Studies of the misprocessing mutations R1202D and E1204K in the drug and organic anion transporter, MRP1 (ABCC1) in cultured HEK cells

by

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Abstract

Multidrug resistance protein 1 (MRP1) is a drug and organic anion transporter of the ATP-binding cassette superfamily. Previous studies showed that opposite charge substitutions of Arg^{1202} or Glu^{1204} in transmembrane helix (TM) 16 cause a >80% reduction in MRP1 levels when expressed in human embryonic kidney (HEK) cells. These substitutions disrupt the folding and/or assembly of MRP1 which targets it for degradation. Attempts were made to enhance levels of the R1202D and E1204K misprocessing mutants by incubating transfected HEK cells at 30 °C or 27 °C. At both temperatures, cells expressed both fully glycosylated and underglycosylated mutants at levels 60–70% lower than wild-type MRP1 in cells grown at 37 °C. The subcellular localization patterns of the two mutants were similar to wild-type MRP1 at all three temperatures, with most of the transporter at the plasma membrane at 37 °C, and in the endoplasmic reticulum at 30 °C or 27 °C. Thus, although poorly expressed, the R1202D and E1204K mutants retained the ability to traffic to the plasma membrane. Attempts were also made to enhance R1202D and E1204K levels by exposing transfected HEK cells to chemical chaperones. Dimethyl sulfoxide and glycerol increased E1204K levels by 20-30% but decreased or had no effect on R1202D and wild-type MRP1. 4-Phenylbutyric acid had little or no effect on either wild-type or mutant MRP1. Thus both mutants were relatively resistant to rescue by chemical chaperones. Finally, a “second-site rescue mutation” approach was taken, guided by an atomic homology model of MRP1. Mutations of Tyr^{1133} alone decreased MRP1 levels, like R1202D; however, although substituting TM15-Tyr^{1133} with Phe, His and Ala in R1202D was predicted to re-establish TM15-TM16 bonding interactions, levels of this mutant did not increase. E1204K levels were also not improved by substituting TM17-Val^{1248} with Asp or Glu although these substitutions were predicted to re-establish TM16-TM17 bonds disrupted in E1204K. These results suggest that the bonding interactions of Arg^{1202} and Glu^{1204} with other amino acids predicted by the MRP1 homology model used in this study are insufficient to predict the critical helix-helix interactions necessary for stable MRP1 expression in mammalian cells.
Co-Authorship

This thesis was written by me with the guidance and assistance of my supervisor Dr. Cole. All experiments in this thesis were performed by me unless otherwise indicated.
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<th>Definition</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloblastic leukemia</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BiP</td>
<td>Binding immunoglobulin protein</td>
</tr>
<tr>
<td>BRIC2</td>
<td>Benign recurrent intrahepatic cholestasis type 2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSEP</td>
<td>Bile salt export pump</td>
</tr>
<tr>
<td>CD</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CL5</td>
<td>Cytoplasmic loop 5</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DJS</td>
<td>Dubin-Johnson syndrome</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E₁3SO₄</td>
<td>Estrone 3-sulfate</td>
</tr>
<tr>
<td>E₂17βG</td>
<td>17β-Estradiol 17-(β-D)-glucuronide</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</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>Hrd1</td>
<td>Hypoxia responsive domain-1</td>
</tr>
<tr>
<td>HRP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>Hsp</td>
<td>Intrahepatic cholestasis of pregnancy</td>
</tr>
<tr>
<td>ICP</td>
<td>Inwardly rectifying potassium channel</td>
</tr>
<tr>
<td>Kir</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LDL</td>
<td>Leukotriene C₄</td>
</tr>
<tr>
<td>LTK₄</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAb</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MDR</td>
<td>Myelodysplastic syndromes</td>
</tr>
<tr>
<td>MDS</td>
<td>Mouse embryo fibroblasts</td>
</tr>
<tr>
<td>MEF</td>
<td>Multidrug resistance protein 1</td>
</tr>
<tr>
<td>MRP1</td>
<td>Membrane spanning domain</td>
</tr>
<tr>
<td>MSD</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>MTX</td>
<td>Nucleotide binding domain</td>
</tr>
<tr>
<td>NBD</td>
<td>Organic cation/carnitine transporter</td>
</tr>
<tr>
<td>OCTN2</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAGE</td>
<td>4-Phenybutyric acid</td>
</tr>
<tr>
<td>4-PBA</td>
<td>Progressive familial intrahepatic cholestasis</td>
</tr>
<tr>
<td>PFIC</td>
<td>Perfluorooctanoic acid</td>
</tr>
<tr>
<td>PFO</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Pgp</td>
<td>Persistent hyperinsulinemic hypoglycemia of infancy</td>
</tr>
<tr>
<td>PHHI</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PMSF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td><em>Pseudoxanthoma elasticum</em></td>
</tr>
<tr>
<td>PXE</td>
<td>Small cell lung carcinoma</td>
</tr>
<tr>
<td>SCLC</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS</td>
<td>Sulfonylurea receptor</td>
</tr>
<tr>
<td>SUR</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TM</td>
<td>Trimethylamine N-oxide</td>
</tr>
<tr>
<td>TMAO</td>
<td>Unconjugated bilirubin</td>
</tr>
<tr>
<td>UCB</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>UPR</td>
<td>Vincristine</td>
</tr>
<tr>
<td>VCR</td>
<td>Wild-type</td>
</tr>
<tr>
<td>WT</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1 Introduction and Literature Review

