superfamily</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>Adenosine 5′-(β,γ-imido)triphosphate</td>
</tr>
<tr>
<td>ATP-γ-S</td>
<td>adenosine 5′-O-(thiotriphosphate)</td>
</tr>
<tr>
<td>β-gus</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast Cancer Resistance Protein</td>
</tr>
<tr>
<td>BmrA</td>
<td><em>Bacillus</em> Multidrug Resistance ATP</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan Fluorescent Protein</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
</tr>
<tr>
<td>CL</td>
<td>cytoplasmic loop</td>
</tr>
<tr>
<td>CSM</td>
<td>cysteine scanning mutagenesis</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>E217βG</td>
<td>17β-estradiol-17β-(d-glucuronide)</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione disulfide</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HisP</td>
<td>NBD of the histidine transporter from <em>Salmonella typhimurium</em></td>
</tr>
<tr>
<td>HlyB</td>
<td>Haemolysin B</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>LmrA</td>
<td>Multidrug Resistance Protein of <em>Lactococcus lactis</em></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LTC₄</td>
<td>leukotriene C₄</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrug resistance</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug Resistance Protein</td>
</tr>
<tr>
<td>MSD</td>
<td>membrane spanning domain</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NBD</td>
<td>nucleotide binding domain</td>
</tr>
<tr>
<td>NBS</td>
<td>nucleotide binding site</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>N-glycosylation</td>
<td>N-linked glycosylation</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NNAL</td>
<td>4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>SAC</td>
<td>substrate assisted catalysis</td>
</tr>
<tr>
<td>SDR</td>
<td>structurally diverse region</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SUR</td>
<td>sulfonylurea receptor</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with Antigen Processing</td>
</tr>
<tr>
<td>TB</td>
<td>transport buffer</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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Chapter 1

INTRODUCTION

1.1 Multidrug resistance

1.1.1 Emergence of Multidrug Resistance

Multidrug resistance (MDR) is a complex phenotype that describes the broad resistance to multiple structurally and functionally unrelated drugs (7). MDR is the focus of extensive research because of its impediment of the successful treatment of malignant and infectious disease and as such, the ability to predict and circumvent MDR would greatly improve current drug-directed therapies (7;8). Indeed, a substantial proportion of all human cancers develop MDR and this often results in treatment failure. MDR can be intrinsic or acquired, although the mechanism by which this occurs is not fully understood. In the cancer setting, classical MDR, as first described mediated by P-glycoprotein (9), arises following exposure to a specific compound and is characterized by cross-resistance to four classes of natural product drugs: the anthracyclines, taxanes, epipodophylotoxins, and the Vinca alkaloids. In clinical settings, MDR is a multi-factorial phenotype likely resulting from a combination of cellular modifications involving an integrated network of several pathways, altogether resulting in cancer cell survival (10). While various biochemical mechanisms and networks contribute to MDR, perhaps the most widely implicated and studied is that resulting from altered membrane transport, in particular by members of the ATP-binding cassette (ABC) superfamily of transporters (11).

1.2 The ATP-binding Cassette Superfamily

1.2.1 Overview of ABC Transporters

ABC transporters form the largest and most widely expressed superfamily of transmembrane (TM) proteins in the human genome. Genes are classified into this superfamily based on presence of three motifs in the ATP-binding domains: the Walker A and Walker B motifs (12), found in all ATP-binding proteins, and the Signature ‘C’ motif, unique to proteins in this family
The ABC transporter family consists of ubiquitous TM proteins, which couple ATP catalysis to the transport of substrates across biological membranes (11). Energy for transport is derived from several steps in ATP catalysis including binding, hydrolysis, and release of ADP·Pi, as discussed in (15). ABC transporters are vital to any living organism due to their central role in the translocation of a plethora of substrates that range from simple ions to complex chemotherapeutic drugs and their conjugates (11). The human ABC transporter superfamily consists of 49 genes, subdivided into 7 branches, denoted A through G based on primary structure similarity and phylogeny (13;16). Since the discovery of the first eukaryotic ABC transporter, the archetypal P-glycoprotein (P-gp), (17), this family has received increased attention because of its involvement in clinical treatments and physiological processes, along with implications in genetic disease. In fact, no fewer than 18 members of this family have been implicated in various human diseases (16). Examples include Tangier disease (ABCA1) (18-20), Stargardt disease (ABCR/ABCA4) (21;22), Dubin-Johnson syndrome (MRP2/ABCC2) (23), pseudoaxanthoma elasticum (MRP6/ABCC6)(24-27), cystic fibrosis (CFTR/ABCC7) (28;29) and congenital hyperinsulinemia (SUR1/ABCC8)(30). In addition, several members of the ABC superfamily have been shown to confer drug resistance such as P-gp (ABCB1) (17), the breast cancer resistance protein (BCRP/ABCG2) (31;32) and the multidrug resistance proteins (MRP) in the ABCC subfamily (reviewed in (33)).

1.3 Multidrug Resistance Proteins

1.3.1 MRP1 Discovery

MRP1 was first cloned in 1992 (34), initially identified based on its elevated expression in the multidrug resistant small cell lung cancer line H69AR. This cell line had been selected from the parental H69 cell line by repeated exposure to the anthracycline, doxorubicin. The H69AR cell line displayed cross-resistance to a wide range of structurally and functionally unrelated drugs, a phenotype that was originally thought to be attributable to over-expression of the
distantly-related MDR protein, P-gp. However, increased P-gp expression was not observed in these cells and the MDR phenotype was consistently unaltered by P-gp inhibitors. Subsequently, the cDNA for MRP1 was cloned via differential hybridization and its expression was found to be increased 100- to 200-fold in the selected H69AR cells versus the parental H69 cell line (34).

1.3.2 Physiological Role and Clinical Significance of MRP1:

MRP1 is ubiquitously expressed with elevated levels in the lung, testes, kidney and blood-organ barriers (34-37). While a role for MRP1 in normal physiology has yet to be clearly defined, its widespread tissue distribution is indicative of an important physiological role for this protein. Studies in MRP1 null mice have revealed some possibilities (38;39) and in general, MRP1 has been proposed to play a protective role to the cells in which it is expressed (36). This is thought to be achieved through extrusion of the wide range of cytotoxic compounds MRP1 transports (34;40-42) and/or through its involvement in redox homeostasis via its ability to transport glutathione (GSH) (43) and its oxidized form (GSSG) (44). Lastly, a possible role in the maintenance of the barrier function at blood-organ barriers is indicated by the elevated expression levels of MRP1 at these sites (34;35;45-47).

Studies with MRP1 null mice have revealed some expected and unexpected physiological roles for MRP1. These mice are viable, healthy and fertile, and display no physical or histological abnormalities (38;39). That MRP1-knockout mice showed an increased sensitivity to inflammatory stimuli attributed to the decreased cellular excretion of leukotriene C₄ (LTC₄) (39) lent additional support of this glutathione conjugate as the major physiological substrate of MRP1 (48;49). In addition, mrp1⁻/⁻ mice displayed an increased sensitivity to the epipodophyllotoxin, etoposide (38;39), which is often used in the chemotherapeutic treatment of cancer. Sites which were particularly sensitive to etoposide included the testes and placenta, pointing to an important role for MRP1 in the maintenance of the barrier function of these sanctuary sites (38;39;47). Consistent with a protective role for MRP1, the ability of MRP1 to transport the conjugate of
aflatoxin B1, a highly mutagenic and carcinogenic mycotoxin, originally led researchers to propose that MRP1 may protect cells from chemical carcinogenesis resulting from conjugation of aflatoxin B1-8,9-epoxide (50). However, studies with MRP1-null mice identified no protective role for MRP1 against the carcinogenic properties of this compound and the expression of functionally redundant ABC proteins was suggested to offer protection to these animals (51). Furthermore, in support of its role in tissue defense, MRP1 has also been demonstrated to transport a glucuronide-conjugate of the nicotine-derived tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (52). In addition to the protective roles proposed for MRP1 in normal physiology, in a pathological setting, the protection of normal cells from the cytotoxic effects of antineoplastic agents can be inferred from MRP1s ability to confer MDR to cancer cells (34;40;41).

The ability of MRP1 to transport both GSH and GSSG has led researchers to propose involvement of MRP1 in the maintenance of the redox state of the cell (43;44). This notion has been supported by both MRP1 knockout mice and cell lines that overexpress this transporter. In \textit{mrp1} \textsuperscript{-/-} mice, increased GSH levels were found in some tissues where MRP1 is normally expressed at high levels, but remained unaltered in tissues where MRP1 expression is not observed in wild-type animals (38). Interestingly, transport of GSSG during periods of oxidative stress has been attributed to MRP1 (53) and expression levels have been shown to increase following exposure to oxidative stress inducing agents (54). A complex relationship exists between MRP1 and GSH/GSSG, the full scope of which has not yet been fully elucidated. Unfortunately, an in-depth review is outside of the scope of this paper but can be found in (33;55;56) and most recently, (57).

Thus far, no specific genetic disorder has been linked to MRP1. Thus, the main clinical significance of MRP1 arises from its role in conferring MDR to cancer cells. Elevated levels of MRP1 protein or mRNA have been detected in almost every tumour type examined (35;58).
However, only in some of the above cases was a correlation drawn between MRP1 overexpression and negative clinical outcome (for example, (59)).

1.3.3 Substrate Specificity and Transport Function

The previously characterized MDR phenotype of the H69AR cells from which MRP1 was originally cloned provided a basis from which a large number of MRP1 drug and heavy metal oxyanion substrates were initially discovered; many of these have now been confirmed using MRP1-transfected cells (40-42;60). Furthermore, \textit{in vitro} studies utilizing inside-out membrane vesicles have also identified many MRP1 substrates, either through their ability to be transported directly or their ability to inhibit the transport of established substrates (43;48;50;61-63). Thus, MRP1 has been demonstrated to confer resistance to natural product drugs such as the anthracyclines, epipodophylotoxins and \textit{Vinca} alkaloids (41), as well as heavy metal oxyanions of antimony and arsenic (40). MRP1 overexpression has also been correlated with resistance to the antifolate, methotrexate, and direct transport was subsequently demonstrated (64). Further to its role in resistance, MRP1 is a primary active transporter of glutathione-, glucuronate-, or sulfate-conjugated drugs, which are often the products of phase II cellular detoxification of hydrophobic xeno- and endobiotics. Examples of these conjugates include the proinflammatory mediator, LTC$_4$ (48;61), the cholestatic hormone, estradiol-17β-glucoronide (E$_2$17βG) (63) and estrone sulfate (65).

1.3.3.1 MRP1 and GSH

Initial attempts to directly demonstrate MRP1 transport of unmodified compounds to which it confers resistance were unsuccessful, leading investigators to propose a mechanism of co-transport (43;62). GSH was originally proposed to be the co-transported molecule based upon the observation that cellular depletion of GSH greatly reduced drug efflux (66-69). Since that time, the complex relationship of MRP1 with GSH has slowly been revealed, but remains incompletely understood (reviewed in (55) and (57)).
GSH itself is a poor substrate for MRP1 with a reported Km of >1 mM (70;71). Its oxidized form, GSSG, however, is a much better substrate and is transported with a higher \( V_{\text{max}} \) and a Km of \( \sim 100 \, \mu \text{M} \) (44). Both reciprocal and non-reciprocal stimulation of transport has been observed for GSH and MRP drug substrates. For example, a mechanism of co-transport with GSH has been shown for substrates including vincristine such that transport of both GSH and the second MRP1 substrate is increased (70;72). In contrast, GSH is able to stimulate transport of MRP1 drug substrates in the absence of its own efflux (52;65). Interestingly, an inverse relationship also exists for the bioflavonoids and phenylalkamines (e.g. Verapamil) that modulate transport of organic anions by MRP1, such that GSH transport is enhanced without concomitant transport of these compounds (73-75). Interestingly, GSH-dependence has also been observed for the transport of many glucuronide- and sulfate-conjugates, and has even been demonstrated for a glutathione conjugate (52;65;76;77). Despite the above knowledge, the relationship between MRP1 and GSH remains complex and further work is needed in order to elucidate the precise nature of the interactions between this transporter and its diverse substrates.

1.3.4 Overview of the MRP/ABCC Subfamily

MRP1 is a member of the MRP/ABCC branch of the ABC transporter superfamily that to date includes 13 members (33;57). This sub-family comprises three classes that include the multidrug resistance proteins (MRP) 1-9, the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR/ABCC7) and the sulfonylurea receptors, SUR1A/ABCC8 and SUR2/ABCC9. Most of the MRP members of the ABCC family have been shown to confer the MDR phenotype to cells in which they are expressed (33). The widespread tissue distribution, specific subcellular localization and redundancy of the substrate profiles within the MRP subfamily are indicative of important physiological protective roles for these proteins. It should be noted that while the substrate profiles of the MRP members overlap, none are identical (33;78). CFTR is functionally different from the rest of the ABCC subfamily and indeed, the entire superfamily of ABC
transporters in that it operates as an ion channel for the translocation of chloride, for which ATP binding and hydrolysis has been shown to play a role in gating of the protein (79;80). The sulfonylurea proteins also differ from the other members of the ABCC branch and no direct transport function has been attributed to either of these proteins. Instead, the SUR proteins form obligate hetero-octamers with the pore-forming tetramer of the K⁺ channel subunit, Kir6.2, and control membrane potential through regulation of the passage of potassium ions through the resultant membrane pores (81-84). In this manner, the SUR/Kir6.2 hetero-octamers play a key physiological role by controlling insulin release from pancreatic β-cells (85).

1.4 Structure of ABC Transporters

1.4.1 Organization of ABC-transporter Domains

The minimal functional unit of ABC transporters requires a combination of two membrane-spanning domains (MSD) and two nucleotide-binding domains (NBD) (11). Interestingly, these domains do not have to be covalently linked for function and many ABC transporters are formed by the association of separately encoded domains. For example, many prokaryotic ABC transporters such as the E. coli ABC transporter responsible for maltose import, malEFGK (86), are referred to commonly as complexes, reflective of the fact that they are composed of separate polypeptides that contain the functional domains. In eukaryotic ABC transporters, the four domains are typically encoded either as one or two polypeptides. In the latter case, each polypeptide is typically composed of one MSD and one NBD, as is the case for mammalian transporters TAP1/2 (ABCB2/ABCB3) and members of the ABCG subfamily, often described as ‘half transporters’ (87-90). The separation of the domains of ABC transporters into separate polypeptides has been mimicked experimentally and in this manner, P-gp(ABCB1), MRP1(ABCC1) and CFTR(ABCC7) that are encoded in a single polypeptide, have all been successfully expressed as ‘half-transporters’ (91-93). In each of these cases, the co-expressed half molecules have been demonstrated to form functional transporters in the membrane through non-
covalent associations not observed when either half is individually expressed. Thus, regardless of the number of polypeptides in which the domains of ABC transporters are encoded, the association of all four domains is a strict functional requirement.

The four domains of ABC transporters are typically arranged in a tandem repeat such that the MSD precedes the cytoplasmic NBD (NH$_2$-MSD-NBD) (Fig. 1-1) (11). This NH$_2$ to COOH-terminal arrangement of the MSDs and NBDs is maintained in half transporters, both prokaryotic and eukaryotic. However, some half-transporters have an inverse arrangement of the domains such that the amino-terminal NBD is followed by the MSD (i.e. NH$_2$-NBD-MSD) (31). Further deviation from the canonical domain organization is displayed by select members of the ABCC subfamily and homologous proteins in lower species that contain an additional MSD, located NH$_2$-terminal to the standard core domains of the ABC transporters (Fig. 1-1) (33).

**1.4.2 Arrangement of ABC-transporter Domains within the ABCC Subfamily**

Members of the ‘C’ branch of ABC transporters consist of a combination of two or three MSDs, the number of which characterizes the short and long members of this subfamily and two NBDs (33). The ‘long’ members of the ABCC subfamily have an additional MSD located NH$_2$-terminal to the core structure of ABC transporters and the organization of the domains in these proteins is NH$_2$-MSD-(MSD-NBD)$_2$ (Fig. 1-1). The ‘long’ members of the ABCC subfamily include MRPs 1, 2, 3, 6 and 7, as well as both SUR1 and SUR2 (26;94-101). The remainder of ABCC proteins are often referred to collectively as the ‘short’ members of this subfamily since they conform to (MSD-NBD)$_2$ (33) (Fig. 1-1). CFTR differs from the rest of the ABCC subfamily and ABC transporters in general in that it contains an additional protein kinase-dependent regulatory domain, located intracellularly between NBD1 and MSD2 (28).

**1.4.3 High-Resolution Structures of Complete ABC Transporters**

High-resolution structures of several bacterial transporters have been described including those for BtuCD, MsbA, the maltose transport complex and *Sav1866* (102-106). BtuCD, the
Figure 1-1. Topology of ABC proteins.  

A, The figure illustrates the typical 6 TM helices located in each of the two MSDs typical of most ABC transporters, with the exception of some members of the MRP/ABCC branch of this superfamily. In addition, the typical NH₂-MSD-NBD tandem repeat is illustrated and relative locations of conserved motifs in the NBDs identified.  

B, The topology model presented in the lower panel illustrates the location and 5-TM helix model for the additional MSD observed for members of the ABCC subfamily including MRPs 1, 2, 3, 6 and 7, and both SUR proteins, SUR1 and SUR2.
Figure 1-1
bacterial transporter responsible for vitamin B$_{12}$ translocation, was the first full-transporter for which a high-resolution structure was determined (102). However, it is difficult to extrapolate this data directly to other members of the superfamily since BtuCD has a greater number of TM $\alpha$-helices in each of its two MSDs than the typical transporter. Despite this difference, comparison of the overall shape of BtuCD to an 8 Å structure of P-gp revealed that the overall shape of these transporters is very similar in that there is extensive contact between the halves and the long axes of each half line up when placed side-by-side (107).

The next full transporter to be structurally characterized was MsbA from *Escherichia coli* (103). However, this alpha-carbon trace did not appear to represent a physiological state of the protein based on the knowledge of the nucleotide binding domains at that time (for discussion see (108)). In relatively quick succession, the same group proceeded to crystallize MsbA from two other species, *Vibrio cholera* (104) and *Salmonella typhimurium* (109), the latter in complex with both nucleotide and substrate. In addition to the problems associated with the relative orientation of the NBDs in these structures, a splayed ‘V’ arrangement of the MSDs within the membrane was observed that did not agree with the previously determined low-resolution eukaryotic structures; in the MsbA structures, the MSDs appear closed at the extracellular face, while low-resolution structures of MRP1 and P-gp appear to open at this location. However, when interpreting and comparing structural data, it is important to consider the limitation of this approach in that crystal structures represent a single conformation in the transport cycle of the protein and does not preclude others. Most recently, a complete high-resolution structure for the ABC transporter from *Staphylococcus aureus*, Sav1866 was determined and found to be critically different from the reported structures for MsbA but in agreement with cross-linking data and low-resolution structures obtained for P-gp (106). In this new structure, the MSD:MSD interface is out of line with the NBD:NBD interface as a result of the dramatic twisting of the helices from opposite MSDs. An additional consequence of this is that each MSD comes into close contact
with both NBDs. The difference between the crystallized structures of *Sav1866* and MsbA led investigators to re-examine the latter structures, which have since been retracted (110), representing a major setback in the field of ABC transporters. Unfortunately, this error which affected both the handedness and topology of the models was not identified until after these structures had been used as the template in several homology modeling studies, including those for MRP1 (111), ABCG2/BCRP (112) and by multiple groups for P-gp (for examples refer to (113-116)). However, the structure for *Sav1866* has since been used to generate models of MRP1 (117), P-gp (118;119), CFTR (120) and ABCG2/BCRP (121) and analyses have shown that both biochemical and cross-linking data can be accounted for in these models. Despite setbacks, high-resolution structures of bacterial ABC transporters have greatly contributed to our knowledge of ABC transporters and facilitated the construction of homology-based molecular models for eukaryotic transporters. The unfortunate error made in analyzing the MsbA structures and subsequent modeling, illustrates an obvious need for biochemical means with which to validate the structures, one such example being the use of cysteine scanning mutagenesis (CSM).

### 1.4.4 Three-Dimensional Organization of MRP1

Despite the vast amount of biochemical data that has accrued supporting the structure and function of eukaryotic ABC transporters, a precise definition of their three-dimensional organization continues to elude researchers due to a lack of high-resolution structures. What is known regarding the structure of these polytopic transmembrane proteins has been based on low-resolution structures and, as mentioned above, homology-based models. To date, low-resolution structures have been determined for P-gp (ABCB1) (107;122), TAP1/2 heterodimer (ABCB2) (123), CFTR (ABCC7) (124), SUR1 (ABCC8) (125) and MRP1 (ABCC1) (126). The first projection structure of MRP1 was determined by single particle analysis and electron crystallography and resolved to ~22 Å (126). The overall shape of the molecule is described as having a large extracellular pore with an approximate diameter of ~35 Å, sealed on one side by
two NBDs and was in agreement with biochemical evidence available at the time of crystallization. The location of MSD0 was not determined at this resolution, although some deviation from 2-fold pseudosymmetry was observed, as would be expected from the presence of this domain. Interestingly, both monomers and dimers of MRP1 were crystallized, although no functional implications were proposed (126). Furthermore, the low-resolution structure of MRP1 was in agreement with that of the distantly related P-gp (102), the only other available at that time, and has since been shown to be similar to structures attained for other eukaryotic transporters (123;124). However, these transporters were all found to exist as monomers. Despite a lack of primary sequence homology, the overall topologies and three-dimensional arrangements of TM helices of these transporters appears to be conserved at low resolution. This conservation of form validates the use of high-resolution crystal structures as templates for homology modeling of eukaryotic ABC proteins, although a need for biochemical confirmation of these models remains of utmost importance.

1.5 MRP1 Topology

1.5.1 Membrane-Spanning Domains of ABC Transporters

The MSDs of ABC transporters are highly hydrophobic and are typically composed of six $\alpha$-helices in each of two MSDs (totally 12 TM helices) (11). These helices span the membrane anchoring ABC transporters and extend into the cytoplasm. However, some ABC transporters do not conform to this topology and have a greater number of TM $\alpha$-helices in each of their two MSDs, such as BtuCD (102). Moreover, members of the human ABCC subfamily, the cadmium resistance factor, Ycf1, in *C. elegans*, Yor1/Ysr1 in *S. cerevisiae* and GSH transporters in *Arabidopsis thaliana* have been found to contain a third MSD NH$_2$-terminally located to the typical core structure (26;94-101;127;128).

Functionally, the MSDs form the translocation pore and are the site of substrate binding, and have been shown experimentally to exhibit considerable conformational changes during the
transport cycle (for example in P-gp (129-135)). Primary sequence within the MSDs of ABC transporters is not well conserved, likely a reflection of the diverse nature of substrates that this family transports (11). Intracellularly, the TM helices are joined by cytoplasmic loops (CL), which have been implicated in interdomain communication between membrane-bound and cytosolic domains in several transporters (102;106;119;120;136-142). While little is known about the precise mechanism of transport, it is apparent that association and communication between the MSDs and the NBDs must be tightly regulated for efficient substrate transport.

1.5.2 Experimental Determination of MRP1 Topology

Subsequent to its original cloning, computer-assisted hydropathy analysis of the predicted sequence of MRP1 and alignment of primary sequence with the predicted structure of LtpgpA (an ABC transporter from *Leishmania tarentolae* and the most closely related to MRP1 at the time (143)) suggested the presence of numerous TM helices and two cytoplasmic domains containing motifs associated with the ABC superfamily (34). This original topology was proposed to have 12 TM segments in an 8+4 arrangement and two intracellular NBDs. This was later modified to place these 12 TM helices in the more typical 6+6 configuration on the basis of alignments of hydropathy profiles of MRP1 with its murine ortholog and members of the ABC superfamily including CFTR, P-gp, SUR1, and Ycf1 (46;144-146). This more recent topology model also identified an additional hydrophobic domain consisting of the first 220 amino acids of MRP1 arranged in 4-6 TM helices not present in either P-gp or CFTR, but seen in the SUR proteins and Ycf1 (95). Hydrophobicity determinations, glycosylation studies and epitope insertions have since lead researchers to conclude that this domain, now denoted MSD0, is organized into 5 TM helices with an extracellular NH$_2$-terminus (94;98;101;147). The proposed arrangement of 5 TM helices with an extracellular NH$_2$-terminus is in agreement with the suggested topology for the equivalent domain in the related transporter SUR1, which was also confirmed by mapping of glycosylation sites (100). In contrast, results from more recent studies using novel antibodies
directed against the NH$_2$-tail of MRP1 have been interpreted to indicate either the intracellular localization of this terminus or that it is buried in the membrane (148). The internalization of this tail may play a role in regulating the function of the transporter, although this seems unlikely given that MRP1 remains functional upon removal of this entire domain (3;149;150).

Evidence for the topology of the two remaining MSDs, denoted MSD1 and MSD2, was also obtained from glycosylation and epitope insertion studies (94;98;101;151). Epitope mapping for MRP1-specific monoclonal antibodies MRPr1 (amino acids 238-247), QCRL-1 (amino acids 918-924), MRPm5 (amino acids 1063-1072) and MRPm6 (amino acids 1511-1520) also provided evidence for the intracellular location of certain regions (145;152;153). Dynamic molecular homology modeling of MRP1 based on existing ABC transporter structures helped further define the TM helix-loop boundary, although these definitions were put into question upon retraction of the template structures utilized for modeling (110;111). However, this has been rectified with a new homology model of MRP1 that was generated using Sav1866 as the template, and described in (117).

1.5.3 MSD0

The precise function of MSD0 in MRP1 continues to evade researchers, while functional roles for this domain have been identified in other ABC transporters such as SUR1, MRP2 and Ycf1. For instance, the interaction between MSD0 of SUR1 and Kir6.2 has been shown to be absolutely required for plasma membrane (PM) targeting of the complex and channel gating (154;155). In addition, this domain plays a critical role in membrane targeting of MRP2 and Ycf1 and is required for apical and vacuolar localization of these proteins, respectively (156;157). Early studies which examined the role of the NH$_2$-terminus of MRP1 indicated that both MSD0 and CL3 were important for MRP1 activity and localization, but later studies implicated CL3 more specifically (3;4;158;159). However, a functional role for MSD0 in MRP1 cannot be ruled out since point mutations and alterations to this region are not without effect. Indeed,
mutagenesis of certain proline, cysteine and tryptophan residues within MSD0, as well as deletion of the entire first TM helix, have been shown to alter the activity and function of MRP1 (1;2;4;160;161). In addition, one group was able to photolabel this domain with an iodoarylazido derivative of LTC4 implicating this region in the binding of substrate and presumably as part of the translocation pore, although these findings have not yet been corroborated (162). Recent work in MRP1 has suggested that MSD0 is not an absolute requirement for PM localization of the protein but may play a part either in retention in or recycling to, the PM (150). In this work, ~55% of MRP1 lacking MSD0 was retained in intracellular compartments (150). Interestingly, MSD0 becomes an absolute requirement for correct PM localization of MRP1 in the absence of elements in the COOH-tail given that mutant proteins lacking both regions were intracellularly retained, indicating that these two regions of MRP1 contain redundant trafficking signals (150). In addition, the use of MRP1 MSD0-CFP fusion proteins demonstrated that, when independently expressed, this domain displays clear PM localization, lending further support to the theory that MSD0 contains signals important for the correct cellular localization of MRP1 (150).

In contrast, the cytoplasmic loop which links MSD0 to MSD1 (CL3) has been clearly demonstrated to be necessary for the transport activity of MRP1 (3;4;158). MRP1 lacking CL3 in its entirety is unable to transport substrates such as LTC4 and, while this loop itself is not labelled, photolabelling with [3H]LTC4 is dependent upon its presence (158;159;163). Consistently, MRP1 lacking CL3 displays reduced levels of vanadate-dependent nucleotide trapping (158). In these instances, levels of transport and nucleotide trapping were restored by co-expression of CL3 with MRP1 core regions. Regions of CL3 have been implicated in trafficking to basolateral membranes in polarized cells (159). Moreover, MRP1 and P-gp hybrid proteins were used to demonstrate that CL3 acts as a distinct domain associating with both hydrophobic membrane regions and specifically with the core of MRP1; deletion of only 10 amino acids within this loop was significant to disrupt this association (158). Truncation mutants further defined the role of
CL3 in that it contains the NH₂-proximal structural and functional boundary for MRP1, namely Cys208 (159).

1.5.4 MSD1 and MSD2

Functionally, the MSDs of MRP1 form the translocation pore and are the site of substrate binding as established through photoaffinity labelling and extensive mutagenesis studies (33;164). As mentioned above, multiple studies have also revealed that the some of the CLs may also contribute to the translocation pore of MRP1. Photoaffinity labelling experiments have used several photo-reactive substrates and their derivatives or inhibitors of MRP1 in conjunction with partial proteolysis or co-expressed fragments of MRP1 and have identified regions from both halves of MRP1 as contributing to the substrate binding. Three different patterns of labelling have emerged from these studies, dependent upon the identity of photo-reactive label used. Consistently, TMs 10, 11, 16 and 17 have been labelled by the majority of compounds examined (165-170). Identification of these regions is consistent with comparable regions in P-gp found to form the substrate binding site (129;171-176) and is supported by multiple mutagenesis studies in MRP1 which found certain residues within these TMs to be crucial for substrate binding and/or specificity (reviewed in (164) and (33)). Additional regions that have been identified through photolabelling studies include MSD0, regions of CL3, TMs 6, 7 and 12-15 and other CLs (162;169;170;177;178). Data obtained through extensive mutagenesis supports some of these photolabelling results for the TM helices (reviewed in (33) and (164)). Substrate binding by MSD0 is inconsistent with earlier work and has yet to be confirmed by mutagenesis or other studies (3;159). A precise binding site in MRP1 is difficult to define given that point mutations in almost every TM helix in the core MSDs of MRP1 have been shown alter transporter function. This is consistent with the observation based on the Sav1866-based homology model of MRP1, that dependent upon the depth within the membrane different sets of the TM helices of MRP1 comprise the translocation pore (117). A model which potentially accounts for the experimental
data regarding MRP1’s binding site has been derived from other multiple drug binding proteins such as QacR from *Staphylococcus aureus*, as discussed in (164). The binding pocket of this protein is described as containing multiple and somewhat overlapping mini pockets for specific substrates within a larger, flexible pocket, allowing for substantial changes in shape and volume upon ligand interaction (179;180). Thus, the drug binding site for MRP1 has been proposed to be multipartite in nature with different substrates making different molecular contacts with the transporter within this binding site (164).

Similarly to other ABC transporters, the TM helices of the MSDs of MRP1 are joined intracellularly by loops, of which there are seven in MRP1. With the exception of CL3, no direct function has been assigned to these loops until recently, when CL5 and CL7 were implicated in the crucial role of signaling between membrane-bound and cytoplasmic domains (137). In CFTR, proximity of the equivalent loop to CL5 of MRP1, to NBD2 has been shown by cross-linking between an endogenous cysteine in this loop and an introduced cysteine in the NBD (120). An interdomain signaling role for CL7 is supported by its high degree of sequence conservation with the second coupling loop in the Sav1866 structure, shown to interact with the cytoplasmic NBD (106). Further, in P-gp, cysteine cross-linking showed the close proximity of equivalent regions (119). While the Sav1866 structure and thus, the MRP1 model contradict the previously accepted notion that the MSD makes direct contact only with the NBD of the same subunit (11), the work described above in addition to other studies has supported the idea that an MSD may make contacts with both NBDs. In further support of this function for CL7, mutation of a residues within this loop of MRP1 had substrate-specific effects on protein expression, transport activity and ATP catalysis (138;140;141). Similar roles in interdomain signaling have been put forth for both CL4 and CL6 based on results from mutagenesis. Point mutations within these loops, including Arg433Ser (a naturally occurring mutation) and Asp1084, conferred substrate-specific effects to MRP1 (139;142). The latter mutation also abolished ADP-trapping,
specifically at NBD2, indicative of reduced hydrolytic activity of MRP1. Taken together, the data reiterate the need for and functional importance of all domains of MRP1.

1.6 The NBDs of ABC transporters

1.6.1 Classification and Function of ABC NBDs

In contrast to the MSDs, the NBDs of ABC transporters are relatively highly conserved across the superfamily (11;15). However, within the ‘C’ subfamily, the sequences between the two NBDs of a single transporter differ substantially (34). Indeed, there is greater structural similarity between comparable NBDs of different members of the ABCC subfamily than the NBDs within a single transporter (34;181). Despite these differences, the NBDs of all ABC transporters act in conjunction as the molecular motors of these transporters providing the energy for transport derived from the binding and hydrolysis of ATP (15). The precise nature of the cooperation between the two NBDs of a single transporter has yet to be defined and the stoichiometry of ATP hydrolysis remains a matter of debate. For transporters such as P-gp in which both NBDs are capable of hydrolyzing ATP, it is thought that ATP binding may be stochastic and ATP hydrolysis occurs at both NBDs sequentially, in an ‘alternating sites’ or ATP-switch model for catalysis (182;183). However, in transporters such as MRP1 where the NBDs are non-equivalent, it is unclear whether or not both NBDs retain the ability to hydrolyze ATP and evidence in support of both arguments has been presented (141;184-189). For example, functional asymmetry has been observed in MRP1 such that NBD2 appears to be the major site of hydrolysis and NBD1 appears to bind ATP with greater affinity (184-186;190;191). However, functional asymmetry has also been proposed for NBD dimers that both retain the ability to hydrolyze ATP (192-194). In both cases, the stoichiometry of ATP hydrolysis is experimentally difficult to establish since hydrolysis of one or two ATP molecules is virtually indistinguishable. Numerous studies have sought to clarify these points, crucial to understanding the mechanism of action of ABC transporters, although no consensus has been reached.
Examination of the sequence similarity of these domains yields three common characteristics used by researchers to classify genes into this superfamily (14;195). Firstly, ABC transporters share two well conserved motifs with a vast majority of other ATPases, namely the Walker A and Walker B motifs (12). These motifs play essential roles in the binding and hydrolysis of ATP (15;196). However, it is the third conserved motif located in the NBDs of ABC transporters which is unique to this superfamily, viz. the Signature ‘C’ sequence (14). This stretch of highly conserved amino acids (ALSGGQ) is located between the Walker A and B motifs and completes the nucleotide binding site formed by the Walker A and B motifs. In addition to these three motifs, the NBDs of ABC transporters also share some other conserved motifs important in the mechanism of ATP hydrolysis, transduction of signal between cytoplasmic NBDs, and membrane bound domains. These are designated the H-, Q-, and D- loops for the highly conserved amino acid contained within.

1.6.2 High-resolution Structures of ABC Transporter NBDs

High-resolution crystal structures for several isolated bacterial and more recently, eukaryotic NBDs have greatly aided the understanding of the mechanism of ATP binding and hydrolysis by these motor domains. These structures have revealed that the architecture of the monomeric ABC transporter NBD is highly conserved across species and, as first demonstrated in the structure of HisP from Salmonella typhimurium, has an overall L-shaped fold (Fig. 1-2) (197). This domain is further divided into two subdomains: the α-helical subdomain, unique to ABC-transporters, and the α/β subdomain, also called the catalytic or F1-like domain for its similarities to the canonical structure of the F1-ATPases (197;198). The catalytic subdomain contains the Walker A and B motifs, as well as the H- and D-loops, and is joined via the Q-loop or hinge region to the α-helical domain which contains the signature sequence and a region of structural diversity (SDR) (Fig. 1-2) (15;199). Since the first structural determination of an ABC transporter NBD, the field has expanded rapidly. Several more structures of prokaryotic monomeric NBDs are now
Figure 1-2. Structure of MRP-NBD1 bound to ATP/Mg2+. Ribbon representations of the crystal structure of MRP1-NBD1 to 1.5 Å resolution (200) (RCSB Brookhaven Protein Data Bank accession number 2cbz). Figures were generated using Pymol (DeLano, W.L. The PyMOL Molecular Graphics System (2002) Web site http://www.pymol.org). A, the top view of MRP1-NBD1 is shown with the approximate division between the two subdomains indicated (dashed line). The location of the Walker A (yellow), Walker B (green) and Signature ‘C’ (red) motifs within the α-helical and catalytic, α/β subdomains are shown, as well the bound ATP and Mg2+ cofactor. In this view, the interface between NBD1 and NBD2 would be located along the bottom of the image such that the Signature motif and D-loop (orange) are positioned on this face and the ATP bound by the Walker motifs of NBD1 would be sandwiched between the NBDs. B & C, Head-on view of the interface between the NBDs illustrating the relative positioning of residues involved in ATP binding and hydrolysis described in the text including the putative catalytic dyad, Asp793 (teal) and His827 (blue) (H-loop), the Q-loop, Q713 (black), and the conserved aromatic residue, W653 (olive). In both B & C, the top of the image corresponds to the top of the NBD, where it presumably forms an interface with the MSDs and/or intracellular loops.
available, in addition to several eukaryotic NBDs including human TAP1, murine CFTR-NBD1 and human MRP1-NBD1 (199-206). These structures all conform to the bi-lobed L-shaped architecture, now recognized as the canonical fold for this domain. In addition, several dimeric NBDs are now available in complex with various nucleotides which have shed a great amount of light on the co-operativity of the NBDs (207-213). To date, only one dimeric, eukaryotic NBD structure is available, a homodimer of human TAP1 (214); however, in its native state, TAP1 forms a heterodimer with TAP2 comprising the TAP transporter associated with antigen processing in the endoplasmic reticulum (ER) (87-89).

1.6.3 Structural Analysis of ATP-Binding by ABC-NBDs

Prior to solving the structure of the NBD of an ABC transporter, identification of residues involved in the co-ordination and hydrolysis of nucleotide was obtained through biochemical means. High-resolution structures have greatly enhanced our current knowledge of the residues involved in the binding of ATP and have provided a scaffold for interpretation of biochemical data. Initial examination of NBD structures identified residues involved in the binding of ATP within and proximal to both the Walker A and Walker B motifs, and the H- and Q-loops (Fig. 1-2) (reviewed in(15)). Mutation of residues both within, and proximal to, the Walker motifs, including the invariant lysine in Walker A and the catalytic glutamate in Walker B, have been demonstrated in several ABC transporters to greatly reduce functionality of these transporters (for examples see (191;215-224)). In addition, mutating the conserved histidine of the H-loop has been observed to reduce ATPase activity to steady-state levels and abrogate transport function in both prokaryotic and eukaryotic transporters (208;217;225;226). Furthermore, non-specific π-π stacking interactions between a highly conserved aromatic residue and the adenine moiety of ATP were also observed in several of these high-resolution structures, and is supported by several mutagenic studies in which non-conservative substitution of this residue altered ATP-binding characteristics in multiple ABC transporters (viz. P-gp, MRP1) (190;227).
The Signature ‘C’ motif unique to ABC transporters has been demonstrated to be involved in the hydrolysis and binding of nucleotide, as well as the dimerization of the NBDs (217; 228-233). However, functional implications for the Signature ‘C’ motif were not evident in monomeric structures of NBDs and only became apparent upon the determination of dimeric configurations of NBDs. The ABC-like Rad50cd NBD was the first to provide structural evidence for cooperativity of the NBDs, and the already experimentally assumed role of the Signature ‘C’ sequence in the binding of ATP (211). This dimer presented the first arrangement of two NBD monomers such that the helical lobe from one monomer contacts the catalytic lobe of the second monomer in what is referred to as a ‘head-to-tail’ configuration. With this orientation, the Signature ‘C’ motif from one monomer (the trans monomer) is in juxtaposition with the Walker A motif from the second monomer (the cis monomer), to which ATP is bound. In this way the Signature sequence completes the two nucleotide binding sites (NBS) formed by the NBD dimerization. Several more NBDs from ABC transporters have since been crystallized in the dimeric form including those in the full-transporters, confirming the head-to-tail configuration as being physiologically relevant (207-210; 212-214). Additional conserved domains were observed in these structures to mediate and/or stabilize NBD dimerization. These include the H-, Q- and D-loops, the latter of which was seen to interact with a serine residue in the Walker A motif of the opposite monomer (208).

There is a significant amount of structural data supporting conformational changes within NBDs upon ATP binding and hydrolysis that has been obtained by comparison of apo and nucleotide-bound NBD structures. For instance, localized rearrangements of side-chains within the NBSs have been observed upon ATP binding (203; 208; 213; 214). Furthermore, the mobility of conserved domains, both the Walker A and Signature ‘C’ motifs, and the D-, H- and Q-loops, were observed to be more restricted in the nucleotide-bound state than in the apo form, as was observed for human TAP1 (205) and bacterial MJ1267 (202). Consequently, an induced-fit
mechanism was proposed for ATP binding and has been augmented by observations from more recently solved high-resolution structures in which rigid-body rotation of the $\alpha$-helical subdomain relative to the catalytic subdomain is observed (202;205;207;210-214;234). This induced-fit mechanism involves the movement of the D-loop and the Q-loop of the cis monomer upon ATP binding, the latter of which mediates the rigid-body rotation of the $\alpha$-helical subdomain towards the NBS on the same NBD. It is this intradomain rotation that concomitantly acts to bring the Signature ‘C’ motif inwards to contact the $\gamma$-phosphate of ATP bound by the trans NBD monomer. This rigid-body rotation presumably facilitates closure of the dimer interface by optimizing the positioning of the $\alpha$-helical domain relative to the catalytic domain and consequently modifying the dimer interface such that the two faces of the NBDs are now complementary (15). However, in contrast to these data, murine CFTR-NBD1 was crystallized in the presence of ATP and ADP, and no significant differences were observed between the two structures with respect to the relative locations of the domains and important motifs (206).

1.6.4 Proposed Mechanisms for Catalysis from ABC-NBD Structures

The high level of both sequence and structure conservation amongst ABC transporter NBDs suggests a common mechanism of hydrolysis. Two separate mechanisms have been proposed based on existing X-ray structures and biochemical data, namely general base (GBC) and substrate-assisted catalysis (SAC) (presented in (15;196)). No consensus has been reached on this, but both mechanisms have confirmed the importance of certain residues, such as the catalytic role for the Glu residue immediately following the Walker B motif (herein referred to as the catalytic Glu), and the conserved His of the H-loop. As mentioned above, experimentally, the role for these residues has been confirmed by mutagenesis in several ABC proteins. In GBC, the catalytic Glu extends into the active site and abstracts a proton from the transition state. More recently, SAC was proposed upon identification of the H-loop His as the ‘linchpin’ of hydrolysis in the structure of HlyB (208). In this mechanism, a hydrogen bond is formed between the
sidechain of the catalytic Glu and the backbone of the H-loop His to form a ‘catalytic dyad’, acting to position the His residue in a conformation that allows it to interact with the γ-phosphate oxygen of ATP and co-ordinate a water molecule. However, these models have been proposed in homodimers of bacterial NBDs, whereas several eukaryotic NBDs are non-equivalent, both in function and at the level of amino acid sequence. In fact, several eukaryotic transporters including MRP1, CFTR, and TAP1 have evolved degenerate sites in which the catalytic Glu residue is mutated, resulting in functional asymmetry of the NBDs (141;184-189;200;206;214) (discussed in detail in the following section). This natural mutation of the catalytic dyad, however, does not rule out either mechanism since presumably the identity and length of the sidechain at this position is equally important to both abstract a proton from the transition state and form a hydrogen bond with the peptide backbone of the H-loop histidine. Upon examination of the plethora of data pertaining to the mechanism of ATP hydrolysis by the NBDs of ABC transporters, it becomes apparent that the mechanism and stoichiometry by which ATP catalysis occurs remains a matter of debate. It should be taken into account that the mechanisms of ATP hydrolysis put forth above are based on structural studies in isolated NBDs (i.e. in the absence of the MSDs) and extensive evidence suggests significant and reciprocal communication between the MSDs and the cytoplasmic NBDs (for examples see (134;135;184;185;187;235-237)). Clearly, further biochemical and structural evidence are needed to resolve this debate.

1.7 Nucleotide Binding Domains of MRP1

1.7.1 Asymmetry of the NBDs of MRP1

The NBDs of MRP1, like those for all other ATP transporters, provide the energy for translocation of its substrates across cellular membranes (34). This energy is derived from mechanical and chemical energy generated from the binding and hydrolysis of ATP, respectively (15). The NBDs of MRP1 are non-equivalent both in sequence and in function (34); NBD1 has been demonstrated to preferentially bind ATP, while hydrolysis occurs mainly at NBD2 (184-
This asymmetry is consistent across the ABCC subfamily and it appears as though comparable NBDs from separate members of this family are more similar than between the NBDs of a single transporter (34;181). Distinctive features of the NBDs appear to have important functional consequences with respect to ATP binding and hydrolysis and contribute to their non-equivalence. For instance, the nucleotide binding site (NBS) of NBD1, consisting of the Walker A and B motifs from NBD1 and the Signature ‘C’ sequence from NBD2, is non-canonical; the catalytic Glu adjacent to the Walker B motif is mutated to Asp and the first Gly of the Signature motif is mutated to a Val (LSGGQ→LSVGQ) (34). Mutation of the catalytic Glu to Asp is also observed in TAP1 and has been structurally characterized (214). In this half transporter, it was purported that the shorter Asp residue no longer extends into the active site and is thus poorly placed to abstract a proton in general base catalysis. The importance of the length of this side-chain at this position in MRP1 was further emphasized by experiments in which reciprocal mutation (Asp ↔ Glu) in the NBDs of MRP1 resulted in complete abrogation of transport and an increase of ATP binding at NBD2, while binding of ATP by NBD1 was unaltered (191). Furthermore, ATP hydrolysis was increased at NBD1 and concomitantly decreased at NBD2 as reflected by ADP trapping experiments, providing evidence for the nature and importance of the cooperation between these domains. The same group that characterized the Glu→Asp deviation in TAP1 went on to structurally analyze the result of substitution of the first glycine of the LSGGQ with the bulkier valine, which is found in both NBD2 of MRP1 and TAP2. This was observed in TAP2 to result in a steric clash with the Mg\(^{2+}\) cofactor (214).