1.1 The ATP-binding Cassette Superfamily of Proteins

The vast majority of ATP-binding cassette (ABC) proteins are polytopic membrane proteins which can be found in all species; they bind and hydrolyze ATP as a source of energy to carry out their functions (1). In humans, there are 49 ABC proteins which have been subdivided into 7 subfamilies, denoted as ABCA to ABCG, and are involved in many different physiological processes (2). These variously include the cellular efflux of bioactive molecules (e.g. eicosanoids, bile acids, and steroid conjugates), the modulation of cellular absorption, tissue distribution, and cellular elimination of nutrients and their metabolites as well as preventing accumulation, and aiding elimination of drugs and chemical toxins and their metabolites from a variety of tissues in the human body (3). In addition to these physiological and pharmacological functions, certain ABC transporters, most notably multidrug resistance protein 1 (MRP1) (encoded by ABCC1), P-glycoprotein (encoded by ABCB1) and breast cancer resistance protein or ABCG2 (encoded by ABCG2), are expressed in human tumour cells and can lead to a multidrug resistance (MDR) phenotype in vitro and are associated with MDR in vivo (4). Furthermore, a number of genetic disorders are caused by mutations in human ABC transporters which impair the activity or proper expression and/or cellular localization of these proteins (Table 1.1). Some of these disorders, including Dubin-Johnson Syndrome (DJS) (caused by mutations in MRP2/ABCC2) (5), Pseudoxanthoma elasticum (PXE) (caused by mutations in MRP6/ABCC6) (6), persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (caused by mutations in SUR1/ABCC8) (7) and cystic fibrosis (CF) (caused by mutations in CFTR/ABCC7) (8), are often due to the absence of the respective proteins at the appropriate cellular location (described in more detail below in Section 1.4). The functional core structure of
Table 1.1. Human disorders and diseases caused by mutations in human ABC transport proteins