Further contributing to the asymmetry between the NBDs of MRP1 is a deletion of 13 amino acids within MRP1-NBD1 located between the Walker A and B motifs that are present in NBD2 and other ABC transporter NBDs (34). The functional significance of this deletion remains unclear, but it is known to alter folding of this domain since insertion of this sequence from P-gp inactivated MRP1 (184). The high-resolution structure of the bacterial transporter BtuCD
suggests that this region is involved in communication between cytoplasmic and membrane-bound domains, indicating that transduction of signal between NBD1 and MSDs likely occurs via an alternative mechanism in transporters with this deletion (102). In contrast, the two main residues within the NBDs of Sav1866 that were identified to interact with the MSD associated intracellular loops reside outside this 13 amino acid deletion and therefore remain in NBD1 of the ABCC proteins (106).

1.7.2 Structural Studies of MRP1-NBD1

Although extensive functional data has provided substantial evidence for the importance of conserved motifs and residues in MRP1, until recently, no structural data was available to offer support. Two groups have been successful in expressing soluble polypeptides of both NBDs of MRP1 in insect cells and E. Coli (184;238;239). In the latter study, solubilized NBD1 was used in Trp fluorescence and NMR experiments and, consistent with earlier mutagenesis studies, the close interaction between Trp653 (equivalent to the conserved aromatic residue in other ABC transporters) and the adenine ring of ATP was demonstrated (190;239). Subsequently, the same group applied NMR to characterize the NBD1/NBD2 heterodimer of MRP1 (240). ATP-dependent influence of NBD2 on NBD1 was detectable and was shown to involve Gly771, located within the Signature ‘C’ sequence. In addition, these results suggest that, at least for MRP1, additional structural domains are required for stable dimerization of the NBDs (240).

Recently, Ramaen et al. (200) determined the X-ray structure of NBD1 of MRP1 and this L-shaped structure conformed to the canonical bi-lobed structure of ABC transporter NBDs (Fig. 1-2). Interestingly, the MRP1-NBD1 structure very closely resembled the CFTR-NBD1 structure (r.m.s.d 1.45Å), even within what is known as the structural diverse region, a highly variable subdomain of the α-helical lobe identified previously in bacterial transporters postulated to be involved in NBD-MSD communication (199). The ATP-binding site of MRP1-NBD1 was observed to closely mimic the binding of ATP observed in other high-resolution NBD structures.
Interactions include \(\pi-\pi\) stacking between Trp653 and the adenine moiety of ATP, as well as interaction of the \(\gamma\)-phosphate of ATP with residues in the Walker A motif and Q-loop (Fig. 1-2). However, residues in the catalytic dyad, Asp793 and His827, adopt unusual, non-active conformations in the MRP1-NBD1 structure. These residues appear to point away from the \(\gamma\)-phosphate of ATP, an orientation which interestingly, resembles the nucleotide-free conformation of the dyad presented in the MalK NBD dimer (207). Modeling of the MRP1 NBD1/NBD2 dimer was also undertaken and led researchers to propose that this non-productive conformation of the catalytic dyad in NBD1 acts to stabilize a productive orientation of the canonical catalytic site of NBD2 (200).

1.8 The Catalytic Cycle of MRP1

Biochemical and structural data, as well as homology-based modeling studies and analogy to the mechanism of other ABC transporters, have led researchers to propose a general model of the catalytic cycle of MRP1, as follows (33). MRP1 exists in the membrane in an open, unliganded state such that neither substrate nor nucleotide is bound. In this state, the NBDs are in a monomeric conformation to the degree allowed by the constraints imposed by the MSDs. Binding of substrate (e.g. LTC₄) at a high-affinity site induces conformational changes in the protein, which are presumably transduced, likely \textit{via} interaction of MSD-associated intracellular loops with the Q-loop and/or surrounding regions of NBD1, enhancing the binding of ATP on this domain. However, the nature of this interaction is unclear since regions other than the Q-loop were found to interact with the coupling helices of the ICLs of \textit{Sav1866} (106;117). The bound ATP on the NBS of NBD1 helps to establish and stabilize a close interaction between the NBDs, referred to as the semi-closed dimer in distantly related bacterial ABC transporter NBDs (207). This interaction facilitates binding of a second ATP at the composite NBS of NBD2, and the two bound nucleotides likely contribute substantially to the stabilization of the closed dimer conformation of the NBDs (6). It has been proposed that the conformational changes of ATP
binding to the first and second NBSs act to bring the Signature ‘C’ motif in proximity to complete
the binding site on each NBD and to reorder the face of the NBD such that it is now in a
complementary conformation to the opposite NBD. If the NBDs of MRP1 mimic bacterial
dimers, these changes could be via rigid-body rotation of the α-helical subdomain of NBD1
towards the catalytic subdomain mediated by the D- and Q-loops, which acts to bring the
Signature ‘C’ motif of NBD1 in closer proximity to the γ-phosphate of the bound ATP on the
NBD2 (15). Thus, the demonstrated co-operative binding of two ATP molecules by the NBDs
completes formation of a closed dimer, through conformational changes in both NBDs which are
transmitted to the MSDs, resulting in decreased affinity for substrate (187;191). This low-affinity
state of MRP1 is maintained through hydrolysis of ATP by NBD2, provided that ADP·Pi is not
released and ATP remains bound at NBD1. Substrate is consequently released extracellularly
from the low affinity state, and ADP·Pi is released from NBD2. It is unclear if ATP is released
from the non-canonical NBS of NBD1 or if ATP is hydrolyzed at this site and release of ADP·Pi
occurs. Without answers to this question, it remains an area of speculation how MRP1 resets
itself from this point back to the original, unbound state and largely hinges on the whether or not
NBD1 has any catalytic activity.

1.9 Thiol-Directed Techniques for Probing Higher Order Structure of Polytopic
Membrane Transporters

1.9.1 Cysteine-Scanning Mutagenesis

Cysteine scanning mutagenesis is a biochemical technique for obtaining structural data which
involves the introduction of one or more cysteine residues into an otherwise cysteine-less variant
of the protein of interest. However, CSM has also been undertaken in proteins such as CFTR in
which at least some of the endogenous cysteines remained, although this is not ideal since
interpretation of data can be difficult and caution should be exercised (241). Typically, however,
CSM is done in cys-less template and as such requires that any endogenous cysteines be
substituted to preclude their reaction with sulphydryl reagents. Further, the resulting cysteine-less template must remain functional for interpretation of data in a biologically significant context. In the application of CSM, residue(s) at locations of interest are replaced by cysteines and examined by the plethora of thiol-specific reagents commercially available of varying reactivities, membrane permeabilities, lengths and fluorescence. The locations at which cysteine residues are introduced are typically chosen based on predicted topography, proximity to important motifs, predicted secondary structures or, more recently, on homology-based models of the protein of interest. Individually introduced cysteine residues can be probed for their reactivity towards fluorescent, thiol-specific compounds of different membrane accessibilities, and alterations in reactivity upon interaction with other proteins or substrates (135;242-244). The introduction of two cysteines allows the use of thiol-specific cross-linkers of known and varying lengths as molecular rulers in order to determine protein dimensions (245). CSM offers many advantages, most notably that it can be performed on functional molecules within intact cells (243). This technique is especially useful in the study of polytopic TM proteins given the inherent difficulty of crystallization of these proteins. Indeed, CSM has been applied with varying degrees of success to several polytopic TM proteins including lac permease (246).

1.9.2 Application of CSM to the ABC Transporter Superfamily

Prior to the determination of a high-resolution structure of an entire ABC-transporter, many researchers in this field successfully applied CSM to members of this superfamily. CSM has also been used to validate some of the high-resolution structures that have emerged (113;119). ABC transporters to which CSM has been successfully applied have ranged from bacterial proteins such as LmrA (247) and maltose transporter (136;248;249), to the more extensively examined eukaryotic proteins P-gp/ABCB1 (250;251) (reviewed in(252)), CFTR/ABCC7 (93;241), TAP1/ABCB2 (253), and more recently, BCRP/ABCG2 (254). In most cases, with the exception of LmrA which is naturally devoid of cysteines, the endogenous cysteines were substituted with
either the more conservative serine, or with alanine without a major impact on function. In some cases, the nature of the amino acid substitutions were chosen based on modeling and primary sequence alignments to homologs or related ABC transporters (241;253). In the case of P-gp, mutation of the seven endogenous cysteines was not overly detrimental to the activity of this transporter, but was not without effect (242;243;250). Initial attempts to apply CSM to CFTR proved difficult since the cysteine-less protein did not fully mature (241). Nonetheless, thiol cross-linking analysis was undertaken in a wild-type background with proper controls in place to differentiate between reactions of reagent with endogenous or with introduced cysteines (241). Two separate groups later generated cysteine-less CFTR templates by substitution of 16 of the 18 endogenous cysteines with serine (93;255). The remaining two endogenous cysteines were substituted with leucine given that mutation to other amino acids markedly diminished function of the channel. Application of CSM to these ABC transporters has proven fruitful and provided researchers with structural data pertinent to protein topology, dimensions, packing of TM helices, location of substrate binding sites and accessibility of domains, to name a few (131;172;176;241-243;245;247;256-268). CSM has also been pursued to probe the NBD dimer interface of P-gp, CFTR and the bacterial maltose transporter complex in different nucleotide bound states (93;136;269). In addition, this technique has been used to identify changes in local conformation which occur in the MSDs and NBDs upon substrate and ATP binding, and ATP hydrolysis (129;130;132-134;235;256;270). Specific residues or regions important for transmission of conformational changes between the MSDs and the NBDs occurring during the catalytic cycle have also been discovered (129;136;244).

1.10 Development of Cysteine-less MRP1

1.10.1 Endogenous Cysteines of MSD0 in MRP1

Initial studies of the twenty-five endogenous cysteines of MRP1 examined the importance of these residues within the additional TM domain of MRP1, MSD0 (1;2). Two cysteines, Cys7 and
Cys32, predicted to be located in the extracellular NH$_2$-terminal tail of MRP1 were mutated to alanine and mutants were found to be routed to the correct cellular locations in a mature form as determined by SDS-PAGE, confocal microscopy, and glycosylation studies (1). Investigators went on to further conclude that cysteines at these positions were important for the maintenance of the integrity of both the NH$_2$-tail and MSD0 since mutants Cys7Ala and Cys32Ala reduced MRP1 function and displayed both altered reactivity to antibody by FACS and altered sensitivity to trypsin digestion. Furthermore, wild-type MRP1$_{1-281}$ (encompassing MSD0 and CL3) expressed in the absence of the MRP1 core formed DTT-sensitive oligomers (1). These oligomers were also observed when Cys7 was mutated but to a much lesser extent, implicating this cysteine in the formation of intermolecular disulfide bonds. However, this higher molecular weight species was not observed when the core domain and MRP1$_{1-281}$ were co-expressed. Taken together, it was proposed that proper folding of MSD0 requires the presence of the core domain and, that in its absence, results in an altered conformation which allows Cys7 to mediate formation of intermolecular disulfide bonds.

Subsequently, our group mutated the remaining five uninvestigated cysteines in MSD0, as well as the two cysteine residues located in CL3, to both serine and alanine in the full-length transporter (MRP1$_{1-1531}$) (2). Interestingly, a single nucleotide polymorphism (SNP) that results in serine substitution of Cys43 located in TM1 has been identified and was included in this analysis (1). During the course of these experiments, the effects of mutating Cys7 and Cys32 were also examined but not all of the findings of Yang and co-workers described above were corroborated (271) (Dr. Elaine Leslie, unpublished data). Mutation of the five native cysteines in the TM helices of MSD0 and the two in CL3 did not greatly alter levels of expression of MRP1 although transport of substrates such as LTC$_4$, E$_2$17βG and GSH ranged from 50-150% of wild-type (2). In addition, PM localization was observed for all mutants in HeLa cells with the majority of the mutants also displaying minor amounts of intracellular staining. However, serine
substitution of Cys43 was not well tolerated by full-length MRP1 and PM trafficking of this mutant was severely disrupted. Further, MRP1 with mutated cysteines located in CL3, (Cys208 and Cys265) both displayed altered cellular localization, regardless of the nature of the substitution and with a greater extent of intracellular accumulation observed for Cys265 mutants. Examination of the resistance profiles of the MSD0/CL3 cysteine mutants revealed that mutant Cys43Ser and not the more conservative mutant Cys43Ala, selectively reduced resistance to vincristine and arsenite (2). Limited trypsinolysis studies were suggestive of structural changes in MRP1 as a consequence of mutation of Cys208 and Cys265. In general, it was concluded that Cys→Ala substitutions in MSD0 of MRP1 are preferred over the more conservative serine substitution. Moreover, the feasibility of conducting any future CSM studies may be impaired by the difficulty in obtaining a functional, cys-less template.

During investigation of the functional and structural role of CL3, Cys265 was deleted altogether (159). Two conserved helical elements are predicted to be located within this intracellular loop, between amino acids ~216-231 and 247-267. Deletion of a portion of this second loop, including Cys265, did not affect biogenesis and the protein was observed at increased levels over wild-type. However, significant defects in basolateral membrane localization were detected, indicating that this region is critical for correct PM localization, although when expressed in Spodoptera frugiperda 21 cells the protein retained normal transport activity. The NH2-proximal functional and structural boundary of CL3 was defined as Cys208, given that truncation past this point impairs maturation and sorting of the protein in the ER, followed by rapid degradation (159). It was concluded that deletion of MSD0 up to Cys208 impairs the structural integrity of CL3 through disruption of the first helical element within this domain. Combining the results of these two studies leads to the conclusion that both Cys208 and Cys265 are important for maintaining the structural integrity of predicted helical elements in
CL3. However, the identity of the amino acid may be more important at position 265, given that intact protein appears more tolerant of mutation of Cys208.

1.10.2 Endogenous Cysteines Within the Core Domains of MRP1

In addition to the SNP described above, a second has been identified in MRP1 that also results in serine substitution of an endogenous cysteine residue, specifically at position 1047 (located in CL6). Letourneau et al. (271) investigated whether this mutation resulted in any effect on the activity and biogenesis of MRP1. Mutant Cys1047Ser, predicted to be located within CL6, did not have any significant effects on either membrane localization or function of MRP1. In a separate investigation, our group examined two vicinal cysteine residues, Cys1205 and Cys1209, located within TM16, and found no observable effects on either substrate specificity or overall MRP1 activity (272). Of the seven cysteines located within the NBDs, only Cys682 resides within a conserved motif, the Walker A motif of NBD1, and has been previously investigated. Alanine substitution of this cysteine resulted in increased affinity for ATP and increased LTC₄ transport activity (189).

1.10.2.1 Expression of Cys-less MRP1 Lacking MSD0 in Yeast

Taken together, the data above indicate that the cysteine residues of MRP1 play some role in the biogenesis, trafficking and transport activity of MRP1, compromising the generation of a functional, cysteine-less transporter for use in CSM studies. However, CSM may still be a feasible method to probe the higher order structure of MRP1 since proteins retaining as little as 50-80% wild-type function have been examined by this technique (273;274), although extra caution must be taken in interpretation of results. Previously, investigators took advantage of the ability of MRP1 lacking MSD0 both to be expressed in the PM and retain reasonable levels of transport function and replaced the 18 endogenous cysteines in CL3 and the core domain with alanine (5). This cysteine-less template of MRP1 lacking MSD0 (MRP1 NΔ204) was expressed in *S. cerevisiae* and, in this system, the 18 endogenous cysteine residues were not found to be
essential for membrane expression of MRP1. The cys-less mutant was expressed at levels comparable to wild-type and functional studies revealed that cys-less MRP1 N\Delta204 retained LTC\(_4\) transport levels that were approximately 60% of wild-type MRP1 N\Delta204 (5). Despite the retention of function of this cys-less MRP1 template, it was not further pursued by this group. Taking into account the above data, it becomes apparent that the most successful template of MRP1 for future CSM studies would be one lacking MSD0, although the deleterious effects of mutating Cys265 in CL3 would have to be circumvented, likely through expression in insect cells.

### 1.11 Research Objectives

In lieu of high-resolution structural information for MRP1, alternative methods are required to ascertain knowledge about the structure of the protein. The method proposed here is CSM. Ideally, application of CSM involves the development of a template completely devoid of endogenous cysteines into which cysteine or paired cysteines can be introduced. It is of utmost importance that the cys-less template remain functional to ensure the interpretation of results in a biologically significant context. The aim of the current research was to further the application of CSM as a means of determining the higher-order structure of MRP1 and isolate individual cysteines or regions of cysteines important in maintaining the structural and functional characteristics of wild-type MRP1.

First steps to achieving this goal were aimed at determining if the endogenous cysteines of MRP1 were involved in disulfide bridges and if mutation of these residues altered function. The possibility that the twenty-five endogenous cysteines of MRP1 were forming disulfide bonds was excluded in intact and co-expressed halves of wild-type MRP1 (Chapter 3). Since cysteines within MSD0 have been previously investigated and were found to significantly affect the structural and functional properties of MRP1 (1;2) and MRP1 lacking this domain remains functional (3;4), this domain was omitted from initial analyses of the endogenous cysteines of
MRP1 and attention was instead directed towards the endogenous cysteines in the core domains. Expression of a functional, cys-less, intact version of MRP1 lacking MSD0 has been described previously (5). Here, we sought to further develop this template and take advantage of the ability to co-express MRP1 as two half molecules (91;163), presuming that use of a dual construct would render interpretation of future cross-linking experiments less ambiguous. To this end, all 18 endogenous cysteines in MSD0-less MRP1 expressed as two half molecules were replaced with alanine and functional assays showed impaired transport (Chapter 3). Subsequently, systematic alanine substitution was done by individual MRP1 half-molecules and then by domain and resultant cys-less MRP1 mutants functionally characterized. Transport assays revealed that mutation of cysteines in CL3, MSD1 and/or MSD2 had detrimental effects on MRP1 transport activity. However, complete wild-type transport activity was retained in MRP1 NΔ204 dual lacking cysteines in both NBD1 and NBD2 and this construct was used for introduction of cysteines to be used for future thiol cross-linking (Chapter 3).

Upon determining the template validity of cys-less NBD1/2 MRP1 NΔ204, paired cysteines were introduced. Two residues, Arg775 and Thr1329, located on opposite faces of the NBD1:NBD2 heterodimer were selected for mutation to cysteine based on a previous finding in the related ABC transporter, CFTR/ABCC7, that comparable residues were coupled through hydrogen bonding (6). These residues form a hydrogen bond in the transition state of CFTR and given their locations relative to the Walker A and Signature C motifs in NBD1 and NBD2, respectively, their proximity would likely permit formation of a disulfide bridge between cysteines introduced at these positions. Functional assays revealed that introduction of cysteines at either of position 775 or 1329 greatly reduced the transport activity of MRP1 and while ATP-binding by these mutants was not altered, trapping of nucleotide was diminished at both NBDs (Chapter 3).
Little conclusive evidence is available regarding the functional and structural importance of MSD0 of MRP1. In efforts to rectify this situation, endogenous cysteines within this domain were mutated to alanine and its suitability as a template for CSM was then examined. Given that a specific function has yet to be attributed to MSD0 of MRP1, transport assays cannot be used for template validation. Instead, this was achieved by analyzing whether cys-less MSD0 retained the ability of wild-type MSD0 to traffic independently to the PM and associate with the remainder of MRP1 (150). Thus, cellular localization assays were done on both independently expressed mutant MSD0 and following its co-expression with MRP1 core regions (MRP1\textsubscript{204-1531}) (Chapter 3). Alanine substitution of the seven endogenous cysteines in MSD0 was not without effect and cellular trafficking assays, co-expression studies and SDS-PAGE analysis suggested an altered conformation of this domain. In a previous study, the importance of Cys7 and Cys32 was demonstrated and mutation of these residues altered function of full-length MRP1 (1); these results, however, were not corroborated by our group (Dr. Elaine Leslie, unpublished data). Consequently, the potential role of these cysteines was evaluated in wild-type and cys-less MSD0 expressed individually and in full-length MRP1 (Chapter 3). Interestingly, mutation of these cysteines in wild-type MSD0 individually expressed was sufficient to disrupt the PM targeting of this domain but similar mutations in a full-length MRP1 had no effect on the cellular localization of the transporter. However, somewhat unexpectedly, re-introduction of these cysteines was not able to restore wild-type PM localization of the independently expressed cys-less MSD0, suggesting the remaining cysteines in MSD0 are required for correct cellular localization. A disulfide bonding pair of Cys7 and Cys32 was suggested by these experiments in MSD0 and further supported by limited trypsin digestion of these mutants in full-length MRP1.
Chapter 2
MATERIALS AND METHODS

2.1 Materials

[14, 15, 19, 20-3H]LTC4 (166.8 Ci mmol⁻¹) and [6,7-3H]E₂₁₁βG (53 Ci mmol⁻¹) were purchased from (NEN Life Science Products) Perkin Elmer (Montreal, QC, Canada). 8-azido [γ-32P]ATP (9.9 Ci mmol⁻¹) and 8-azido [α-32P]ATP (14.9 Ci mmol⁻¹) were from Affinity Labeling Technologies Inc. (Lexington, KY, USA). The monoclonal antibody anti-calnexin, nucleotides (ATP, AMP and ATP-γ-S), DMEM, FBS, Grace’s media, N-ethylenemaleimide, poly-L-lysine, propidium iodide, RNaseA, BeSO₄, and NaF were all obtained from Sigma-Aldrich (Oakville, ON, Canada). MAb MRPr1 and mAb MRPm6 were purchased from Alexis Biochemicals (San Diego, CA, USA). Fluorescently-conjugated secondary antibodies Alexa 488, Alexa 546, and Alexa 594 (Molecular probes©), Hoescht 33432, geneticin, and Lipofectamine2000™ were from Invitrogen Corporation (Burlington, ON, Canada). Mutagenesis kits, QuickchangeII® Mutagenesis kit and Transformer™ Site-Directed Mutagenesis kit were obtained from Stratagene (La Jolla, CA, USA) and Clontech (Mountain View, CA USA), respectively. Fugene6™, Complete EDTA-free protease inhibitors and hygromycin B were purchased from Roche (Indianapolis, IN, USA). All restriction endonucleases, PNGase F, and the large (Klenow) fragment derived from Escherichia coli (E. coli) DNA polymerase I were purchases from New England Biolabs Ltd. (Pickering, ON, Canada). The expression vector pECFP-N1 was a generous gift from Dr. Peter Greer at Queen’s University, Canada.

2.2 Generation of MRP1 Constructs

The cloning of intact and half transporter (dual) constructs of wild-type MRP1 and the NH₂-proximal truncation (MRP1 NΔ204) into pFastbac (pFB) and pFastbac dual (pFB dual) (Invitrogen, Burlington, ON, Canada) expression vectors has been described previously (4;91;163;184). The dual-expression vector in which amino acids 1-203, encompassing MSD0,
with a COOH-terminal FLAG epitope tag was co-expressed with the MRP1 core domain, amino acids 204-1531, (pFBdual-MRP1$_{1-203}$FLAG/204-1531) was created with the assistance of Ruth Burtch-Wright.

The Transformer™ Site-Directed Mutagenesis kit was used to generate cysteine-free MRP1$_{1-1531}$ in which all 25 endogenous cysteines were mutated to alanine (pFB-MRP1$_{cys-less}$) by Dr. Dawei Zhang in vector pFB. In addition, cys-less MRP1 lacking MSD0 (cys-less MRP1 NA204) in pFB was provided by Dr. Chris Westlake. Preliminary examination of cys-less protein expression levels in Sf21 cells and transport studies suggested problems in the cloning and mutagenesis of these original constructs (data not shown). Subsequent sequencing (ACGT Corp., Toronto, ON, Canada) revealed two spontaneous mutations, K332M, previously shown to selectively decrease LTC$_4$ transport while maintaining expression levels (275), and a stop codon that resulted in prematurely truncated protein following Cys1479. To facilitate cloning into pFB dual for co-expression studies and for correction of these mutations, MRP1$_{204-932}$cys-less and MRP1$_{932-1531}$cys-less were constructed into pFB. PCR was used to create pFB-MRP1$_{204-932}$cys-less with pFB-MRP1$_{204-1531}$cys-less as the template. The forward primer (5’-CATGGTCATCAGCAGCATCGT-3’) was designed upstream of the unique EcoRI site in MRP1 coding sequence (1955-1965) and the reverse primer (5’-TCATCTAGAGTTATGCGGTGCTGTTGTGGTGCCTGC-3’) contained an introduced XbaI site (underlined) directly following the stop codon. The resulting PCR product was cleaved with EcoRI and XbaI to produce an 840-bp fragment encompassing MRP1$_{653-932}$cys-less. A three-step ligation was performed using the 840-bp fragment from the PCR reaction, pFB-MRP1$_{204-1531}$cys-less digested with SalI and EcoRI corresponding to MRP1$_{204-653}$cys-less and pFB digested with SalI and XbaI as the backbone. For this and all subsequent constructs, the fidelity of PCR products, junctions between ligated fragments and identity of variant constructs was confirmed by sequencing (ACGT Corp., Toronto, ON, Canada). Site-directed mutagenesis (Quickchange II,
Stratagene, La Jolla, CA, USA) was performed using pFB-MRP1<sub>204-932cys-less</sub> as the template to generate pFB-MRP1<sub>204-932cys-less K(M)332K</sub>.

Two separate PCR reactions were performed on the template pFB-MRP1<sub>204-1531cys-less</sub> to obtain the cys-less variant of the COOH-proximal half, pFB-MRP1<sub>932-1531cys-less</sub>. The first PCR product was created with the following forward and reverse primers, 5’-TCTGAATTCCGCTAGGGCGGCCGCATGGCACGAACTGCAGAAAGCTGAGG-3’ and 5’-AAGCGGAGGTCGTGCAGGC-3’, respectively. The forward primer introduced EcoRI, NheI and NolI sites (underlined) and a consensus Kozak sequence (bold). This PCR product was digested with EcoRI and the resulting 1.1-kb fragment corresponding to MRP1<sub>932-1294cys-less</sub> was ligated into the appropriately digested pFB-MRP1<sub>932-1531</sub>, resulting in pFB-MRP1<sub>932-1531(932-1294cys-less)</sub>. The second PCR product was created using primers 5’-TCTGAATTCCGCTAGGCGCCGCATGGCACGAACTGCAGAAAGCTGAGG-3’ (forward), which includes EcoRI and NheI sites (underline) and a consensus Kozak sequence (bold) and 5’-AAGCGGAGGTCGTGCAGGC-3’ (reverse). A 1.2-kb fragment corresponding to MRP1<sub>1144-1531cys-less</sub> was excised by digesting the PCR product with XhoI and KpnI and subsequently ligation into pFB-MRP1<sub>932-1531</sub> digested with the same enzymes. Thus, pFB-MRP1<sub>932-1531(1144-1531cys-less)</sub> was created and used as a template for site-directed mutagenesis (Quickchange II, Stratagene, La Jolla, CA, USA) to remove the stop codon that introduced during initial mutagenesis reactions. Finally, pFB-MRP1<sub>932-1531cys-less</sub> was constructed by digestion of pFB-MRP1<sub>932-1531(1144-1531cys-less)</sub> with XhoI and KpnI and this 1.2 kb fragment was ligated into pFB-MRP1<sub>932-1531(932-1294cys-less)</sub> which had been similarly digested.

Wild-type and cys-less dual-expression constructs were cloned in a similar fashion to pFBdual-MRP1<sub>204-932/932-1531</sub> (MRP1 NΔ204 dual) (163), such that the NH<sub>2</sub>-half was under the control of the polyhedrin (P<sub>Ph</sub>) promoter and the COOH-half was under control of the p10 (P<sub>p10</sub>) promoter (App. I). Briefly, the cys-less NH<sub>2</sub>-proximal half of MRP1 was moved into the pFB
dual vector already containing the wild-type COOH-terminal fragment by digestion of vector and insert (pFB-MRP1\textsubscript{204-932cys-less}) with XbaI, resulting in pFBdual-MRP1\textsubscript{204-932cys-less/932-1531} (cys-less NH\textsubscript{2}). Dual-expression of wild-type MRP1\textsubscript{204-932} with MRP1\textsubscript{932-1531cys-less} (pFBdual-MRP1\textsubscript{204-932/932-1531cys-less} or cys-less COOH) was generated by digestion of pFB-MRP1\textsubscript{932-1531cys-less} with XbaI and ligation of excised fragment into pFB dual that already contained wild-type MRP1\textsubscript{204-932}. Dual-expression of MRP1 lacking MSD0 and completely devoid of cysteines in both halves was generated by digestion of empty vector (pFB dual) and insert (pFB-MRP1\textsubscript{932-1531cys-less}) with NheI and KpnI. The cys-less NH\textsubscript{2}-proximal half of MRP1 was then cloned into this vector using Sall and XbaI creating pFBdual-MRP1\textsubscript{204-932cys-less/932-1531cys-less} (Cys-less MRP1 N\textDelta204 dual).

The COOH-proximal half of MRP1 with Cys\rightarrow AlAs in NBD2 (amino acids 1294-1531) was first cloned into pFB by ligation of insert (1.1 kb) and linearized vector generated by EcoRI digestion of pFB-MRP1\textsubscript{932-1531(1144-1531cys-less)} (vector) and pFB-MRP1\textsubscript{932-1531} (insert). For co-expression studies, the resultant construct (pFB-MRP1\textsubscript{932-1531(1295-1531cys-less)}) was digested with BssHII and blunt ends generated by digestion with the Klenow fragment. This DNA was subsequently digested with KpnI and the resultant 1.8-kb fragment was ligated into pFB dual which had been subjected to digestion with SmaI and KpnI to generate pFBdual-MRP1\textsubscript{932-1531(1295-1531cys-less)}. Co-expression of the wild-type NH\textsubscript{2}-proximal half with the COOH-half molecule of MRP1 lacking cysteines within NBD2 was achieved by digestion of vector (pFBdual-MRP1\textsubscript{932-1531(1295-1531cys-less)}) and insert (pFB-MRP1\textsubscript{204-932}) with RsrII and XbaI. This generated pFBdual-MRP1\textsubscript{204-932/932-1531(1295-1531cys-less)} (Cys-less NBD2). Unlike the remainder of the dual-expression constructs, the construct in which Cys\rightarrow AlAs substitutions were made only in NBD1 (Cys-less NBD1, residues 653-932) was cloned directly into pFB dual due to the relative simplicity of the cloning steps required. Briefly, pFB-MRP1\textsubscript{204-932cys-less} was digested with EcoNI/XbaI and the resulting 840-bp fragment was ligated into the backbone resulting from EcoNI/XbaI digest of pFBdual-MRP1 N\textDelta204, creating pFBdual-MRP1\textsubscript{204-932(653-932cys-less)/932-1531}. Cys-less NBD1/NBD2...
in MRP1 \(\Delta204\) (pFBdual-MRP1\(_{204-932(653-932\text{cys-less})/932-1531(1295-1531\text{cys-less})}\)) was generated by digestion of vector (pFBdual-MRP1\(_{932-1531(1295-1531\text{cys-less})}\)) and insert (pFBdual-MRP1\(_{204-932(653-932\text{cys-less})/932-1531}\)) with \(Rsr\)II and \(XbaI\) and subsequent ligation.

### 2.2.1 Promoter Switch Cloning

As described in the results section, expression of the cys-less COOH-proximal half molecules was severely impaired. Therefore, the decision was made to re-clone the MRP1 half-molecules such that the NH\(_2\)- and COOH-proximal halves were under the control of the \(P_{p10}\) and \(P_{PH}\), respectively. This ‘promoter switch’ (PSW) cloning, described in detail below, was completed for all constructs in which either the entire COOH-terminal half or just MSD2 were cysteine-less. Wild-type MRP1 \(\Delta204\) dual was also re-cloned in a similar fashion as a control and is denoted PSW MRP1 \(\Delta204\) dual. Generation of promoter switch constructs was completed with assistance from Monika Vasa.

For creation of the PSW constructs, the wild-type or cys-less NH\(_2\)-proximal half was inserted first into the multiple cloning site (MCS) downstream of the p10 promoter in pFB dual. Empty vector was digested with \(SmaI/NheI\). Inserts were generated by digestion of either pFB-MRP1\(_{204-932}\) or pFB-MRP1\(_{204-932\text{cys-less}}\) with EagI, treatment with the large (Klenow) fragment derived from \(E.\ coli\) DNA polymerase I to fill in the 3’ overhang, and digestion with \(XbaI\). The control construct for PSW proteins, PSW MRP1 \(\Delta204\) dual or PSW pFBdual-MRP1\(_{204-932/932-1531}\) was created by linearizing vector PSW pFBdual-MRP1\(_{204-932}\) by digestion with \(NspV\). This was followed by generation of blunt ends with the Klenow fragment and finally, digestion with \(SalI\). Insert for this control was excised from pFB-MRP1\(_{932-1531}\) by sequential digestion with \(Acc65I\), the Klenow fragment and \(SalI\), followed by a final ligation. Next, PSW MRP1 \(\Delta204\) dual was used as the vector backbone to co-express wild-type NH\(_2\)-half molecule with the COOH-proximal half, cys-less between residues 932-1294. This was accomplished by ligation of fragments from \(EcoRI\) digestion of both vector and insert (pFB-MRP1\(_{932-1531(932-1294\text{cys-less})}\)) creating PSW pFBdual-
Co-expression of the same COOH-half molecule with the cys-less NH₂-proximal half was slightly more involved such that vector (PSW pFBdual-MRP1₂₀₄-9₃₂) and insert (pFB-MRP1₉₃₂-₁₅₃₁) were cut initially with BstBI and Acc65I, respectively, and ends blunted by digestion with the Klenow fragment. DNA was then digested with SpeI (vector) and NheI (insert) and ligated. This created pFBdual-MRP1₂₀₄-9₃₂/9₃₂-₁₅₃₁ (PSW cys-less NH₂/MSD2). Similarly, the completely cys-less COOH-proximal half was inserted under the control of the PH promoter into the PSW vectors from above, into which wild-type or cys-less NH₂-proximal half had already been inserted. Insert for both of these constructs was obtained by digestion of pFB-MRP1₉₃₂-₁₅₃₁ with Acc65I, generation of blunt ends by digestion with the Klenow fragment and subsequent digestion with NheI. Vector backbones PSW pFBdual-MRP1₂₀₄-9₃₂ and PSW pFBdual-MRP1₂₀₄-9₃₂ were digested with XbaI and BstBI, respectively, followed by digestion with the Klenow fragment to produce blunt ends. Vectors were then digested with either NotI (wild-type) or SpeI (cys-less) depending on the cysteine status of the NH₂-proximal half. This cloning yielded PSW pFBdual-MRP1₂₀₄-9₃₂/9₃₂-₁₅₃₁ (PSW cys-less COOH) and PSW pFBdual-MRP1₂₀₄-9₃₂/9₃₂-₁₅₃₁ (PSW cys-less NΔ204 dual).

In order to ascertain the role of cysteines located within MSD0 (amino acids 1-203), PCR was utilized to create COOH-terminally fused CFP proteins as described previously (150). Cys-less MSD0 was generated by PCR on template pFB-MRP1₂₀₄-1₅₃₁ using forward primer 5’-TATGCTAGCCGCCCATGCGCTCCGGGGCTTCGCCAGCGGATGG-3’ which introduces a NheI site (underlined) and a consensus Kozak sequence (bold) and the reverse primer described previously in (150)). The resulting product was digested with NheI/AgeI for insertion into pECFP N1 vector that had been similarly digested. PCR was used for re-introduction of Cys7 using the same set of primers as above but with the codon at this position in the forward primer reverted to wild-type sequence (gcc→tgc). The Quickchange II Mutagenesis™ kit was employed for
mutagenesis of Ala32Cys, Cys7Ala and Cys32Ala in cys-less and wild-type pE MRP1_{1-203}-CFP, respectively. Full-length MRP1 with mutations Cys7Ala, Cys32Ala and the double mutant, Cys7/32Ala, in the mammalian expression vector pcDNA3.1(-) were generous gifts from Dr. Susan Cole.

2.3 Recombinant baculovirus production and viral infection

Transformation of pFB and pFB dual plasmid constructs, isolation of recombinant bacmid DNA and subsequent transfection of Sf21 cells were carried out as described previously (91). Approximately 72 hours post-infection, or when 90% of cells had lifted from the plate, cells were harvested for whole cell lysate preparation and analysis of protein expression levels. Simultaneously, first generation baculovirus was collected and amplified to produce second generation virus. This second generation virus was used for large scale infection of Sf21 cells which were harvested for membrane vesicle preparation after washing cell pellets twice with homogenization buffer (50mM Tris pH 7.5, 250mM sucrose, 0.25mM CaCl$_2$). Prior to freezing at -70°C, pelleted cells were layered with 10 mL homogenization buffer.

2.4 Mammalian Cell Expression and Localization of MRP1 proteins

Human embryonic kidney 293 (HEK293) cells were transfected with constructs cloned into the pECFP-N1 and pcDNA3.1(-) vectors using Fugene6™ or Lipofectamine2000™ according to the manufacturer’s protocols. For crude membrane preparations, larger scale transient transfections were done and cells harvested three days post-transfection. Stable cell lines expressing MSD0-CFP fusion proteins were generated by selection with geneticin (800 μg mL$^{-1}$). For rescue experiments, stable cell lines of either MRP1$_{204-1531}$-YFP or MRP1$_{204-1531}$ were transiently transfected with wild-type and variant pE MRP1$_{1-203}$-CFP. For all confocal microscopy studies, cells were seeded on poly-L-lysine-coated glass coverslips at 2.5x10$^5$ cells/well and transfected 12 hours later. Medium was removed 36 hours post-transfection and cells were washed with 1xPBS pH 7.4 containing 0.1 mM each of MgCl$_2$ and CaCl$_2$ (PBS*).
Cells were fixed with ice-cold 95% ethanol. Nuclei were stained with either Hoescht 33342 or cells were treated with RNase (0.02 mg/mL, 1 hour) followed by propidium iodide. This was completed directly following fixation for cells containing only CFP- and YFP-fusion proteins, or following incubation with fluorescent secondary antibodies and associated wash steps for cells requiring further processing. In the latter case, after fixation, cells were washed in triplicate (1xPBS) and exposed to block solution (1% bovine serum albumin (BSA)/1xPBS pH 7.4) (10 min, RT). Since the epitope for mAb MRPr1 remained intact in all MRP1 core variants used, this antibody was used for immunofluorescent detection of this protein (1 hr RT) in conjunction with Alexa 488 or Alexa 594 goat anti-rat immunoglobulin (1 hr, RT). The endoplasmic reticulum (ER) was visualized using a mAb to calnexin, a membrane-bound protein associated with the rough ER (1 hr, RT) with secondary antibody, Alexa 546 goat anti-rabbit immunoglobulin. The fluorescent secondary antibodies, the nuclear stain and MRP1 variant CFP and YFP fusion proteins were visualized using a Leica TCS SP2 dual photon confocal microscope.

2.5 Crude Membrane and Membrane Vesicle Preparations

Crude membranes were isolated from Sf21 or HEK293 cells as described previously (41). In order to prevent disulfide exchange or the formation of disulfide bonds not present in native protein, N-ethylmaleimide (NEM) (5 mM) was added in some experiments as specified, to the PBS wash of cell pellets (276). For isolation of crude membranes, cell pellets were thawed on ice and resuspended in lysis buffer (10mM Tris pH 7.5, 10mM KCl, 1.5mM MgCl₂, and EDTA-free complete protease inhibitors). Cells were mechanically disrupted using 100 strokes in a Tenbroeck homogenizer and cellular debris, nuclei and undisrupted cells were removed by centrifugation (500 x g, 4ºC, 15 min). Membranes were isolated by ultracentrifugation (100000 x g, 4ºC, 20 min) and resuspended in transport buffer (TB) (50 mM Tris pH 7.5, 250 mM sucrose).

For transport and photolabelling studies, membrane vesicles were generated as described in (43;91). Briefly, Sf21 frozen cell pellets were thawed on ice and resuspended in 10mL of
homogenization buffer. Membranes were disrupted by nitrogen cavitation (200 psi, 5 min, 4°C), and cellular debris and unexploded cells removed by centrifugation (750 x g, 15 min, 4°C). Plasma membrane vesicles were isolated by ultracentrifugation (100000 x g, 1.5 hr, 4°C) on a sucrose cushion (35% w/v sucrose, 50mM Tris pH 7.5, 1mM EDTA). The interface between the two phases was collected and washed twice by centrifugation. Pelleted membrane vesicles underwent final resuspension in TB. Protein concentrations were measured using a Bio-Rad Bradford assay with bovine serum albumin as a standard.

2.6 Immunoblotting and Glycosylation studies

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10% gels for full-length and dual-half proteins according to Laemmli method (277). Gradient gels (7-15%) were utilized for protein separation in photolabelling experiments for increased resolution of proteins spanning a broad range of molecular weights (91;238). Low percentage gels (5%) were used for separation of higher-molecular weight species. Proteins were transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA) using 25 mM Tris-base, 192 mM glycine, and 20% methanol buffer. MRP1 was detected using monoclonal antibodies (mAb) MRPr1, MRPm6 and/or QCRL-1, previously characterized that recognize linear epitopes within CL3, the COOH-terminal region and the linker region between NBD1 and MSD2, respectively (145;152). Monoclonal antibodies were used in combination with horseradish peroxidase secondary antibodies utilizing a Supersignal® West Pico chemiluminescence substrate (Pierce Biotechnology, Inc., Rockford, IL, USA) and AGFA film (Electromedical Equipment Co. Ltd., Richmond Hill, ON, Canada). Membrane proteins were deglycosylated by incubation with peptide N-glycosidase F (PNGase F) according to the manufacturer’s protocol and as described previously (91;150).
2.7 Quantitation of MRP1 protein by immunoblotting

The Bio-Dot® SF Microfiltration apparatus (Bio-Rad, Mississauga, ON, Canada) was used to generate ‘slot blots’ in order to avoid any size-dependent differences in transfer efficiency. Briefly, total membrane protein was determined by Bradford assay as described above and serially diluted protein samples from each membrane preparation were transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). Wild-type NΔ204 MRP1, its co-expressed halves and the PSW control (PSW MRP1 NΔ204 dual) were included on each blot. Membrane-bound proteins were probed using anti-MRP1 mAb MRPr1 or MRPm6 as described above. Densitometry of film images was performed using a ScanMaker i900 scanner (Microtek, Carson, CA) and ImagePro Software. Expression of each mutant MRP1 protein was determined relative to the appropriate wild-type protein. Linearity between total membrane protein loaded and resultant signal was established (data not shown). For cysteine mutant MRP1 proteins expressed as half-molecules, the levels of expression of each half-molecule compared to the equivalent wild-type fragment were determined, as described previously (278). Where expression of the either half of the cysteine mutant protein differed from wild-type by more than ±10%, normalization of mutant proteins to wild-type was done using the expression levels of the least abundant half molecule. Similarly, the expression level of each half-molecule of the wild-type dual constructs was normalized to the equivalent signal obtained from the intact protein.

2.8 LTC4 and E217βG Transport Assays in Sf21 Membrane vesicles

Uptake of [3H]LTC4 (50 nM) and [3H]E217βG (400 nM) was measured at 23 and 37 °C, respectively, as described previously (63;91;188). Briefly, 4 µg (LTC4 uptake) or 6 µg (E217βG uptake) of Sf21 membrane vesicles were incubated with substrate in the presence of 4 mM ATP or AMP and 10 mM MgCl2 in TB and uptake measured using a rapid filtration technique. ATP-dependent transport was determined by subtracting uptake in the presence of AMP from uptake in the presence of ATP. In addition, transport due to MRP1 was calculated by subtraction of uptake
values from insect cell membranes derived from cells infected with virus encoding β-glucuronidase (β-gus) from uptake levels from membranes derived from cells infected with MRP1-encoding baculovirus.

2.9 Photolabelling of MRP1 with azido-[γ-32P]-ATP

8-Azido-[γ-32P]ATP photoaffinity labelling was performed as previously described (184;191). Briefly, Sf21 membrane vesicles (20 µg total protein) were resuspended in TB (10 µL) containing 5 mM MgCl₂ and 5 µM 8-azido-[γ-32P]-ATP. After 5 min on ice in a 96-well plate, the membranes were irradiated for 7 min on ice in a UVP CL-1000 Ultraviolet Cross-linker (λ = 302 nm). Reactions were stopped by the addition of ice-cold buffer (150 µL of 50 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, and 5 mM MgCl₂) to each sample-containing well of the plate. The membranes were transferred to 1.5-mL tubes and pelleted by centrifugation (14000 rpm, 4°C). A second wash step with centrifugation was performed. The pellets were resuspended in Laemmli’s buffer (3x) containing dithiothreitol (100 mM final) and membrane vesicles were electrophoresed on gradient SDS-PAGE (7–15%). Gels were dried (2 h, 65°C) and autoradiographed using AGFA Curix Ultra UV-G Plus medical X-ray film with an intensifying screen. At least three independent experiments were performed using membrane vesicles derived from separate infections of Sf21 cells.

2.10 Beryllium fluoride-induced Trapping of 8-Azido-[α-32P]-ATP by MRP1

Beryllium fluoride ADP trapping was performed as previously described (188;191). Briefly, Sf21 Membrane vesicles (20 µg of protein) were resuspended in TB (10 µL) containing 5 mM MgCl₂, and 5 µM 8-azido-[α-32P]-ATP and incubated at 37°C for 15 minutes in the presence or absence of 200 µM beryllium fluoride. The reaction was started by addition of 8-azido-[α-32P]ATP and stopped by transfer to ice and dilution with ice-cold buffer (150 µL of 50 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, and 5 mM MgCl₂). Unreacted nucleotides were then removed by
two centrifugation steps (14000 rpm, 4°C). Pellets were resuspended in ice-cold buffer (14 μL), transferred to a 96-well plate and irradiated for 7 min on ice in a UVP CL-1000 Ultraviolet Cross-linker (λ = 302 nm). Vesicle proteins were then diluted in Laemmli’s buffer (3x) containing dithiothreitol (100 mM final) and both SDS-PAGE and autoradiography completed as described above.