<table>
<thead>
<tr>
<th>Human ABC gene</th>
<th>Disorder caused by mutations</th>
</tr>
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<tbody>
<tr>
<td>ABCA1</td>
<td>Tangier disease (Familial hypoapoproteinemia)</td>
</tr>
<tr>
<td>ABCA3</td>
<td>Neonatal respiratory distress syndrome/pulmonary surfactant deficiency, interstitial lung disease</td>
</tr>
<tr>
<td>ABCA4</td>
<td>Stargardt/fundus flavimaculatus; retinitis pigmentosa; cone-rod dystrophy; age-related macular degeneration</td>
</tr>
<tr>
<td>ABCA12</td>
<td>Congenital ichthyoses</td>
</tr>
<tr>
<td>ABCB2</td>
<td>Behcet’s disease (immune deficiency)</td>
</tr>
<tr>
<td>ABCB3</td>
<td>Behcet’s disease (immune deficiency)</td>
</tr>
<tr>
<td>ABCB4</td>
<td>Progressive familial intrahepatic cholestasis-3 (PFIC-3)</td>
</tr>
<tr>
<td>ABCB7</td>
<td>X-linked sideroblastic anemia</td>
</tr>
<tr>
<td>ABCB11</td>
<td>Progressive familial intrahepatic cholestasis-2 (PFIC-2); benign recurrent intrahepatic cholestasis type 2 (BRIC2); intrahepatic cholestasis of pregnancy (ICP)</td>
</tr>
<tr>
<td>ABCC2</td>
<td>Dubin-Johnson syndrome (DJS)</td>
</tr>
<tr>
<td>ABCC6</td>
<td><em>Pseudoxanthoma elasticum</em> (PXE)</td>
</tr>
<tr>
<td>ABCC7</td>
<td>Cystic fibrosis (CF)</td>
</tr>
<tr>
<td>ABCC8</td>
<td>Persistent hyperinsulinemic hypoglycemia of infancy (PHHI)</td>
</tr>
<tr>
<td>ABCC9</td>
<td>Cantú syndrome</td>
</tr>
<tr>
<td>ABCC11</td>
<td>Earwax type; axillary osmidrosis</td>
</tr>
<tr>
<td>ABCD1</td>
<td>Adrenoleukodystrophy</td>
</tr>
<tr>
<td>ABCG2</td>
<td>Gout</td>
</tr>
<tr>
<td>ABCG5</td>
<td>Sitosterolemia</td>
</tr>
<tr>
<td>ABCG8</td>
<td>Sitosterolemia</td>
</tr>
</tbody>
</table>
mammalian ABC transporters typically consists of two membrane spanning domains (MSDs) and two nucleotide binding domains (NBDs) (9). MRP1 belongs to the ABCC subfamily, members of which have a lower sequence homology between their two NBDs compared to the two NBDs in other ABC subfamily proteins (e.g. P-glycoprotein (Pgp) encoded by \textit{ABCB1}) (2). In addition to MRP1 (\textit{ABCC1}), the human ABCC subfamily consists of 12 other members, including MRP2 (\textit{ABCC2}), MRP3 (\textit{ABCC3}), MRP4 (\textit{ABCC4}), MRP5 (\textit{ABCC5}), MRP6 (\textit{ABCC6}), MRP7 (\textit{ABCC10}), MRP8 (\textit{ABCC11}), MRP9 (\textit{ABCC12}) and the pseudogene \textit{ABCC13}, as well as the cystic fibrosis transmembrane conductance regulator CFTR (\textit{ABCC7}) and the sulfonylurea receptors SUR1 (\textit{ABCC8}) and SUR2 (\textit{ABCC9}) (Fig. 1.1) (2). Most MRPs transport organic anions whereas CFTR is a cAMP-gated chloride channel, and SUR1 and SUR2 are regulators of inwardly rectifying potassium channels (Kir6.2 and Kir6.1, respectively). No transport function for MRP9 has yet been reported (10). The current review will focus on the topic of this thesis, the multidrug and organic anion transporter MRP1.