2.11 Limited trypsinolysis of full-length MRP1

Limited trypsinolysis of MRP1-enriched membranes was carried out as described previously (2;145;279). Membranes were diluted in 50 mM Tris pH 7.4 (1.5 mg of protein ml⁻¹) and treated with diphenylcarbamyl chloride-treated trypsin at trypsin:protein ratios of 1:30000 to 1:15 (w/w) for 15 min at 37 °C. Reactions were stopped by removal of aliquots into Laemmli sample buffer containing leupeptin (16.7 μg ml⁻¹) and phenylmethylsulfonyl fluoride (10 mM), with or without DTT (100mM). Samples (5 μg of total protein) were resolved on a 5-15% gradient SDS-PAGE and transferred to Immobilon-P, as described above. Tryptic fragments of MRP1 were detected by primary mAbs MRPr1 and QCRL1, whose linear epitopes lie within CL3 (amino acids 238–247) and the linker region connecting NBD1 and MSD2, respectively (145;152).
Chapter 3

RESULTS

3.1 Endogenous cysteine residues do not contribute to the formation of naturally occurring disulfide bridges in MRP1

Prior to the application of CSM to MRP1, the possible involvement of the twenty-five cysteines in disulfide bridges in the native structure had to be precluded. To this end, wild-type MRP1 and the NH$_2$-terminally truncated protein, NΔ204, were expressed in Sf21 cells. These two MRP1 proteins were also expressed as separate halves that have been previously shown to form non-covalent associations resulting in a functional transporter (91). For distinction from wild-type, intact MRP1, proteins expressed as half molecules will herein be referred to as MRP1 dual or NΔ204 dual. Migration of these MRP1 polypeptides in the presence or absence of reducing agent (100mM DTT) was compared by SDS-PAGE and detected with monoclonal antibodies directed against the NH$_2$- (MRPr1) and COOH-terminal halves (MRPm6), as described in Materials and Methods. The amount of protein trapped at the interface between the stacking and resolving gels increased in the absence of DTT and this observation was consistent for all constructs examined. No difference in mobility was observed between the presence or absence of DTT upon detection with either antibody for both intact and dual MRP1 and MRP1 NΔ204 (Fig. 3-1, A) and these proteins and half molecules migrated to expected molecular weights. Intact MRP1 proteins were detected at 170 and 150 kDa, consistent with previously reported sizes of the full-length and N-terminally truncated proteins, respectively, upon expression in insect cells (91;159). In addition, to the species at 170 and 150 kDa, minor amounts of partial proteolytic cleavage products of the intact proteins were observed. Apparent molecular weights of ~100 and 80 kDa were observed for the NH$_2$-proximal half of MRP1 dual and NΔ204 dual, respectively (Fig. 3-1, A, top blot), and the COOH-half of both dual constructs migrated to
Figure 3-1. Role of endogenous cysteine residues of wild-type MRP1 in disulfide bridges assessed by SDS-PAGE. Panels A-C, Immunoblots of crude membranes from Sf21 cells infected with baculovirus for co-expressed, wild-type MRP1 fragments. Cells were harvested in the presence NEM to prevent disulfide exchange or the formation of disulfide bonds irrelevant to native protein structure as a result of oxidation during protein preparation procedures. Protein concentrations were determined by the Bradford assay and, for A & C, protein (1.5 μg) was diluted in Laemmli buffer with (+) or without (-) DTT (100mM). A, Detection of MRP1 proteins with MRP1 specific mAbs MRPr1 (top blot) and MRPm6 (lower blot) directed against the NH2- and COOH-proximal halves, respectively. Full-length and NH2-terminally truncated MRP1 (NΔ204) were expressed both as a single polypeptide (intact) and as dual-half fragment (dual). The location of MRP1 half molecules is indicated (block arrows) and the interface between the stacking (4%) and resolving gel (10%) shown (—). B, Crude membranes of MRP1 NΔ204 dual were prepared as described above except samples were exposed to increasing amounts of DTT (0-100mM) in Laemmli buffer. The COOH-terminal peptide was visualized using MRPm6 (block arrow). The presence of three distinct higher-molecular weight species is highlighted: (i) at the interface between the stacking (4%) and resolving gel (10%) (—), (ii) at ~200kDa (double-ended arrow) and (iii) at ~150 kDa (arrow). C, Detection of the co-expressed MRP1 fragments MRP1204-1531 and MRP11-203FLAG with mAb anti-FLAG (right) and MRPr1 (left), respectively. Bands corresponding to intact MRP1 peptides are indicated (block arrows). Bands corresponding to MRP1 degradation products are also detected at ~75 kDa. Additionally, lower mobility bands observed in the absence of DTT using an Anti-FLAG mAB are identified (double-ended arrow).
Figure 3-1
an apparent molecular weight of 65 kDa (Fig. 3-1, A, lower blot). Locations of these fragments are indicated by block arrows. Taken together, the data indicate that neither the intact MRP1 polypeptides nor the half molecules of MRP1 appear linked by disulfide bonds.

In addition to the band representative of the COOH-half-molecule, minor amounts of high molecular weight species are present in the lower blot of Figure 3-1, representative of previously reported oligomers of the COOH-proximal half (91;191). These oligomers are observed regardless of reducing agent, albeit at almost undetectable levels in the presence of DTT, suggesting that this interaction is not mediated solely if at all by disulfide bonds. While the size of this band is more consistent with heterodimers of the NH2- and COOH-half molecules, this can be excluded since no band of a similar size was observed when blots were probed with MRP1 (Fig. 3-1, A, top blot). To more fully investigate the apparent homodimerization of the COOH-half-molecules, MRP1 NΔ204 dual was subjected to SDS-PAGE after titration with DTT (1-100 mM). The resultant immunoblot was probed with MRPm6 (Fig. 3-1, B) for detection of the COOH-proximal half alone. Two higher molecular weight species were identified and observed across the DTT titration. The first appears to be retention of protein at the stacking and resolving gel interface similar to that observed in Figure 3-1 A and the second, minor higher molecular weight species seen at an apparent molecular weight of ~150 kDa (Fig. 3-1, B, arrow) appears to be the same species as that observed in Figure 3-1 A and described previously (91). At that time, this 150 kDa species was concluded to be homodimers of the COOH-proximal fragment of MRP1 resulting from hydrophobic interactions given that its level was observed to increase with less rigorous denaturation. In addition to the two species previously observed, a third higher molecular weight species (double-headed arrow) began to appear when protein samples were treated with ≤10 mM DTT (Fig. 3-1, B, double-headed arrow). The apparent size of this species could be consistent with trimers of the COOH-proximal half molecule although interactions with non-MRP1 proteins cannot be excluded based on these results.
Co-expressed fragments of MRP1 representing MSD0 and its core domain (residues 204-1531) also form associations (150) and a similar approach to that described above was used to investigate cysteine-mediated interactions between these MRP1 fragments. Briefly, co-expressed fragments corresponding to MRP1 MSD0 and the core domain were subjected to SDS-PAGE in the presence or absence of DTT (100mM) and resultant immunoblots examined for shifts in mobility. For detection of the core domain, immunoblots were probed with the mAb MRPr1. This revealed a significant amount of protein at the interface of the stacking and resolving gels and degradation product at ~75 kDa, but the banding pattern for the core domain was otherwise identical in the presence and absence of reducing agent (Fig. 3-1, C). Since there are no commercially available antibodies directed against MSD0 of MRP1, detection of this domain was achieved through COOH-terminal fusion of a FLAG epitope (MSD0-FLAG). Immunoblots that were initially probed with MRPr1 were stripped and re-probed with an anti-FLAG mAb. Interestingly, the mobility of MSD0-FLAG was altered in the absence of DTT such that, in addition to the major species observed at ~20-25 kDa, three additional very minor bands were observed that migrated to apparent molecular weights between 50-75 kDa. The size of the major species is consistent with both the predicted size of MSD0-FLAG (http://www.expasy.ch/tools/pi_tool.html) and previous reports of this peptide. Differential glycosylation is unlikely to account for the additional bands observed since proteins were expressed in Sf21 cells. These cells differ from mammalian cell lines in the pathways related to processing of N-linked oligosaccharides and generally produce proteins with less complex glycosylation. The size of these higher molecular weight species would be consistent with trace amounts of dimers or oligomers of this domain. However, the possibility that these bands are the result of interactions of this domain with non-MRP1 proteins cannot be excluded. Finally, in the absence of DTT, no band was detected near the expected molecular weight of an associated complex of MRP1 MSD0 and MRP1 core (~170 kDa) with either antibody, indicating that SDS is
sufficient to disrupt interactions between these domains and MSD0:core MRP1 interactions are not mediated by the cysteine residues.

3.2 The expression of the COOH-terminal half of MRP1Δ204 dual lacking cysteines in MSD2 is greatly impaired under the control of the p10 promoter.

Previously, mutation of individual cysteines located within MSD0 of MRP1 was shown to have some deleterious impact on the trafficking, conformation and function of the full-length, intact transporter (2). In that study, cysteines were substituted with either the more conservative serine or with alanine and in general, the latter was preferred due to less deleterious effects. Deleterious serine substitution of some of the endogenous cysteines in the distantly related ABC transporter, CFTR, has also been observed (93;241). Taking into account these results and since it has been shown previously by our group and others that that the MSD0 region of MRP1 is not required for MRP1 transport activity (3;4;159), a cys-less variant of MRP1 lacking MSD0 was created by Lee and Altenberg (5). This protein retained ~60% function when expressed in *S. cerevisiae* but has not been used for the application of CSM. Here, our goal was to generate a dual-half, cys-less template of MRP1 lacking MSD0 for CSM studies. Dual-half expression of MRP1 was selected over the expression of the intact protein under the presumption that, in future CSM experiments, formation of covalent interactions (i.e. disulfide bridges) between the two half molecules would result in a greater mobility shift than would be observed using the intact protein. To this end, cloning was undertaken that allowed the mutation not of the individual cysteines of MRP1, but rather mutation of all cysteines contained within a particular domain (i.e. MSD1 or NBD1). The resultant wild-type and cys-less MRP1 NΔ204-dual constructs are depicted in Figure 3-2. These constructs were initially cloned into pFB dual (an expression cassette which allows the simultaneous expression of both halves of MRP1 dual) similar to wild-type MRP1 NΔ204-dual, as described previously (163). Levels of expression of wild-type and cysteine mutant proteins were determined as described in *Materials and Methods* using mAbs to each half
Figure 3-2. Expression of wild-type and various cysteine mutant MRP1 NΔ204 dual constructs. A, Schematic of completely or partially cysteine-less halves of MRP1 lacking MSD0 (NΔ204 dual). Wild-type (blue) and Cysteine-less (red) domains within each proximal half are illustrated. Locations of endogenous cysteines which were mutated to alanine are indicated. B, Immunoblot of crude membranes (2 μg from Sf21 cells) following amplification of first generation baculovirus is shown of the constructs in A. MRP1 NH2- and COOH-proximal halves were detected by mAbs QCRL-1 and MRPm6, respectively, and the location of these halves is indicated (block arrows). Double-headed arrows highlight the absence of the cysteine-less COOH-proximal half (lower band, lanes 7, 10 & 11) even when the NH2-proximal half is expressed at levels comparable to wild-type (upper band). C, As in B, except 10μg crude membrane protein was loaded for constructs lacking endogenous cysteines in the COOH-proximal half (lanes 2-4) and for detection of this half alone (block arrow), this blot was probed with the mAB MRPm6.
Figure 3-2
molecule, QCRL-1 (epitope 918-924) and MRPM6 (epitope 1511-1520) (145;152); neither epitope for these antibodies contains cysteine residues. In all constructs, the NH₂-proximal half was expressed at levels comparable to wild-type (Fig. 3-2, B, top band). However, when equivalent amounts of total membrane protein were examined, no band corresponding to the COOH-half was detectable for MRP1 NΔ204 dual constructs in which the entire COOH-terminal half lacked cysteines (Fig. 3-2, B, lanes 7, 10 and 11). Even upon overexposure (data not shown), the cysteine-less COOH-half molecules remain undetectable with no obvious degradation products present. Upon loading 20 times more total membrane protein for these constructs, a band corresponding to the cysteine-less COOH-half molecule was detected (Fig. 3-2, C, lanes 2-4). In contrast, when Cys→Ala substitutions were made only within NBD2 and the five endogenous cysteines within MSD2 remain untouched, this partially cys-less COOH-half was detectable at levels similar to wild-type (Fig. 3-2, B, lanes 5, 6 and 8). While these data indicate that Cys→Ala substitution in MSD2 severely inhibits the biogenesis of the COOH-proximal half, parallel work in our laboratory with an intact version of the similar construct indicated that this may not necessarily the case (Dr. Lei Qin, unpublished data). Sequencing of the 5’ region of these constructs including the p10 promoter also did not reveal any obvious explanations for this lack of expression. Cloning was undertaken to ensure that inequality of the two promoters in the dual-expression cassette was not contributing to the lowered expression levels so that all constructs in which either all cysteines in the COOH-terminal half or just those cysteines within MSD2 were mutated to alanine were placed under control of the opposite promoters. The NH₂- and COOH-proximal half molecules that were originally cloned under control of the polyhedron and the p10 promoters, respectively, were re-cloned such that expression was now driven by the p10 and polyhedrin promoters, respectively. Wild-type MRP1 NΔ204 dual was cloned similarly and denoted ‘promoter switch’ (PSW) MRP1 NΔ204 dual. Figure 3-3 provides a schematic of the typical (A) and promoter switch (B) arrangement of the two halves of MRP1 in pFB.
Figure 3-3. Expression of wild-type and various cysteine mutant MRP1 NΔ204 dual promoter switch constructs. A, Schematic of wild-type (blue) and partially cysteine-less (red) MRP1 NΔ204 dual constructs. Polyhedron (PH) or p10 promoters from the dual-expression cassette, pFB dual, that drive the expression of the NH$_2$- and COOH-proximal halves, respectively, are identified. B, Schematic of ‘promoter switch’ MRP1 NΔ204 dual constructs. Wild-type (blue) and completely or partially cysteine-less (red) domains of MRP1 lacking MSD0 are illustrated. Promoters, polyhedron (PH) or p10, driving expression of each half of MRP1 are indicated. C, Immunoblot of membrane vesicle protein (0.5 μg) from Sf21 cells expressing wild-type and various cysteine mutants of MRP1 NΔ204 following PSW cloning. Constructs from B are distinguished from those in A by the nomenclature PSW. The NH$_2$- and COOH-halves of MRP1 were detected with MRP1 mAbs MRPr1 and MRPm6 and the location of these is indicated (block arrows).
Figure 3-3
Comparison of the expression levels and transport activity of MRP1 NΔ204 dual and PSW MRP1 NΔ204 dual was done to validate the use of the latter construct as a positive control in future experiments for similarly cloned mutants. Levels of expression of each half molecule were determined by comparison with intact MRP1 NΔ204 and revealed virtually identical amounts of each fragment (App. I, B). Further, similar levels of transport of LTC₄ and E₂17βG were observed (App. I, C). However, since PSW NΔ204 dual displayed slightly lower levels of E₂17βG transport, this construct was used as the positive control in subsequent experiments for similarly cloned PSW cysteine mutants. Subsequently, expression levels of PSW MRP1 NΔ204 dual with Cys→Ala mutations in just MSD2 or the entire COOH-half were found to be greatly improved (Fig. 3-3, D, lower blot, lanes 4-7) and sufficient for functional characterization.

3.3 Endogenous cysteines within CL3 and the core domains of MRP1 are essential for transport function in Sf21 cells.

Once able to express enough of each cys-less fragment, functional consequences of Cys→Ala substitutions in MRP1 NΔ204 dual were examined by vesicle transport studies. As outlined above, MRP1 NΔ204 dual and the promoter switch counterpart, PSW MRP1 NΔ204 dual, were used where applicable as positive controls in these experiments and are clearly indicated on separate graphs. Densitometry revealed that the half molecules of the cys-less mutants were expressed at levels ranging from 0.4- to 4.4-times wild-type (Fig.3-4, A). Transport levels have been normalized to account for observed differences in MRP1 expression levels. Further, these levels are shown as percent wild-type and are the means and standard deviations of three independent experiments (Fig. 3-4, B and C).

Alanine substitution of all endogenous cysteines within either half molecule or in both halves together diminished transport of both substrates, albeit to a greater extent for LTC₄. PSW MRP1 NΔ204 dual completely devoid of cysteines retained only 18% of the LTC₄ transport activity displayed by wild-type, while 62% of E₂17βG transport levels were achieved by this mutant (Fig.
Figure 3-4. Alteration of the transport activity of MRP1 NΔ204 dual as a result of alanine substitution of endogenous cysteine residues. A, Representative slot blot probed with mAbs MRPr1 and MRPm6 to ascertain expression of the MRP1 half molecules. Membrane vesicles were prepared from Sf21 cells expressing wild-type MRP1 NΔ204 dual, its promoter switch counterpart and various cysteine mutant constructs. Levels of expression of wild-type and mutant dual constructs were estimated by densitometry as described in Materials and Methods. Values of each half molecule relative to the appropriate wild-type MRP1 fragment are indicated below each band and similar values were obtained from at least one additional infection. The amount of total membrane protein loaded is also shown (above blot). B & C, Levels of [3H]LTC4 (B) and [3H]E217βG (C) uptake by membrane vesicles in A were determined and corrected to take into account observed differences greater than ±10% in MRP1 protein expression. MRP1-dependent transport was determined by subtraction of uptake resulting from membrane vesicles infected with baculovirus for β-gus (empty vector control) from uptake from vesicles infected with wild-type and mutant MRP1 proteins. Transport levels are shown as percent wild-type. In panels B & C, the graph on the left includes all cys-less variants (shaded bars) for which MRP1 NΔ204 dual (black bars) acted as positive control where the graph on the right includes all PSW cys-less variants (shaded bars) for which PSW MRP1 NΔ204 dual (black bars) acted as the positive control. Bars represent means ± S.D from three independent experiments using vesicles derived from at least two separate infections.
Figure 3-4
Similarly, PSW MRP1 NΔ204 dual lacking cysteines in only the COOH-half molecule retained 51% and 73% of LTC₄ and E₂₁⁷βG transport levels, respectively. This apparent substrate-specific effect was not observed when Cys→Ala replacements are made only in the NH₂-half molecule and transport of both substrates was virtually abolished to just ~10% wild-type function (Fig. 3-4, B and C). Thus, cysteine residues in both halves of MRP1 NΔ204 dual are required for full transport activity of the protein.

In order to delineate which groups of cysteines were contributing to the reduced transport activity, the five endogenous cysteines within MSD2 were substituted with alanine (cys-less MSD2). Replacement of endogenous cysteines within this domain also displayed the substrate specific influence described above for constructs lacking cysteines in the entire COOH-half molecule. Uptake of [³H]E₂₁⁷βG by this construct was comparable to, or slightly increased from wild-type levels while a nearly 40% reduction in [³H]LTC₄ uptake was seen for this construct. Since cys-less MSD2 demonstrated increased transport activity of both substrates when compared to the completely cys-less COOH-half, it was co-expressed with cys-less NH₂ to see if this increased function was able to compensate for the almost complete loss of function of cys-less NH₂ NΔ204. Co-expression of the cys-less NH₂-half molecule with the COOH-half molecule lacking cysteines only in MSD2 resulted in LTC₄ and E₂₁⁷βG transport levels of 21% and 45% compared to wild-type, respectively, representing an increase over the levels displayed by the cys-less NH₂ mutant (Fig. 3-4, B and C). Thus, substitution of cysteines in the NH₂-proximal half of MRP1 is sufficient to disrupt transport of both substrates and Cys→Ala substitutions in both halves of MRP1 seem to more severely alter transport of LTC₄. Conversely, alanine substitution of the cysteines in MSD2 of MRP1 NΔ204 dual selectively increased the levels of E₂₁⁷βG transport.
3.4 Endogenous cysteines located in the NBDs of MRP1 are dispensable for transport of 
LTC₄ and E₂₁βG in S²¹ cells.

In order to more clearly define cysteines important for activity of MRP1, endogenous 
cysteines located in the nucleotide binding domains, NBD1 (amino acids 653-932) and NBD2 
(amino acids 1299-1531) were mutated to alanine. Resultant constructs were expressed in S²¹ 
cells and MRP1 protein levels were determined to range from 0.4- to 1.0-fold wild-type (Fig 3-5. 
A). Transport values were normalized to account for differences in MRP1 expression and are 
shown as the mean of three independent experiments. All partially cys-less constructs retained 
the ability to transport LTC₄ and E₂₁βG (Fig. 3-5, B). For these mutants, transport levels of 
E₂₁βG were indistinguishable from wild-type. MRP1 NΔ204 dual lacking cysteines in NBD1, 
NBD2 or in both NBDs displayed increased levels of LTC₄ transport, between ~1.5- and 2.0-fold 
wild-type. Given that Cys→Ala substitution in the NBDs did not reduce transport activity of 
MRP1, these constructs show promise for use as template for future CSM studies on this 
transporter.

3.5 Alanine substitution of endogenous cysteines within the NBDs is sufficient to alter the 
nucleotide binding profile of MRP1 NΔ204 half-molecules.

Having observed a general increase in the transport levels displayed by dual constructs 
lacking cysteines in either or both NBDs, interactions of these cysteine mutants of MRP1 with 
nucleotidide were analyzed. ATP-binding was examined by photolabelling with 8-azido-[γ-
³²P]ATP under non-hydrolytic conditions (4°C) of wild-type MRP1 NΔ204 dual and the mutant 
lacking endogenous cysteines in both NBDs (Fig. 3-5). It has been demonstrated extensively by 
our group and others that labelling of MRP1 under these conditions occurs preferentially at 
NBD1 and to a lesser extent at NBD2. Also, labelling at both NBDs can be strongly competed by 
the poorly hydrolysable analogue, ATP-γ-S. Consistently, ATP labelling occurred preferentially 
on NBD1 and to a lesser extent on NBD2 of both the wild-type and the cys-less NBDs mutant of
Figure 3-5. Functional characterization of alanine substitutions of endogenous cysteine residues within the NBDs of MRP1 NΔ204 dual. A, Expression levels of MRP1 NΔ204 dual constructs lacking cysteines within either NBD1, NBD2 or both NBD1/2 were examined by slot blot as described in the legend for Figure 3-4. B, Transport assays for [3H]LTC4 and [3H]E217βG were completed on membrane vesicles for proteins in A (MRP1 NΔ204 dual (black bar), all cys-less mutants (shaded bars)), as described in the legend for Figure 3-4. C and D, Representative autoradiographs resulting from photolabelling of MRP1 NΔ204 dual and the equivalent construct in which Cys→Ala substitutions have been made in both NBDs (cys-less NBD1/2) with 8-azido-[32P]-nucleotides. Protein levels were adjusted for observed differences in MRP1 expression and then subjected to SDS-PAGE on 7-15% gradient gels. Autoradiographs were obtained following exposure of dried gels to X-ray film (6-24 hrs, RT) following treatment as described in detail below. Similar results were obtained in at least three independent experiments using membrane vesicles derived from separate infections of Sf21 cells. The positions of labelled endogenous proteins (E) and labelled MRP1 NH2- and COOH-half molecules are indicated. C, at 4°C, membranes (20 μg) were incubated with 8-azido-[γ-32P]-ATP (5 μM) in the absence (−) or presence (+) of ATP-γ-S (500 μM) in TB with 5 mM MgCl2. Samples were cross-linked (λ = 302 nm) and unincorporated nucleotides were removed by centrifugation. D, Under hydrolytic conditions (37 °C), membranes (20 μg) were incubated with 8-azido-[α-32P]ATP (5 μM) in the presence (+) or absence (-) of trapping agent, BeFx, in TB with 5 mM MgCl2. Unincorporated nucleotides were removed by centrifugation prior to cross-linking (λ = 302 nm).
### Figure 3-5

#### A.

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#### B.