1.2 Physiological and Pharmacological Roles of MRP1

MRP1 was first cloned by Cole and co-workers in 1992 and was identified as being responsible for the MDR phenotype of the small cell lung carcinoma (SCLC) cell line H69AR (11; 12). The \textit{ABCC1} (formerly called \textit{MRP1} or \textit{MRP}) gene was mapped to chromosome 16p13.1 and was predicted to encode a protein with 1531 amino acids (11). In most cells, MRP1 locates to the plasma membrane, especially in mammalian epithelial cells. In mammalian cells, MRP1 is both N-glycosylated (at Asn\textsuperscript{19}, Asn\textsuperscript{223} in MSD0, and Asn\textsuperscript{1006} in MSD2) and phosphorylated, and it usually exhibits an electrophoretic mobility of \(~\text{190 kDa}\) (13-17). As mentioned above, in addition to the two NBDs and two MSDs typical of the majority of ABC
**Figure 1.1.** Phylogenetic tree of the human ABCC transporter subfamily. Phylogram demonstrating the relative similarities among members of the human ABCC subfamily of ABC transporters. Multiple amino acid sequence alignments and the phylogram were generated using ClustalW v2.0 software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Shown in parentheses are the common protein names for each ABCC transporter. The pseudogene ABCC13 has been omitted from this analysis.
proteins, MRP1 (and MRP2, 3, 6, 7, SUR1, SUR2) are distinguished by the presence of an additional MSD at the NH₂-terminus which is predicted to have 5 transmembrane helices (TMs) with an extracellular NH₂-terminus (Fig.1.2) (17). These 5-domain MRPs are often referred to as the ‘long’ MRPs (2).

1.2.1 Tissue distribution and subcellular localization

MRP1 protein and/or mRNA is ubiquitously expressed throughout the body. \(ABCC1\) mRNA can be detected in lung, testis, skeletal muscle, heart, kidney, pancreas, ovary, thymus, spleen, prostate, peripheral blood mononuclear cells and at lower levels, in the brain and liver (2). MRP1 protein expression can be easily detected in testis and lung, but also in the skin, esophagus, small and large intestine, myocardium, pancreas, macrophages, muscle cells and at lower levels in the liver (2). Interestingly, some differences in tissue distribution of MRP1 among various species have been reported (18; 19). For example, the canine liver shows relatively high levels of \(ABCC1\) mRNA relative to human liver (20). Further, some gender differences in expression have also been reported, e.g., female mice exhibit higher \(ABCC1\) mRNA levels in kidney and liver than do male mice (21).

\(ABCC1\) mRNA or protein is also expressed at several tissue-blood barriers in the body, including the blood-testis barrier, placenta-blood barrier, the blood-brain barrier and the cerebrospinal fluid (CSF)–blood barrier (22-24). MRP1 is believed to protect these organs from xenobiotics by limiting their absorption or transporting them back into the blood thus limiting their accumulation in the organ. In terms of subcellular localization, MRP1 normally localizes to the basolateral membrane in polarized mammalian epithelial cells, such as canine kidney MDCK and human colon Caco-2 cells (15; 25). Exceptionally, MRP1 has been found at the apical surface of human brain capillary endothelial cells (26-28). In addition to the plasma membrane
Figure 1.2. A general membrane topology model of MRP1. A topology model of MRP1 showing MSD0, MSD1, MSD2, NBD1, and NBD2 coloured in orange, blue, purple, green, and red, respectively. MSD, membrane spanning domain; NBD, nucleotide binding domain; A, Walker A motif; B, Walker B motif; C, the active transport signature motif of ABC proteins.
(14), perinuclearly localized lysosomes in HeLa cells (31), trans-Golgi vesicles in GLC4/Adr
cells (30) and mitochondria in murine heart after doxorubicin treatment (32), but the functional
relevance, if any, is unknown.

### 1.2.2 Substrate specificities of MRP1

In addition to chemotherapeutic agents and toxicant metabolites that are discussed below
in Sections 1.2.5 and 1.2.6, MRP1 also transports many organic anions in an ATP-dependent
manner. The most common substrates of MRP1 are summarized in Table 1.2. Most substrates
have only been identified in vitro and their physiological relevance in whole organisms remains
to be confirmed. In general, exogenous substrates of MRP1 are often products of phase II drug
metabolism, that is, metabolites of xenobiotics conjugated to GSH, glucuronide and sulfate.
MRP1 is also capable of transporting unconjugated xenobiotics, such as methotrexate (MTX) and
aflatoxin B₁, although far less efficiently than GSH-conjugated aflatoxin B₁ epoxide (33).