**LTC₄ Uptake (mg wildtype after 3 min)**

- Nt204 Dual
- Cysless NBD1
- Cysless NBD2
- Cysless NBD1/2

**E-17G Uptake (mg wildtype after 5 min)**

- Nt204 Dual
- Cysless NBD1
- Cysless NBD2
- Cysless NBD1/2

#### C.

**ATP-γ-S**

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#### D.

**BeFx**

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**8-azido [γ-³²P] ATP (4°C)**

- NH₃-half
- COOH-half
- E

**8-azido [α-³²P] ATP (37°C)**
NΔ204 dual (Fig 3-5, C). However, labelling on NBD1 was observed to be slightly decreased from wild-type levels on cys-less NBD1. ATP-γ-S strongly competed 8-azido-[γ-32P]ATP labelling on both wild-type and cys-less NBD1 and NBD2. Similar results were obtained in at least two to three independent experiments.

In order to ascertain whether Cys→Ala substitutions in the NBDs altered ATP hydrolysis, ADP trapping experiments were performed under hydrolytic conditions (37°C) using 8-azido-[α-32P]ATP in the presence of absence of beryllium fluoride (BeFx). BeFx has been used as a trapping agent with ABC transporters and mimics the pre-hydrolysis, ground state with bound ATP and Mg2+ by occupying the space normally taken up by the γ-phosphate (280;281). In wild-type MRP1, BeFx-dependent trapping of nucleotide occurs preferentially at NBD2, the major site of ATP hydrolysis. This photolabelling pattern was observed on both wild-type and cys-less NBDs in the NΔ204 dual constructs (Fig 3-5, D) and the intensity of the photolabelling pattern was comparable between the wild-type and cys-less NBDs. Interestingly, under these conditions, low-level photolabelling of cys-less NBD1 was detected in the absence of BeFx where similar labelling of wild-type NBD1 was not detected. Taken together, the transport data and photolabelling studies indicate that MRP1 NΔ204 dual with complete alanine replacement of cysteines in both NBDS is a suitable template for CSM of these domains.

3.6 Introduction of C775 and C1329 into cys-less NBD1/2 MRP1 NΔ204 dual significantly reduces transport activity.

Preliminary CSM and cross-linking experiments were focused on the NBDs of MRP1 given that MRP1 NΔ204 dual with complete Cys→Ala substitution in both NBDs was proven as a viable, functional template for this method. Residues R775 in NBD1 and T1329 in NBD2 of MRP1 were chosen for mutation to Cys based on a previous finding in the related ABC transporter, CFTR/ABCC7, that comparable residues were evolutionarily coupled and hydrogen bonded together (6). These residues were also selected due to their location within, or proximity
to, conserved motifs of the NBDs proposed to interact during the catalytic cycle of MRP1; R775 is located just COOH-terminal to the Signature 'C' sequence of NBD1 and T1329 is located in the Walker A motif (Fig. 3-6, A). For data obtained in cross-linking studies to be interpreted in a biological significant context, the impact of these introduced cysteines on the biogenesis and function of MRP1 was ascertained. Western blot analysis revealed that all mutant constructs were expressed at levels comparable to wild-type and the NH2- and COOH- proximal halves were observed to have the expected apparent molecular weights of 80 kDa and 65 kDa, respectively (Fig. 3-6, B). Oligomers resulting from hydrophobic interactions of the COOH-half described earlier were again noted (Fig. 3-6, B, lower blot). Densitometry revealed that the mutant NH2- and COOH-half molecules were expressed at levels ranging from 0.6- to 0.9-fold wild-type and transport levels were adjusted to account for these differences (Fig. 3-7, A). For introduced cysteine mutants, R775C, T1329C and the double mutant, LTC4 and E217βG transport levels are described relative to the parental protein, cys-less NBD1/NBD2 MRP1 NΔ204 dual, since this protein displayed slightly increased levels compared with wild-type. Introduction of the single and double cysteines greatly attenuated transport of both substrates (Fig. 3-7, B). Mutant R775C displayed LTC4 transport levels that were ~60% of the activity displayed by the parental protein. Similarly, transport of this same substrate by both mutant T1329C and the double mutant, R775C/T1329C, showed greater impairment with levels reduced by at least 80%. Furthermore, both single mutants equally inhibited transport of E217βG by ~80% when compared to the cys-less NBD1/NBD2. The double mutation did not further reduce transport levels of this substrate over either of the single mutations. Due to the limited functionality resulting from the introduction of these cysteines, the use of this construct for cross-linking is questionable.
Figure 3-6. Expression of cys-less NBD1/2 MRP1 NΔ204 dual following introduction of cysteines at positions 775 and/or 1329.  A, Topological model of MRP1 NΔ204 dual with Cys→Ala mutations of both NBDs. Locations of endogenous cysteine residues remaining in the CSM template are shown (©). Wild-type primary sequences of and surrounding the Signature ‘C’ motif of NBD1 and the Walker ‘A’ motif of NBD2 are shown in dropdown text and residues, R775 and T1329, selected for mutagenesis to cysteine underlined.  B, Expression of R775C, T1329C and the double mutant, R775/T1329C, are examined in comparison to the parental protein, cys-less NBD1/2 NΔ204 dual and wild-type MRP1 NΔ204 dual. Membrane vesicles were prepared from Sf21 cells and subjected to SDS-PAGE. Blots were probed with mAbs MRP1 and MRPm6 and the location of the NH₂- and COOH-proximal half indicated (block arrows).
Figure 3-6
Figure 3-7. Functional impact of R775C, T1329C and R775/T1329C on the transport activity, photolabelling and trapping of 8-azido-[32P] nucleotides by MRP1 NΔ204 dual. A, Wild-type (NΔ204 dual) and mutant (cys-less NBD1/2, R775C, T1329C and R775/T1329C) MRP1 expression levels were examined by slot blot as described in the legend for Figure 3-4. B, The ability of mutants R775C, T1329C and the double mutant (open bars) to transport [3H]-labelled organic anions was examined. For transport assays, membrane vesicles from A were incubated with tritiated substrate (50 nM [3H]LTC4 or 400 nM [3H]E217βg) in TB with MgCl2 (10 mM) and either ATP or AMP (4 mM). LTC4 and E217βg assays were performed at 23°C for 3 minutes or 37°C for 5 minutes, respectively. Values are shown as percent wild-type and bars represent means ± S.D from three independent experiments using vesicles from different infections. C & D, Representative autoradiographs resulting from cross-linking of MRP1 mutants R775C, T1329C and R775/T1329C with either 8-azido-[γ-32P]-ATP (Panel C), in the presence (+) or absence (-) of competing amounts of ATP-γ-S (A), or 8-azido-[α-32P]-ATP (Panel D) in the presence (+) or absence (-) of BeFx. Methodology followed for C & D as described in legend for Figure 3-5. Similar results were obtained from at least 2-3 additional vesicle preparations and independent experiments. The positions of labelled endogenous proteins (E) and MRP1 NH2-half and COOH-half molecules are indicated (double-ended arrows).
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B.

![Graphs showing normalized LTC4 and E17/g uptake](image)

C.

![Western blots with different conditions](image)

D.

![Additional Western blots](image)

Figure 3-7
3.7 Introduction of cysteine residues, C775 and C1329, does not alter the nucleotide binding characteristics of MRP1 but appears to increase BeFx-independent trapping.

The influence of the introduced cysteines on nucleotide interactions was investigated given the location of the introduced cysteines residues within the NBDs and in light of their detrimental effect on transport activity of MRP1. Photolabelling experiments to assess the nucleotide binding and hydrolysis capacity of the cys-less NBDs were done on each of the single and double introduced cysteine variants. Briefly, photolabelling was done with either 8-azido-[γ-32P]ATP under non-hydrolytic conditions (4°C) or 8-azido-[α-32P]ATP under hydrolytic conditions (37°C) in the presence or absence of BeFx. Consistent with previous reports of wild-type and as seen for the parental protein, preferential photolabelling on NBD1 was observed for all of the introduced cysteine mutants when 8-azido-[γ-32P]ATP was used under non-hydrolytic conditions (Fig. 3-7, C). For all proteins examined, photolabelling at NBD1 under these conditions was strongly competed with ATP-γ-S. Further, as expected, minimal labelling was detected on NBD2 of the parental and introduced cysteine mutants. The data indicate that the ability of these constructs to bind ATP is relatively unaffected. In contrast, both of the single mutants and the double mutant displayed significantly different nucleotide trapping profiles in comparison to the parental template (Fig. 3-7, C). For these constructs, the intensity of photolabelling at both NBDs under hydrolytic conditions in the presence of BeFx was significantly reduced. Indeed, very little photolabelling occurred at either NBD of the double mutant, R775/T1329C. Moreover, all three cysteine mutants, C775, C1329 and C775/C1329 retained some extent of BeFx-independent trapping on NBD1 as observed for the cys-less parental protein. Taking into account the altered profiles of ATP-binding and hydrolysis with the limited functionality of the introduced cysteine mutants, alternate locations for cysteine introduction within the NBDs of MRP1 should be considered for future CSM-based investigations.
3.8 Cys-less MSD0-CFP does not traffic to the plasma membrane of HEK293 cells when stably expressed in the absence of the core domain of MRP1.

Focus was next directed to the additional NH$_2$-terminal MSD of MRP1, MSD0 and the application of CSM to this domain. Ideally, CSM would provide a means to probe both the intra- and interdomain interactions of MSD0. Since MSD0 has been demonstrated to be largely dispensable for activity of MRP1, template validation of a cys-less variant of MSD0 for CSM cannot be achieved by transport assays and photolabelling experiments. Instead, advantage was taken of the demonstrated ability of wild-type MSD0 to traffic independently to the plasma membrane in HEK293 cells when COOH-terminally fused to CFP for detection (150) presuming that retention of this ability by cys-less MSD0 would strongly suggest correct folding of this domain. Wild-type and cys-less MSD0-CFP fusion proteins (Fig. 3-8, A) were stably expressed in HEK293 cells and examined by confocal microscopy; resultant images are shown in Figure 3-8. Similar to previous reports, the majority of the wild-type fusion protein localized to the plasma membrane, with minor accumulation within intracellular, vesicular-like structures (150). In contrast, trafficking of cys-less MSD0-CFP was impaired and this protein displayed diffuse, intracellular staining. Co-localization studies with the rough ER-associated protein, calnexin, showed that this cys-less CFP-fusion protein is retained within this cellular apparatus as indicated by the white in the merged images of Fig. 3-8.

3.9 Individual alanine substitution of Cys7 or Cys32 is sufficient to disrupt membrane localization of MSD0-CFP.

It has previously been suggested that Cys7 and Cys32 located in the extracellular NH$_2$-tail of MRP1 are important in maintaining the structural integrity of this tail, since individual or double mutation disrupted function of full-length MRP1 while PM localization was unaltered (1). However, similar observations were not obtained when our group expressed individual mutations
Figure 3-8. Cellular localization of wild-type and cys-less MSD0 CFP-fusion proteins by confocal microscopy. A, Topological model of wild-type MSD0 is depicted and the position of the COOH-terminal CFP fusion following MRP1 residue 203 shown. The 5 TM helices are illustrated and within these, the location of endogenous cysteines that were substituted with alanine are indicated (©). B, Confocal microscopy images of stable HEK293 cell lines expressing wild-type or cys-less MSD0-CFP. The endoplasmic reticulum was detected using a mAb against calnexin in conjunction with Alexa 594 (red) and nuclei stained with Hoechst 33342 (blue). Isolated signal from MRP1 MSD0-CFP is shown in the first panel of each row (cyan). Overlays of all three images are shown in the third (field view) and fourth (zoom) panels, with areas of co-localization (white) indicated (yellow arrows). Scale bars can be found in the second panel of each row and represent 30 μm and 16 μm for images of wild-type and cys-less MSD0-CFP, respectively.
Figure 3-8
of these cysteines in HeLa cells (Dr. Elaine Leslie, unpublished work). To further examine the potential role of these cysteines, Ala substitutions were made at each of these positions in wild-type MSD0-CFP and cellular localization was examined. Substitution of either Cys7 or Cys32 was sufficient to disrupt plasma membrane localization of the mutated MSD0 given that no plasma membrane associated protein was detected (Fig. 3-9, A, column 1). Not surprisingly then, no plasma-membrane associated MSD0-CFP was detected for the double mutant, C7/32A (Fig. 3-9, A, column 1). Mutants C7A, C32A and C7/32A all displayed a similar diffuse, intracellular staining pattern indicative of retention in the ER and this was verified by co-localization studies with the ER marker calnexin (Fig. 3-9, A, columns 3 and 4).

Having established that alanine substitution of either Cys7 or Cys32 was sufficient to disrupt trafficking of MSD0-CFP, we next investigated whether re-introduction of these cysteines into cys-less MSD0-CFP would restore the plasma membrane localization of cys-less MSD0-CFP. However, this was not found to be the case and the cellular staining pattern of fusion proteins into which these cysteines were restored was unaltered from the cys-less parent protein (Fig. 3-9, B, column 1). Mutants A7C, A32C and A7/32C in cys-less MSD0-CFP remain trapped in the ER as demonstrated by co-localization with calnexin, similar to the cys-less template into which they were re-introduced (Fig. 3-9, B, columns 3 and 4).

3.10 Cys-less MSD0-CFP fusion proteins are differentially glycosylated in mammalian cells.

The relative expression levels of MSD0-CFP fusion proteins in crude membranes from stably transfected HEK293 cells were estimated by immunoblotting. All constructs were detectable under the conditions used (Fig. 3-10, A) but constructs in which the cysteine at position 7 was mutated to alanine (C7A and C7/32A) were expressed at very low levels. Wild-type MSD0-CFP was observed to migrate with an apparent molecular weight of ~50 kDa. However, MSD0-CFP fusion protein mutants C7A, C32A and the double mutant showed greater mobility and migrated with a size of ~40 kDa. The parental cys-less MSD0-CFP fusion protein and the re-introduced
Figure 3-9. Localization and subcellular distribution of wild-type and cys-less MSD0-CFP fusion proteins with mutations at positions 7 and 32. Confocal images of stable HEK293 cell lines of wild-type MSD0-CFP, cys-less MSD0-CFP and associated alanine substitution or re-introduction mutants of cysteines 7 and/or 32. The top three rows (A) depict the effect on cellular localization of Cys→Ala substitutions in a wild-type MSD0-CFP at positions 7 and/or 32, while the bottom three rows (B) show the effect of re-introduction of cysteines at these positions in cys-less MSD0-CFP (CFP (cyan), calnexin (red), nuclei (blue)). Overlays are presented in the third (field view) and fourth (zoom) panels of each row, with areas of co-localization between signals (white) indicated (yellow arrows). Where applicable, areas within field view that are magnified in zoom view are outlined by in the third panel (yellow square). Scale bars can be found in the second panel of each row and represent 30 μm for all panels with the exception of those for C7A and C7/32A in which scale bars represent 19 μm and 15 μm, respectively.
Figure 3-9
Figure 3-10. Expression and oligomerization of wild-type, cys-less and related mutant MSD0-CFP fusion proteins. A-D, Immunoblots of MSD0-CFP fusion proteins independently expressed in HEK293 cells. Since no commercial mAB specific for MSD0 is available, MRP1 MSD0-CFP proteins were detected using a HRP-conjugated primary mAB directed against GFP and related fluorescent proteins and are indicated. The first 4 lanes of each blot contain mutants A7C, A32C and A7/32C (lanes 1-3) and their parental protein, cys-less MSD0-CFP (lane 4) while mutants C7A,C32A and C7/32A (lanes 6-8) and their parental protein, wild-type MSD0-CFP (lane 5) can be found in the last 4 lanes. Crude membrane preparations from the stable HEK293 cell lines expressing MSD0-CFP fusion proteins were subjected to SDS-PAGE in the presence of DTT (panel A) or treated with PNGaseF and then subjected to SDS-PAGE (panel B). Migration of MRP1 MSD0-CFP fusion proteins in SDS-PAGE were assessed in the presence (C) or absence (D) of DTT (100mM). Total amounts of protein loaded in panels B-D were normalized for differences in expression between MRP1 MSD0-CFP fusion proteins observed in A, D, High-molecular weight species not observed in the presence of DTT are indicated (block arrow).
Figure 3-10
mutants A7C, A32C and A7/32C also demonstrated increased mobility compared to wild-type MSD0 with bands corresponding to these proteins detected at an apparent molecular weight of ~40 kDa. Treatment with PNGaseF, which removes N-linked glycosylation, revealed that this difference in mobility was due to differential glycosylation patterns (Fig. 3-10, B), in support of ER-retention of these mutant proteins.

3.11 MSD0-CFP fusion proteins with alterations at positions C7 and C32 form DTT-sensitive oligomers.

Mutation of Cys7 and Cys32 in independently expressed, wild-type MRP1_1-281, inclusive of MSD0 and CL3, have been previously reported to result in dimerization of this peptide (1). The oligomeric status of the MSD0-CFP fusion proteins was examined by omission of reducing agent and separation by SDS-PAGE. The total amount of protein loaded was adjusted to account for observed differences in MSD0-CFP fusion protein expression. Trapped protein was observed only in the absence of DTT (compare Fig. 3-10, C and D). Mobility of cys-less and wild-type MSD0-CFP was unaltered by the lack of reducing agent (compare Fig. 3-10, C and D) except that wild-type protein apparently trapped at the gel interface appears as more of a smear. In contrast, a significant proportion of cys-less MSD0-CFP with A7C, A32C and A7/32C was observed to migrate to a higher apparent molecular weight of ~120kDa (Fig. 3-10, C and D). Indeed, the proportion of A32C MSD0-CFP protein migrating to this higher molecular weight was so large that the monomer observed in the presence of DTT at ~40 kDa was undetectable. Mutants C7A and C32A also formed these oligomers and bands are seen at 120 kDa (Fig. 3-10, C and D). Taken together, the data indicate that MSD0 completely devoid of cysteine residues is in an altered conformation from wild-type. Further, while alanine substitution of either Cys7 or Cys32 in an otherwise wild-type MSD0 also resulted in an altered conformation of this domain, re-introduction of these in cys-less MSD0 was not sufficient to restore the wild-type MSD0
phenotype. In the absence of the core domain, the altered conformation of the cysteine mutants of MSD0 allowed this domain to form oligomers that are not formed by wild-type MSD0.

3.12 MSD0-CFP fusion proteins are unable to associate with the core domain of MRP1.

In an attempt to increase the amount of cys-less MSD0-CFP protein on the plasma membrane, co-expression studies of this peptide with the core domain were undertaken. Previous work by our group has demonstrated that co-expression of MSD0 with the core domain of MRP1 increased the plasma membrane localization of the MSD0-less protein (150). Co-expression studies have also demonstrated that MSD0 can restore the PM localization of a MRP1 NΔ204-YFP fusion protein, which accumulated in intracellular vesicles when expressed independently (150). Here, co-expression of MRP1 NΔ204-YFP with wild-type or cys-less MSD0-CFP and related mutants was performed to see if plasma membrane localization of these domains would be restored. Confocal microscopy confirmed the accumulation of MRP1 NΔ204-YFP in the previously described large, intracellular vesicular-like structures while the trafficking of MRP1-YFP remained unaffected by the COOH-terminal addition of the fluorescent protein (representative images are presented in Figure 3-11, A and B) (150). When wild-type MSD0-CFP and MRP1 NΔ204-YFP are expressed within a single cell (Fig. 3-11, C, top panel), the majority of both fusion proteins co-localized at the PM. However, localization of YFP- and CFP-fusion proteins was unaltered when the YFP-fused core domain and MSD0-CFP mutants C7A, C32A and C7/32A were co-expressed and each displayed a similar staining pattern to that observed when expressed individually. Cys-less MSD0-CFP was also unable to restore the PM localization of MRP1 NΔ204-YFP (Fig. 3-11, D, row 5) and expectedly, re-introduction of C7 and/or C32 into this protein failed to restore cellular localization of the NH2-terminally truncated MRP1 YFP-fusion protein (Fig. 3-11, D, bottom 3 panels).

In order to preclude the possible involvement of fluorescent protein tags in either the rescue of these constructs (as observed for wild-type) or prevention of association of MSD0 with the
Figure 3-11. Rescue of non-plasma membrane targeted MRP1 NΔ204-YFP by co-expression with wild-type and variant MSD0-CFP fusion proteins.  

A, Merged confocal images of HEK293 cells stably expressing wild-type MRP1 1-1531-YFP and MRP1 NΔ204-YFP fusion proteins (yellow), described previously (150). Nuclei were counterstained with Hoechst 33342 (blue).  

B & C, Confocal images following transient transfection of MRP1 NΔ204-YFP stable cell lines with either wild-type MSD0-CFP and its alanine substitution mutants (panel B) or cys-less MRP1 MSD0-CFP and related introduced cysteine mutants. Isolated signal from wild-type and mutant MSD0-CFP (cyan) and MRP1 NΔ204-YFP (yellow) fusion proteins is shown in the first panel and second panel of each row, respectively. Scale bars can be found in the panel containing signal from yellow channel (middle panels). Overlays of all three channels are found in the last panel of each row. In these panels, areas of co-localization between YFP- and CFP-fusion proteins are represented by white.
Figure 3-11
core domain (as observed for mutant MSD0-CFP proteins), the same experiment was carried out with an untagged version of MRP1 NΔ204, shown previously to increase in plasma-membrane localization when co-expressed with MSD0 (150). Equivalent results were obtained in co-expression studies with untagged MRP1 NΔ204 and YFP-hybrid of this MRP1 fragment (data not shown). Thus, as previously demonstrated, the rescue of plasma-membrane localization by wild-type fusion proteins is attributable to interaction of MRP1-specific domains, not the fluorescent proteins to which they are attached (150). Further, the failure of mutant MSD0-CFP fusion proteins to rescue plasma-membrane trafficking is attributable to the cysteine mutations within this domain and not the fluorescent proteins used for detection.