The pro-inflammatory cytokine leukotriene C₄ (LTC₄) (Fig. 1.3) is so far the best
characterized endogenous substrate of MRP1 and has one of the highest apparent affinities for
MRP1 ($K_m$ approximately 0.1 μM) of all the substrates identified to date (2). LTC₄ is an
arachidonic acid derivative conjugated to GSH that is involved in asthmatic and allergic
reactions, suggesting a role for MRP1 in mediating inflammatory responses (discussed below in
Section 1.2.4). In addition to LTC₄, MRP1 can transport other GSH conjugated fatty acids
including prostaglandin A₂-SG (34) and 4-hydroxynonenal-SG (35; 36). Thus, the location of
MRP1 in numerous tissues and its ability to transport GSH conjugates make MRP1 a ubiquitous
pump of glutathione conjugates; indeed, the existence of a ‘GS-X pump’ was hypothesized even
before MRP1 was cloned (37). 17β-Estradiol 17-(β-(D)-glucuronide) (E₂17βG) (Fig. 1.3) is
another well characterized substrate of MRP1, at least in vitro (38; 39). Despite >90% sequence
identity with human MRP1, murine, dog, cow, and rat Mrp1 transport E₂17βG very poorly (2), revealing significant species differences in MRP1/Mrp1 substrate specificity. Therefore, the Abcc1I(−/−) mouse models cannot be used to predict all of the observed physiological and pharmacological functions of human MRP1 (63). In the presence of reduced glutathione (GSH) or S-methyl GSH, MRPI can efficiently transport estrone 3-sulfate (E₁3SO₄) (Fig. 1.3) and certain other sulfate conjugated steroid hormones (44; 64; 65) (Fig. 1.3). Additionally, MRPI transports both bilirubin mono- and bis-glucuronide as well as conjugated bile salts (e.g. sulfatolithocholy taurine) (39; 66). MRPI also transports folic acid derivatives (1; 67). Finally, both GSH and the oxidized form of GSH (glutathione disulfide; GSSG) can themselves be transported by MRPI, though the affinity for GSSG is much higher (Table 1.2), unless GSH transport is measured in the presence of verapamil, apigenin or certain other phytoestrogens (68-71).

1.2.3 Studies in Abcc1 knockout mice

Mice deficient in Mrp1 (Abcc1I(−/−)) have been generated by at least two independent groups (72; 73). In both cases, these mice are viable, healthy, and fertile with normal histological, hematological and serum-chemical parameters, suggesting that Mrp1 is not essential to these animals in the absence of drug treatments or exposure to toxicants. However, Abcc1I(−/−) mice displayed significantly altered metabolite profiles. For example, in tissues which express higher levels of Mrp1 such as lung, testes and bone marrow cells, changes in the GSH levels (an increase of 20-90%) were more drastic than in tissues known to have low expression of Mrp1 (72). Furthermore, embryonic stem cells derived from normal Abcc1I(+/+) and Abcc1I(−/−) mice showed different levels of baseline GSH export (74). Since there was no detectable increase in
<table>
<thead>
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<tr>
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<tr>
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<td>Leukotriene C₄, leukotriene D₄, leukotriene E₄</td>
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</tr>
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<tr>
<td>4-Hydroxynonenal-SG</td>
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<tr>
<td><em>Glucuronide conjugates</em></td>
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<tr>
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<tr>
<td>Dehydroepiandrosterone 3-sulfate</td>
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<tr>
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<tr>
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<td>(57)</td>
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<tr>
<td>Anticancer drugs</td>
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<tr>
<td>Anthracyclines (daunorubicin, doxorubicin, epirubicin)</td>
<td>(59; 60)</td>
</tr>
<tr>
<td><em>Vinca</em> alkaloids (vinblastine, vincristine)</td>
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<td>SN-38, irinotecan</td>
<td>(62)</td>
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<tr>
<td>Methotrexate</td>
<td>(48)</td>
</tr>
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</table>
Figure 1.3 Chemical structures of major MRP1 physiological substrates

GSH

\[\text{GSH}\]

\[\text{LTC}_4\]

\[\text{E}_2\text{17}\beta\text{G}\]

\[\text{GSSG}\]
the activity of glutamate-cysteine ligase, the rate-limiting enzyme for GSH synthesis (72), these observations are consistent with an important role for MRPI in mediating GSH efflux (75).