3.13 Disulfide-mediated MSD0-CFP oligomers are maintained in the presence of the core domain.

Results described above indicate that cys-less and cysteine mutant MSD0-CFP-fusion proteins examined are unable to form significant interactions with the core domain of MRP1. The influence of co-expression of MRP1 NΔ204 with MSD0-CFP-fusion proteins on the oligomerization status of the latter proteins was assessed. Crude membranes derived from transient transfectants of stable cell lines of MRP1 NΔ204 or MRP1 NΔ204-YFP with MSD0-CFP fusion proteins were analyzed by SDS-PAGE in the presence and absence of DTT, followed by immunoblotting. Since the HRP-conjugated mAb targeted against GFP proteins used cross-reacts with YFP and CFP, and MRP1 NΔ204-YFP migrated to approximately the same molecular weight as the oligomers of MSD0-CFP, interpretation of these immunoblots was equivocal. Therefore, immunoblots from co-expression studies with untagged core and MSD0-CFP are shown (Fig. 3-12 and App. 2). Migration of the core domain was unaltered by co-expression with any of the MSD0 constructs and protein from untransfected cells co-migrated with protein from cells co-expressing MSD0 and MSD0-less MRP1 (App. II). Moreover, as expected based on
Figure 3-12. Influence of co-expression with MRP1 NΔ204 on the expression, maturation and oligomerization of wild-type and variant MSD0-CFP fusion proteins. A-D, Immunoblots of crude membranes prepared from MRP1 NΔ204 stable cell lines transiently transfected with MSD0-CFP fusion proteins probed with HRP-conjugated mAB anti-GFP. The locations of the CFP-fused MSD0 proteins are indicated. Crude membrane preparations of MRP1 NΔ204 stable cell lines transiently transfected with MSD0-CFP were subjected to SDS-PAGE in the presence of DTT (panel A) or treated with PNGaseF and then subjected to SDS-PAGE (panel B). Migration of MRP1 MSD0-CFP fusion proteins co-expressed with core domains of MRP1 in SDS-PAGE were assessed in the presence (C) or absence (D) of DTT (100mM). Total amounts of protein loaded in panels B-D were normalized for differences in expression between MRP1 MSD0-CFP fusion proteins observed in A. D, High-molecular weight species not observed in the presence of DTT are indicated (block arrow).
A. MRP1<sub>204-1531</sub> co-expressed with:

<table>
<thead>
<tr>
<th></th>
<th>A792C</th>
<th>A32C</th>
<th>ATC</th>
<th>Cysless MSD0-CFP</th>
<th>Wildtype MSD0-CFP</th>
<th>in wildtype MSD0-CFP background</th>
</tr>
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<tr>
<td>kDa</td>
<td>1</td>
<td>2</td>
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<td>4</td>
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B. + PNGaseF (37°C), 100mM DTT

C. normalized for protein levels, 100mM DTT

D. normalized for protein levels, no DTT

Figure 3-12
experiments presented in Figure 3-1, no band was observed in the presence or absence of DTT that would correspond to a full transporter formed by the association of MSD0-CFP and the core domain of MRP1. In turn, all MSD0-CFP hybrids migrated to the apparent molecular weights of 50 kDa and 40 kDa, respectively, and were seen to co-migrate following treatment with PNGaseF (Fig. 3-12, A and B), as observed when independently expressed (Fig. 3-10, A and B). Further, no change was observed in banding profiles for cys-less MSD0 and mutants A7C, A32C, A7/32C and C7/32A. In contrast, a few differences were observed for the remaining MSD0-CFP fusion proteins co-expressed with MRP1 NΔ204. For wild-type MSD0-CFP and mutants C7A and C32A the amount of protein apparently trapped at the gel interface increased. Since there is known interaction between wild-type MSD0 and the core domain, this increase in higher molecular weight species cannot be ignored. However, a similar change was not observed in the banding pattern of MRP1’s core domain (App. II) and this was also observed for two mutants for which interaction with the core was not demonstrated (compare Fig. 3-12 and 3-10, C and D). Moreover, an increase in intensity was observed in bands of higher mobility in these lanes indicating that this increase is likely due to overloading of the gel. Taken together, these results indicate that protein preparations procedures are sufficient to disrupt interactions between MRP1’s MSD0 and the MSD0-less core region. Further, since a cys-less MSD0-CFP did not display wild-type PM localization and this cannot be restored by the methods herein, this MRP1 fragment is an unlikely template candidate for future CSM studies.


Investigations were undertaken to determine if oligomerization of MSD0 observed when Cys7 or Cys32 were mutated also occurred when these residues were mutated in the full-length transporter. Confocal microscopy revealed that alanine substitution of Cys7 and/or Cys32 in full-length MRP1 did not alter the cellular localization of this transporter and these mutant proteins
were found on the plasma membrane of HEK293 cells similar to wild-type (Fig. 3-13, A), as found previously by our group (Dr. Elaine Leslie, unpublished) and others (1). Further, ER retention was not indicated since no significant co-localization was observed of wild-type MRP1 or mutants C7A, C32A and C7/32A with calnexin (red signal). A graphical representation of the subcellular localization of these proteins was obtained by analyzing the fluorescence profiles of individual cells (Fig. 3-13, A, i-iv). These profiles were very similar for both wild-type and mutant proteins and revealed essentially no intracellular MRP1. Relative expression of C7A, C32A and the double mutant were estimated by immunoblotting with MRPr1 and all mutants were observed at levels similar to wild-type (Fig. 3-13, B). All mutant and wild-type proteins co-migrated on SDS-PAGE gels in the presence of reducing agent, indicating that they are glycosylated to the same extent, in support of confocal microscopy results and indicating that both wild-type and mutant MRP1 were fully processed. Taken together, these results imply that these mutations do not impair the biogenesis and processing of full-length MRP1. Migration of full-length MRP1 containing C7A, C32A or C7/32A was analyzed by SDS-PAGE and immunoblotting to determine whether the DTT-sensitive oligomers resulting from alanine substitution of Cys7 or Cys32 were artefacts of expressing MSD0 independently, or were biologically relevant in a full-length transporter. SDS-PAGE on low percentage (5%) gels was performed in the presence or absence of reducing agent (100mM DTT) and MRP1 detected with MRPr1. A minor amount of protein was detected near the top of the separating gel in all lanes (Fig. 3-13, C). The intensity and mobility of this band remained relatively unchanged in the absence of DTT for wild-type MRP1 or the double mutant. However, for both of the single cysteine mutants, C7A and C32A, the amount of protein was increased in the absence of DTT. Due to the low resolution inherent to low percentage acrylamide gels, it is difficult to estimate the apparent molecular weight of this low mobility component or ascertain if it is comprised of more
Figure 3-13. Influence of Cys→Ala substitutions in the extracellular NH2-proximal tail on the localization of full-length MRP1 proteins. A, HEK293 cells were transiently transfected with wild-type pcDNA3.1(-)-MRP1 and mutant pcDNA3.1(-)-MRP1 C7A, pcDNA3.1(-)-MRP1 C32A and pcDNA3.1(-)-MRP1 C7/32A expression vectors as indicated and processed for examination by confocal microscopy. MRP1 polypeptides were detected with mAb MRPr1 in conjunction with goat-anti-rat Alexa 488 (green). The endoplasmic reticulum (red) was detected and nuclei (blue) stained as described in Figure 3-8. Merged fluorescent images are presented in the third panel of each row. In these panels, yellow is indicative of co-localization of green and red signals. Typical fluorescent profiles of MRP1 polypeptides (green line), the ER (red line) and nuclei (blue line) were plotted across individual cells from the overlayed image (i-iv) (described previously in (150)). B, MRP1 expression in crude membranes of HEK293 cells transiently transfected with pcDNA3.1(-) containing wild-type and mutant (Cys7Ala, Cys32Ala, Cys7/32Ala) cDNAs was determined by immunoblotting with MRPr1. Equivalent amounts of total protein were loaded and blot shown is representative of at least three separate transfections. C, Crude membranes from B were treated with (+) or without (-) DTT (100mM) in Laemmli’s buffer with heating (65°C for 10 minutes) prior to loading on a 5% gel. The use of siliconized plates allowed the transfer of both stacking (4%) and separating (5%) gels to Immobilon-P membranes and the interface between these shown (—). MRP1 was visualized with mAB MRPr1, HRP-conjugated secondary antibodies and enhanced chemiluminescence techniques. Higher-molecular weight bands unique to mutants Cys7Ala and Cys32Ala in the absence of DTT are indicated (block arrow).
Figure 3-13
than one species. Further investigations are required to ascertain the role of C7 and C32 in full-length MRP1.

3.15 Mobility of tryptic fragments of C7 and C32 mutants shows DTT-dependence.

Given the equivocal results obtained by the above analyses of the possible oligomerization of cysteine mutants of MRP1, limited trypsin digests were performed and tryptic fragments separated in the presence or absence of DTT. If Cys7 and/or Cys32 are mediating dimerization in full-length MRP1, this should be reflected in the tryptic digest pattern such that the fragment corresponding to MSD0 and a portion of CL3 will migrate to a higher molecular weight in the absence of DTT. Furthermore, alteration in the overall conformation of MRP1 by mutants C7A, C32A and C7/32A will be detected by trypsin digestion since previous studies on P-gp, MRP1 and other membrane proteins have demonstrated that changes in conformation can correspond to a change in accessibility of trypsin cleavage sites.

The pattern of tryptic fragments obtained after controlled exposure of MRP1 crude membranes to trypsin and their detection with several regionally directed, MRP1 specific antibodies has been previously well-characterized (2;145) (Figure 3-14, A). MRP1 is initially cleaved within the linker region between NBD1 and MSD2 into two fragments, one that corresponds to the COOH-half of ~75-80 kDa (C1), and another of ~120 kDa that corresponds to the NH₂-terminal half (N1). MRP1 mAb QCRL-1 is able to detect both fragments due to the existence of several hypersensitive sites located within the linker region surrounding the epitope, although the C1 fragment appears to be more readily detected (145). This typical pattern of MRP1 tryptic fragments was observed for all proteins digested and the location of the N1 and C1 fragments indicated in Figure 3-14. For wild-type MRP1 and mutants C7A, C32A and C7/32A, the N1 and C1 tryptic fragments appeared at a trypsin:protein ratio of 1:30000. Furthermore, comparison of the blot on the left with the blot on the right reveals that there is no detectable
Figure 3-14. Limited proteolysis of MRP1 NH2-tail Cys→Ala mutants and detection of tryptic fragments with QCRL-1. Crude membranes from HEK293 cells over-expressing wild-type MRP1 and mutants C7A, C32A and the double mutant of these were digested with DPCC-treated trypsin at increasing trypsin:protein ratios at 37°C for 15 minutes as follows: (1) control, (2) 1:30000, (3) 1:15000, (4) 1:3000, (5) 1:300, (6) 1:30 and (7) 1:15. Reactions were stopped by removal of separate samples into Laemmli’s buffer containing leupeptin and PMSF with (right hand blot) or without DTT (left hand blot). Tryptic fragments were separated on 5-15% gradient gels and detected with MRP1 specific mAb QCRL-1. The designations N1 and C1 refer to discrete proteolytic fragments generated which are described in the text and illustrated in the schematic in A. The block arrows on the blot indicate the trypsin:protein ratio at which the C1 fragment was detected. Location of full-length (FL) MRP1 is also indicated.
Figure 3-14
difference in the pattern of tryptic these fragments in the presence or absence of DTT for all MRP1 proteins.

Further digestion with trypsin yields two smaller tryptic fragments, designated N2 (40-60 kDa) and N3 (~60 kDa) that are the result of cleavage of the N1 fragment within CL3. Cleavage of the N1 fragment in CL3 is just COOH-terminal to the epitope for MRPr1, such that this antibody detects the N2 fragment that corresponds to MSD0 and the first ~40 amino acids of CL3. Immunoblots of tryptic fragments initially probed with QCRL-1 were stripped and re-probed with MRPr1 and resultant images are presented in Figure 3-15. For all proteins, detection of the N1 fragment with this mAb occurs at 1:30000, consistent with results obtained with QCRL-1. Further, for wild-type MRP1 the N2 tryptic fragment appears at trypsin:protein ratios of 1:3000 while the majority of the N1 fragment remained intact. The tryptic digest patterns for mutant C32A and the double mutant, C7/32A, closely resembled that of wild-type such that the N2 fragments were first detectable at a trypsin:protein ratio of 1:3000 and N1 was readily detectable. However, for these mutants, the amount of the N2 fragment generated at this ratio is very low and significantly increased at a higher trypsin:protein ratio of 1:300. The tryptic digest pattern of C7A differs from the other MRP1 proteins examined such that the N2 fragment appeared first at a higher trypsin:protein ratio of 1:300 and very little N1 fragment remained undigested. The tryptic digest pattern observed for wild-type and the double mutant in the presence of DTT is indistinguishable from that obtained in the absence of DTT. However, for mutants C7A and C32A, in the absence of DTT, at 1:300 trypsin:protein the majority of the N2 fragment appears to migrate at a higher molecular weight between 80-120 kDa. The tryptic digestion patterns of C7A, C32A and C7/32A indicate that the overall conformation of these proteins differs from wild-type. Furthermore, C7 and C32 in the single mutant proteins may form disulfide bridges between MRP1 molecules, although disulfide linkages with proximal membrane proteins cannot be excluded.
Figure 3-15. Limited proteolysis of MRP1 NH2-tail Cys→Ala mutants and detection of tryptic fragments with MRP1. Stripped immunoblots from Figure 3-15 were re-probed with MRP1-1. The designations N1 and N2 refer to discrete proteolytic fragments generated which are described in the text and illustrated in the schematic at the top of the previous figure. Double-headed arrows to the left of the blots indicate bands present in absence of DTT (right hand blot) and not in the presence of DTT (left hand blot). Block arrows and arrowheads on the blot indicate the trypsin:protein ratio at which the N1 and N2 fragments were detected, respectively. Location of full-length (FL) MRP1 is also indicated.
Figure 3-15
Chapter 4

DISCUSSION

CSM has been applied to members of the ABC-superfamily including P-gp (reviewed in (252)), TAP1, BCRP and CFTR with great success (93;241;244;250;251;253;254). In the absence of high-resolution structures, this technique has provided a large amount of structural data pertinent to protein topology, dimensions, packing of TM helices, location of substrate binding sites and accessibility of domains (131;172;176;241-243;245;247;256-268). In addition, changes in local conformation which occur in the MSDs and NBDs upon substrate and ATP binding and ATP hydrolysis have been detected by CSM (93;129;130;132-134;136;235;256;269;270). Specific residues or regions important for transduction of conformational changes between the MSDs and the NBDs that occur during the catalytic cycle have also been uncovered (129;136;244). The combination of structural data obtained through CSM and crystal structures of ABC transporters in conjunction with biochemical and functional studies has provided a better understanding of the mechanism by which these transporters work but much remains unanswered.

Initial attempts to create a functional cys-less CSM template of MRP1 were unsuccessful, given that endogenous cysteine residues in MSD0 and CL3 were deemed structurally and functional important (1;2). However, subsequent expression of cysteine-less MRP1 lacking MSD0 yielded a partially functional transporter in yeast (5), although this template was not further pursued by this group. Given these results, with the ultimate goal of applying CSM to MRP1 in its entirety, we investigated the endogenous cysteines within MSD0 and MRP1 half-molecules separately. All 18 endogenous cysteines located within the core regions of MRP1 were mutated to alanine since this less conservative substitution was preferred over serine in the NH2-terminal of MRP1 and the distantly related CFTR (93;241). The validity of co-expressed cys-less, MSD0-less MRP1 half-molecules as CSM templates was assessed by transport and ATP
binding/hydrolysis assays. Dual-half MRP1 lacking MSD0 and completely devoid of cysteines expressed in insect cells displayed reduced transport of the substrates examined. Uptake of LTC₄ was reduced to a greater extent than uptake of E₂₁⁷βG (Fig. 3-4). Reduced transport levels of LTC₄ by cys-less, NH₂-terminally truncated MRP1 have been reported previously (5), where transport of E₂₁⁷βG was not examined. However, cys-less MRP1 expressed as intact protein in yeast retained higher levels of LTC₄ transport than observed here for the equivalent dual-half protein, indicative either that the dual-half protein is more sensitive to slight alterations in conformation than the intact protein, or to processing differences between yeast and insect cell expression systems. In order to delineate individual cysteines or regions of cysteines contributing to the reduced functionality, the 18 endogenous cysteines located within the core regions of MRP1 were substituted with alanine, first in each of the half-molecules and then by domain. Functional studies of MRP1 NΔ204 dual lacking cysteines in either the NH₂- or COOH-half molecules demonstrated that residues within both halves of the protein are important for full function since each of these constructs retained only partial transport activity (Fig. 3-4). Alanine substitution of cysteines located within the NH₂-half molecule inclusive of CL3, MSD1 and NBD1 had a more global and severe effect on MRP1 function and transport of both LTC₄ and E₂₁⁷βG was almost completely abolished. It is interesting that alanine substitution of the cysteines in the NH₂ half-molecule of MRP1 is more detrimental than alanine substitution in both half-molecules and that this appears to be substrate specific. Removal of cysteines may impart increased flexibility to the COOH-half molecule that the equivalent wild-type fragment lacks allowing the cys-less COOH-half molecule to more efficiently associate with an altered conformation of the cys-less NH₂-half molecule. If this was indeed the case, levels of transport would be higher for a completely cys-less dual protein compared to the partially cys-less dual protein since transport is highly dependent on the efficient association of the half-molecules (91). This was indeed the case (Fig. 3-4) but does not readily offer an obvious explanation as to why
this effect was limited to E$_2$17$\beta$G. However, transport of substrates that bind predominantly to the NH$_2$-half of MRP1, such as LTC$_4$ (163;178;278), would be expected to be low regardless of the cysteine status of the co-expressed half if the regions within the NH$_2$-half molecule that comprise the substrate site are altered such that substrate is unable to bind. However, transport of substrates that interact with regions in both halves of MRP1, such as E$_2$17$\beta$G, might be expected to increase with more efficient association of the two halves since contacts are made in both halves of the protein, presuming that loss of contacts in the NH$_2$ half-molecule is compensated for by substrate binding by the COOH half-molecule. Clearly, photo-affinity labelling studies and substrate binding assays would help resolve these questions and should be strongly considered.

LTC$_4$ transport was greatly affected by Cys$\rightarrow$Ala mutations in the COOH-terminal fragment of MRP1 (inclusive of domains MSD2 and NBD2), which reduced transport to levels almost half that of wild-type. Further investigations of cysteines by domain, revealed that wild-type levels of transport were retained by cys-less MRP1 mutants lacking cysteines in either or both NBDs. Taken together, the data indicate that the attenuated function of the cys-less protein can likely be attributed to cysteines within the transmembrane domains or the intracellular loops that connect the TM helices.

Truncated MRP1 dual lacking cysteines only within MSD2 was used to further investigate the endogenous cysteines within this domain. This variant selectively altered transport levels similar to the completely cys-less protein and the protein lacking cysteines in the entire COOH fragment with LTC$_4$ and E$_2$17$\beta$G transport levels at $\sim$40% and $\sim$120% of MRP1 NA204 dual, respectively. Thus it seems that cysteines in MSD2 are important in at least one step of LTC$_4$ transport and future binding assays are warranted. Further, in general it seems that transport of E$_2$17$\beta$G was less affected by mutation of endogenous cysteines to alanine, although the basis for this is unknown.
As a general observation, the most common structural contribution of cysteine residues to protein stability is through the formation of disulfide bridges. Initial experiments described here excluded the involvement of the endogenous cysteines of MRP1 in native disulfide bonds between MRP1 half molecules or between MSD0 and the core domain, since no shifts in mobility were detected in the absence of reducing agent. Since alanine substitution of cysteine removes both the hydrogen bonding capacity and the steric bulk of these residues, these mutations have the potential to alter protein conformation globally and within the local environment. However, the retained ability of various cys-less mutants to transport substrate indicates that alanine substitutions have not caused gross perturbation of protein structure, and supports the lack of cysteine-mediated, native covalent interactions between the co-expressed wild-type fragments of MRP1. Given the location of native cysteines within the TM helices in the homology-based model of MSD0-less MRP1 (117), these residues are unlikely to form covalent interactions with cysteines or functional interactions with any residues of the NBDs (Fig. 4-1). During interpretation of these results in the context of the model of MRP1, it is important to consider that since this model is based on a crystal structure that represents a single structural conformation in the transport cycle it provides only a snapshot of the transporter. In this case, the outward or low-affinity conformation of Sav1866 was captured in the presence of bound ATP (282). Despite this caveat, native, interdomain disulfide bridges do not appear to play a role in stabilizing the tertiary structure of the core of MRP1. These observations should be confirmed by mammalian co-expression of the various cys-less MRP1 half-molecules and examination of cellular localization since insect cells are a well-characterized expression system known to express high levels of proteins that do not traffic correctly and are apparently misfolded in mammalian cells, for example the CFTR mutant ΔF508 (283). Cysteines essential for PM localization of MRP1 have been identified by our group (Dr. Lei Qin, unpublished data), and re-introduction of these cysteines into the cys-less dual constructs used here might restore activity of these mutants. As
**Figure 4-1. Location of the endogenous cysteines within the core domains of MRP1.** The figure illustrates four views of the model of MRP1 based on the crystal structure of the ADP bound form of Sav1866 generated as described in (117) and produced using PyMol (DeLano Scientific LLC, San Carlos, CA). Each view represents a 90° rotation around the y-axis from the preceding image. The locations of the endogenous cysteines in the core domains of MRP1 are illustrated. The domains of MRP1 are coloured as follows: MSD1 (light blue), MSD2 (*pink*), NBD1 (*pale yellow*) & NBD2 (*green*). Cysteine residues are coloured by element as follows with the exception of carbon residues which maintain the colour of the domain in which they reside: nitrogen (*blue*), oxygen (*red*) and sulfur (*yellow*). Hydrogen atoms have been omitted for clarity.
observed for CFTR (241), the presence of endogenous cysteines does not preclude future cross-linking studies but caution should be exercised during interpretation of results.

It remains a possibility that native disulfide bonds formed between cysteines within the same half molecule of MRP1 might have been overlooked, since the resultant shift in mobility would presumably be small and below the detection limit of the assay. Thus, the maintenance of local structures within MRP1 by disulfide bonds between proximal cysteine residues cannot be excluded. However, analysis of the MRP1 model (117) does not reveal any obvious candidate cysteine pairs with the core of MRP1. In MSD0 and CL3 of MRP1, which is not completely included in the Sav1866 model, three candidate cysteine pairs exist based on proximity in primary sequence namely C7 with C32, C43 with C49 and C208 with C265. Cysteine mutations in the MSDs of MRP1 could alter the packing of TM helices as a result of mutation of residue directly involved in a disulfide bridge, or through disruption of the secondary structure of an individual TM helix. Within MSD1 and MSD2 of MRP1, the consequences of Cys→Ala mutations could include global alteration of the translocation pore or alternatively, eliminating a direct interaction with substrate. Unfortunately, little can be derived from primary sequence alignments with related ABC transporters on which CSM has been successful given the limited or absence of homology within the core TM domains and MSD0, respectively, and the large number (25 in total) of cysteine residues in MRP1.

Native cysteine residues located in CL3 and MSD1 were found to be important for the overall activity of MRP1 since a global disruption of MRP1 transport was observed as a result of their mutation to alanine. The two cysteines located within CL3, Cys208 and Cys265, have been extensively probed. Deletion studies revealed that Cys208 defines the NH$_2$-terminal structural and functional boundary for CL3 (159). Additionally, mutation of this residue to serine or alanine increased LTC$_4$ transport without altering the plasma-membrane localization of MRP1 in HeLa cells (91), suggesting that the amino acid identity at this position in MRP1 may not be vital.
Indeed, the identity of the amino acids corresponding to Cys208 in MRP1 is not well conserved across members of both the human ABCC subfamily and evolutionarily diverse ABCC family members from *S. cerevisiae, C. elegans* and plants (178). Taken together, these results suggest that the abrogated function of cysless NH2 MRP1 NΔ204 dual is not likely a result of mutation of Cys208. Cys265 is located within a conserved helical element in CL3 important for maintenance of the structural integrity of this region. Our group has demonstrated that a region encompassing Cys265 can be deleted from full-length MRP1 and the transporter will retain significant levels of LTC4 transport (159). However, since this deletion mutant of MRP1 is mis-localized in polarized cells, the region between 261 and 270 was deemed critical for proper folding and trafficking of the protein. Our group has also shown that full-length MRP1 does not tolerate mutation of Cys265 to serine or alanine given that both plasma membrane trafficking and transport were disrupted (2). Consistent with deletion studies, limited trypsinolysis studies found that the conformation of MRP1 was altered by mutation of Cys265, in support of the importance of this residue for the structural maintenance of CL3 (2). Therefore, the severely reduced function of MRP1 NΔ204 dual lacking cysteines only in the NH2-proximal fragment could be a result of the mutation of Cys265 altering the conformation of the conserved helical element in which it resides. Since our group has also shown that LTC4-labelling of the MRP1 NH2 half-molecule is dependent upon of the presence of CL3 (163), it is possible that mutation of Cys265 is altering the binding characteristics of this substrate. Further, if mutation of this single cysteine is responsible for the reduced activity of this construct by disruption of the local structure of CL3, the impact of disrupting this region would be greater in a dual-expression system and, as observed, one would expect a more severe reduction in activity. This is because in systems of MRP1 dual-expression, even though expression and routing to the plasma membrane of each half is not dependent upon the other, protein function is highly dependent upon the association of the co-expressed MRP1 fragments. One can envision that association of MRP1 half-molecules is in
turn heavily reliant upon the structural integrity of the individual halves. Thus, further evaluation of Cys265 in the context of MRP1 Δ204 dual is warranted.

The remaining cysteines in MSD1 (residues 375, 388, 555 and 563) are limited to TMs 7 and 10, both of which have been reported to contribute to the substrate binding site (165-170;178). TM10 has been consistently labelled by the majority of photo-reactive substrates examined but TM7 was only identified in more recent analysis of photolabelling with LTC4 by MALDI-TOF (178). Additionally, TM7 was found to line the translocation pore of MRP1 at the cytoplasm-membrane interface in the Sav1866-based MRP1 model (117). However, in the low-affinity state represented in the MRP1 model, Cys375 or Cys388 are unlikely to contribute to substrate binding since Cys375 is located at the extracellular end of TM7 where this TM helix does not contribute to the pore and, despite its location near the cytoplasmic end of TM7, Cys388 apparently projects into the lipid bilayer. This is in contrast to the proximal residue, Lys396, which in the model projects directly into the translocation pore, non-conservative mutation of which resulted in substantial and global reduction in transport (275). However, deleterious effects of mutation of Cys375 cannot be excluded since this residue also projects into the pore, and in light of its proximity to Cys1205 located in TM16, as discussed below. As mentioned above, it is important to remember that the model used for interpretation of these results represents MRP1 in the low-affinity state. The pitch and rotation of the TM helices is likely to be different in earlier stages of the catalytic cycle given that conformational changes have been demonstrated biochemically in MRP1 (187;191) and by CSM in the distantly related, P-gp (129;130;132;133;135).

Numerous site-directed mutagenesis studies have been aimed at identifying particular residues within TM10 that may be directly involved in substrate binding or may confer substrate specificity to MRP1 (141;161;284). Not surprisingly, several amino acids within this TM have been demonstrated to globally affect transport or exert substrate specific alterations in MRP1 transport activity including Thr550, Trp553, Thr556, Pro557, Thr564 and Tyr 568 (141;161;284).
While these amino acids cluster on one face of TM10 and generally project into the pore in the MRP1 model based on Sav1866 (117), neither Cys555 nor Cys563 are on this face of the alpha-helix. Cys555 is distally located on the directly opposite TM face in a position that would allow interaction with the lipid bilayer and Cys563 projects towards adjacent TM helix 9. Despite the close proximity of TM9 and TM10 (~5Å) at this membrane depth, examination of the local environment of Cys563 does not reveal any obvious disulfide bonding partners.

The five cysteines within MSD2 (residues 984, 1046, 1105, 1205 and 1209) are located in TM helices 12-14 and 16. In the homology model of MRP1 based on Sav1866 (117), all of these helices contribute residues to the translocation pore dependent upon the depth within the lipid bilayer. Close to the intracellular compartment, TMs 13-16 form part of the translocation pore while the extracellular end of the substrate pathway is lined by MSD2 TM helices, 12, 14 and 17. All of the TM helices that comprise MSD2 have been identified through photolabelling studies to contribute to substrate binding and residues within them have been shown to influence substrate specificity. For example, two residues within TM12 proximal to Cys984, His986 and Ala989, have been shown to confer substrate specificity to MRP1. Helical wheel projections place these residues proximal to one another but on the opposite face of Cys984, in agreement with the MRP1 model that places these three residues at the extracellular end of TM12 with both His986 and Ala989 projecting towards the pore. Cys984 projects directly towards the adjacent TM helix 13, distant from Cys1047. The SH moiety of Cys984 is not close enough to adjacent residues to form hydrogen bonds. Cys1047 is located very near the cytoplasmic interface of TM13 on the outside of the helix such that it projects into the lipid bilayer. A previous study investigated a naturally occurring variant of MRP1 in which this cysteine is replaced by serine and found that neither the expression of MRP1 nor the transport of LTC₄, E₂17βG and methotrexate were affected by this mutation (271). Continuing through the cysteines in MSD2, Cys1105 is located near the extracellular end of TM14 projecting away from the pore formed by MSD1 and MSD2,
towards TM15. The importance of charged residues within TM14 has been highlighted (142). For instance, mutant Asp1084 globally disrupted MRP1 function while mutation of a second charged amino acid, Glu1089, elicited substrate specific effects. However, both of these residues are more centrally located within the helix and project into the pore. Recent work has shown that TMs 12-15 of MRP1 can be exchanged for the equivalent region of MRP3 with little effect on LTC₄ transport (278). Alignment of the primary sequences of MRP1 and MRP3 indicates that the native cysteines in MRP1 within the swapped region are not conserved in MRP3, providing further evidence that these cysteines do not play a significant role in binding and transport of LTC₄. TM 16 contains the remaining two cysteines of MSD2, Cys1205 and 1209, that have been previously mutated with no effect on substrate specificity or overall activity of the transporter (272). Examination of the model reveals that Cys1209 appears to be located on the extracellular side of the TM helix and project into the lipid bilayer. However, due to the twisting interaction of the TM helices (117), Cys1205 appears to be in proximity to Cys375 located within TM7. Indeed, while the distance between these amino acids (~6 Å) exceeds the maximum for stable disulfide formation, one can imagine that these cysteines could form meaningful interaction during the catalytic cycle of MRP1. Interestingly, a role for Glu1204 in communication between MSD2 and NBD2 has been postulated (285). Since transport by MRP1 mutated at position 1204 was selectively disrupted, it was suggested mutation of Glu1204 diminished the coupling between MSD2 and NBD2 in such a manner that some substrates were more affected than others. That this coupling during the catalytic cycle involves not only Glu1204 but all of TM16 including Cys1205 and based on the current findings, could involve stabilization of a transition states via transient disulfide formation between Cys1205 and Cys375. However, the functional implications of such an interaction may be limited since one member of this disulfide binding pair can be mutated without great effect (272).
As mentioned above, MRP1 mutants lacking cysteines in either or both NBDs attained significant levels of transport of both substrates. In fact, LTC₄ transport was increased between 1.5- to 2.0-times wild-type activity. This increase in transport can likely be attributed to mutation of Cys682, located within the Walker A motif of NBD1 since mutation of this residue in a full-length transporter resulted in an increase in LTC₄ transport to ~120% wild-type (189). This increase in transport was attributed in that case to increased affinity of NBD1 for ATP and was supported by ADP trapping experiments with this mutant. Consistently, mutant MRP1 completely devoid of cysteines in NBD1 and NBD2 displayed some degree of tight nucleotide binding by NBD1 in the absence of beryllium fluoride. It is unclear how mutation of Cys682 is affecting the binding of ATP since this residue, unlike others in the Walker A motif, was not seen to hydrogen bond with the phosphates of ATP in the nucleotide-bound structure of this domain (200). Structural analysis of the model of MRP1 (117) does not reveal that the SH moiety of any of the cysteines in NBD1 including Cys682 form disulfide bridges or hydrogen bonds with proximal residues. Thus the effect of this mutation on the conformation of this NBD is not known. Comparison of these experiments with parallel studies utilizing γ³²P-labelled nucleotide would help resolve this question by identifying the nature of the tightly bound nucleotide.

Transport of LTC₄ was most significantly increased by alanine substitution of the four cysteines in NBD2 (residues 1299, 1423, 1439 and 1479) and since these residues do not reside in the conserved motifs, disulfide and hydrogen bonding capacity of these residues was examined. No candidate pairs of bonding cysteines become apparent upon analysis of the MRP1 homology model, but this certainly does not exclude involvement of the native cysteines in NBD2 especially since mutation of these residues was not without effect. It remains unclear why tight binding of nucleotide by NBD1 had a greater influence on one substrate than another since ATP binding presumably follows substrate binding in the proposed catalytic cycle of this transporter (33).
However, substrate-specific alteration of transport by mutations within the NBDs has been observed before.

Initial cross-linking experiments focused on the NBDs of MRP1 given that cys-less mutants of these domains were functional. Two residues, Arg775 and Thr1329, located on juxtaposed faces of the NBD1:NBD2 heterodimer were selected for mutation to cysteine based on a previous finding in the related ABC transporter, CFTR/ABCC7, that comparable residues were coupled through hydrogen bonding (6). That these residues form a hydrogen bond in the transition state of CFTR and given their locations relative to the Walker A and Signature C motifs in NBD1 and NBD2 (Fig. 3-6), respectively, the close proximity of these residues would likely permit formation of a disulfide bridge between cysteines introduced at these positions at some stage of the catalytic cycle. The hydrogen bond formed between Arg775 and Thr1329 is conserved in the MRP1 model (App. III), as expected since a nucleotide-bound template was used. Introduction of cysteines at either of these positions greatly reduced the transport activity of MRP1 and while ATP-binding by these mutants was not altered, trapping of nucleotide was diminished at both NBDs. This is likely due to removal of this essential hydrogen bond, which in turn prevents tight dimerization of the NBDs, as seen in CFTR (6), and thus, inefficient progression through the transport cycle. Statistical analysis by Vergani et al. (6) found that residues corresponding to MRP1 Arg775 and Thr1329 were being evolutionarily co-selected, such that two major subsets of donor-acceptor pairs were identified. In these donor-acceptor pairs, arginine is most frequently paired with serine, and to a lesser extent, threonine, as is the case in both CFTR and MRP1 (6). Since arginine and serine at these positions form a functional donor-acceptor pair, conservative mutation of one member of this pair, for example T1329C, might be expected to retain the hydrogen bonding capacity of wild-type protein. However, this was not the case since the single mutation of threonine to cysteine at this position showed the same detrimental effects on transport and ADP-trapping as did the other introduced cysteine mutants. Therefore, it is likely the
removal of the more polar-OH rather than the shortening of the sidechain of this residue that prevents hydrogen bond formation. It is now established that the nucleotide binding sites of ABC transporters are a composite of the Walker A and B motifs from one NBD and the Signature ‘C’motif of the second NBD. Further, in MRP1, experimental evidence suggests that ATP binding on NBD1 occurs first and promotes binding of ATP by the second NBS. Thus, since the mutations here are both located in the composite NBS on NBD2, they would be expected to more drastically alter nucleotide-protein interactions at the composite site on NBD2 leaving nucleotide binding by NBD1 virtually unaffected. Indeed, this was the case since ATP-binding profile of NBD1 was similar for the parental cys-less NBD1/2 protein and all of the introduced cysteine mutants. The hydrogen bond between the equivalent residues in CFTR is postulated to exist in some but not all of the conformational states of the protein and is thought to stabilize interaction between the NBDs in the ATP bound state (6). Thus, the introduction of cysteines at these positions is likely not drastically altering the conformation of MRP1 but rather destabilizing the transition state and altering function in that way. Thus, the double mutant could be used for future cross-linking experiments to probe the interface of the NBD1:NBD2 heterodimer, although this would be limited to certain MRP1 conformations and increased caution would be required during interpretation of data.

Since very few ABC transporters outside of the ABCC branch contain analogous structures to MSD0, structural data for this domain will likely have to be obtained by means other than homology modeling. Thus, CSM becomes a vital tool in defining the structure of MSD0 and from this, how it forms associations with the core domains. To this end, a completely cys-less MSD0 was expressed in mammalian cells and its validity as a CSM template ascertained by its ability to traffic appropriately to the PM and associate with the core domains of MRP1. Consistent with previous findings (1;2), the seven endogenous cysteines within MSD0 were found to be important in maintaining the three-dimensional structure of this domain since, in
contrast to wild-type MSD0 which is PM-associated, cys-less MSD0 was found in the ER. Further evidence for an altered conformation of cys-less MSD0 was provided by its inability to associate with the core domain of MRP1 as evidenced co-expression experiments.

Attempts to identify individual cysteines necessary for correct folding of MSD0 were initially aimed at Cys7 and Cys32 in the NH$_2$-terminal tail of MRP1 to clarify conflicting previous findings regarding the importance of these residues in preserving the structure of this extracellular region (98;149;286). In addition, primary sequence alignment of MSD0 from human MRP1 to members of the human ABCC subfamily which contain this additional MSD, as well as other mammalian and yeast members, reveals that these two cysteines and particularly Cys32 may be conserved across the subfamily and between species (App. IV). Re-introduction of Cys7 and Cys32 in cys-less MSD0 did not restore PM localization of this mistrafficked protein, and these proteins did not form interactions with the core of MRP1 based on co-expression experiments. Taken together, these results indicate that aberrant folding of this cys-less MSD0 is not solely a result of mutation of Cys7 and Cys32. Re-introduction of the remaining five native cysteines should be considered and, based on previous work that alluded to the importance of Cys43 (2), this residue would be a good candidate with which to begin. Mutation of Cys7 and Cys32 was sufficient to disrupt the membrane localization of MSD0. The effects on cellular localization due to mutation of Cys7 or Cys32 in an otherwise wild-type MSD0 were limited to individual expression of this domain, since full-length MRP1 with either or both of these mutations trafficked appropriately. This is consistent with epitope insertion studies of the extracellular NH$_2$-tail in which these cysteines reside, that found that this region can be significantly altered with no effect on membrane localization or function of full-length MRP1 (98;149;286). However, it remains unclear if, in the absence of the core domain, mutation of Cys7 and Cys32 prevents disulfide bridge formation or if proper membrane insertion is somehow altered by their mutation.

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During the course of these studies, MSD0 MRP1 variant proteins with either mutation or reintroduction of cysteines at positions 7 and 32 were shown to form DTT-sensitive oligomers. In the cys-less background, the altered folding of cys-less MSD0 was thought to account for the formation of these oligomers. However, in wild-type MSD0, it is possible that if Cys7 and Cys32 are disulfide bonding partners, mutation of one but not both of these would leave a free SH group that could result in dimerization of MSD0. This was indeed the case. Additionally, these dimers persisted in the presence of MRP1 core domains. This was thought to be due to the failure of the MSD0 mutant proteins to interact with core regions rather than indicative of such dimers being formed in the native MRP1 structure. However, limited tryptic digestion of full-length MRP1 mutants Cys7Ala and Cys32Ala showed that the conformation of the NH₂-terminal end of MRP1 is altered. Further, the migration of a tryptic fragment corresponding to MSD0 and part of CL3 was markedly decreased in the absence of DTT, suggesting the formation of dimers mediated by these cysteines as observed when MSD0 is expressed alone. This raises the possibility that when one or the other extracellular cysteines is mutated, the other may be free to form intermolecular disulfide bonds.

This would not represent the first time MRP1 has been proposed to exist in the dimeric form. In fact, MRP1 was observed to exist in a homodimeric state in the membranes of erythrocytes and upon formation of two-dimensional crystals for EM, although no functional role was assigned in either case (126;287). More recent studies have used various biochemical means to demonstrate that MRP1 may exist as a dimer (1;288), purportedly mediated by MSD0-CL3, although functional consequences of this remain a matter of debate. Other members of the ABC transporter family have been reported to exist as homodimers such as SUR, CFTR, P-gp, ABCA1 and the half-transporter ABCG2, which has been observed in multiple oligomeric species (125;289-295). However, the field remains uncertain since dimers or oligomers of these transporters are not consistently observed and appear unlikely to have functional impact given the
requirement of four ABC transporter domains for function (11). Our results indicate that
dimerization of MRP1 or at least the COOH-half molecules could be mediated by the cysteines in
this MRP1 fragment and/or by hydrophobic interactions. That these dimers are a consequence of
overexpression of MRP1 in the plasma membrane also cannot be excluded, nor that it is not
homo-dimerization at all but rather interactions of the COOH half molecule with other, proximal,
non-MRP1 membrane proteins. To further define the role cysteines in the formation of COOH-
proximal half oligomers, similar experiments with MRP1 NΔ204 dual lacking cysteines in the
COOH-proximal half would be useful.

The current lack of structural framework for MRP1 poses challenges to the interpretation of
data from mutagenesis studies and design of future experiments. In the absence of a high-
resolution structure of MRP1, CSM has the potential to reveal a great amount regarding the
structure and function of MRP1. Even upon determination of a high-resolution structure of
MRP1, CSM can find application and can be used to verify any homology-based models or high-
resolution structures of MRP1, which are limited by the fact that they provide snapshots of
transporter conformations and are susceptible to misinterpretation, as exemplified by MsbA
(110). Unfortunately, MRP1 contains more cysteines than other ABC transporters to which CSM
has been applied. Observations herein and those from previous studies of the cysteines of MRP1
(1;2), that at least some of the 25 endogenous cysteines in MRP1 can significantly alter the
functional properties of MRP1 may greatly impede creation of a cys-less MRP1 template and thus
the application of CSM to MRP1. However, since CSM has been applied to cys-less template
proteins that retained as little as 50% function (273;274), use of this technique with MRP1 may
still be feasible. Alanine substitution of the cysteines in the cytoplasmic loops and MSDs of
MRP1 was not without effect and future development of a CSM template of MRP1 should focus
on identification of individual cysteines within these regions important in maintaining the
structure and functional properties of MRP1. A modified CSM approach may be considered in
which select cysteines remain in the template. Further, since MRP1 lacking cysteines within its NBDs was demonstrated to be a viable template for CSM, the nature of the interface of the NBD1:NBD2 heterodimer can be probed using this technique. To this end, the MRP1 model should prove useful to select residues on opposite sides of the interface for mutation to cysteine and subsequent crosslinking studies. Thus, the application of CSM to MRP1 remains a possibility and a modified approach will be needed given the detrimental effects of mutation of the endogenous cysteines of this transporter.
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Appendix I

*Validity of the ‘promoter switch’ constructs as means for expression of cysteines-less COOH-half of MRP1 NΔ204 dual.*  

_A,_ Depiction of the plasmids into which the two halves of MRP1 NΔ204 dual were cloned. The polyhedrin and p10 promoters in pFB dual are depicted as *block arrows* (*black*); other elements in the parental plasmid are coloured but names omitted for simplicity (for reference: http://www.invitrogen.com/content/sfs/vectors/pfastbacdual_map.pdf). The typical arrangement of MRP1 NH₂- (red) and COOH-(blue) fragments in pFB dual is represented on the *left* and plasmid maps for ‘promoter switch’ (PSW) clones are represented on the *right._  

_B,_ Expression levels of the NH₂- and COOH-proximal halves of wild-type MRP1 NΔ204 dual and its PSW counterpart were determined by slot blot probed with MRPr₁ and MRPm6. Subsequent densitometry was performed and expression of each half relative to the equivalent half of the intact protein determined; these values are represented below each band. Blot shown is representative of at least two independent experiments with similar values obtained for each MRP1-half molecule.  

_C,_ [³H]LTC₄ and [³H]E₂₁βG transport assays were completed on membrane vesicles from _A_ as described in *Materials and Methods._ Each bar represents an independent experiment and shown as the mean ± S.D from triplicates within each experiment. Transport levels were normalized for differences observed in protein expression and are shown as pmol mg⁻¹ after 3 minutes.
Appendix I
Appendix II

Migration of MRP1 NΔ204 in the presence and absence of reducing agent following co-expression with wild-type and mutant MSD0-CFP fusion proteins. A & B, Immunoblots of crude membranes prepared from a stable cell line expressing MRP1 NΔ204 and this stable cell line transiently transfected with pECFP N1 (empty) or MSD0-CFP fusion proteins probed with MRP1. The locations of the CFP-fused MSD0 proteins are indicated. Crude membrane preparations of MRP1 NΔ204 stable cell lines alone or following transient transfection with MSD0-CFP and subjected to SDS-PAGE in the presence (panel A) or absence of DTT (panel B) (100mM). Total amounts of protein loaded were normalized for differences in expression between MRP1 MSD0-CFP fusion proteins observed in Figure 3-12.
A. 

**MRP1**$_{204-1531}$ co-expressed with:

<table>
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<th>PECFP N1</th>
<th>wildtype</th>
<th>cys-less</th>
<th>MSD0-CFP</th>
<th>C7A*</th>
<th>C32A*</th>
<th>CT/32A*</th>
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<tbody>
<tr>
<td>kDa</td>
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<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+100mM DTT; MRPr1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>-100mM DTT; MRPr1</td>
<td></td>
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*In wildtype MSD0-CFP background*

B. 

<table>
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<th>wildtype</th>
<th>cys-less</th>
<th>MSD0-CFP</th>
<th>A7C**</th>
<th>A32C**</th>
<th>A7/32C**</th>
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**In cys-less MSD0-CFP background**

Appendix II
Appendix III

Hydrogen bond formation between Arg775 and Thr1329 is maintained in the model of MRPI based on Sav1866. The figure illustrates a top-down view of the NBD1 (pale yellow):NBD2 (green) heterodimer interface as viewed from the extracellular face of the membrane. Images were created as described in the legend for Figure 4-1. The location and the approximate distance (~3Å) of the hydrogen bond between Arg775 and Thr1329 are illustrated. Wild-type residues are coloured by element as in Figure 4-1. Using Pymol, mutations Arg775Cys and Thr1329Cys were introduced into the model and the distance between these estimated at ~7Å. Introduced cysteines are coloured red.
Appendix IV

Primary Sequence Alignment of MSD0. Alignment of primary sequence corresponding to MSD0 from selected ABCC subfamily members and various species. The conservation of cysteines (white text on black background) and areas surrounding these residues is highlighted. Conserved residues and domains are shown in bold text and highlighted in grey, respectively. Hs – *Homo sapiens*; Rn – *Rattus norvegicus*; Mm – *Mus musculus*; Sc – *Saccharomyces cerevisiae*. 
Appendix IV