LTC₄ release was also found to be lower by about 3-fold in bone marrow mast cells obtained from Abcc1⁻/⁻ mice while the intracellular levels of LTC₄ were 4-fold higher than in cells from wild-type mice (76), confirming a role for Mrp1/MRP1 in LTC₄ secretion. More importantly, Abcc1⁻/⁻ mice did not respond to the pro-inflammatory stimulus as much as wild-type mice did, and the lack of inflammatory response was reversed by administration of exogenous LTC₄ (76). These observations support a role for MRP1 in mediating inflammation (discussed further below in Section 1.2.4).

The ability of Mrp1 to confer resistance to xenobiotics, including chemotherapeutic agents, has also been reported in vivo. Abcc1⁻/⁻ mice displayed increased sensitivity (4-fold) to vincristine whereas no change in sensitivity to cisplatin or sodium arsenite was observed (72; 73). Also, Abcc1⁻/⁻ mice were hypersensitive (2-fold) to etoposide (VP-16) (72; 73). This was particularly the case in bone marrow, oropharyngeal mucosa, Sertoli cells in the seminiferous tubules of the testis, and the urinary collecting tubules in the kidney where extensive drug-induced damage was observed (72; 73). Additionally, upon exposure to the pesticide methoxychlor, developing spermatocytes and spermatids were damaged in the testis of Abcc1⁻/⁻ mice (77). These observations strengthen the conclusion that MRPI plays a crucial role in protecting at least some cell and tissue types against xenobiotic-induced toxicities. Taken together, these findings demonstrate that although MRPI is dispensable for normal development, it exerts a critical role in GSH homeostasis and tissue defense against xenobiotic toxicities.
1.2.4 Physiological importance of MRP1

The ubiquitous expression and diverse substrate specificity of MRP1 suggest numerous potential physiological functions for this protein. Some of these have been discovered from knockout studies in mouse models (described above in Section 1.2.3). As indicated earlier, the best appreciated physiological role of MRP1 is the LTC₄-mediated inflammatory response (Table 1.2). LTC₄ is produced in mast cells, basophils, eosinophils, dendritic cells, macrophages, neutrophils, platelets, kidney, and brain (78; 79). It has been reported that during latency of human cytomegalovirus (HCMV) infection MRP1 is downregulated to reduce export of cellular LTC₄, which functions to sensitize dendritic cells’ migration to lymph nodes, and thus prevent the migration of HCMV-infected dendritic cells to lymph nodes to generate an HCMV-specific immune response (80). Further, MRP1 has been indicated as a transporter of vitamin B12 (49; 50), sphingosine 1-phosphate (51; 52) and lysophosphatidylinositol (53).

1.2.5 Toxicological roles of MRP1

Many studies have reported both in vitro and in vivo evidence indicating a role for MRP1 in protecting tissues from toxin-induced damage (81). Detoxification processes of many drugs and xenobiotics in cells typically encompass four stages: the uptake of the xenobiotics (Phase 0), followed by oxidation (Phase I) and conjugation with an anionic moiety (Phase II), and finally, the conjugates are extruded from cells and ultimately the body (Phase III) (54). MRP1, as well as other MRPs, particularly MRP2 and MRP4, have been suggested to play a role in the terminal step of elimination (Phase III) (82) and also in absorption (Phase 0) in some cases (3).

In vitro, MRP1 has been reported to transport a variety of compounds of toxicological relevance (Table 1.2). Many of these in vitro studies support the conclusion that MRP1 has a role in the removal of toxins as GSH, glucuronide or sulfate conjugated metabolites from cells (Table
1.2). For instance, aflatoxin B₁ is a potent liver and lung carcinogen, and when conjugated to GSH, is transported by MRP1 with high affinity (apparent $K_m$ 0.19 µM) (55). However, an *in vivo* study using *Abcc1*(+/+) and *Abcc1*(−/−) mice showed that aflatoxin B₁ exposure induced a similar number of lung and liver tumours, indicating that, at least with the mouse strain used and under the conditions tested, Mrp1 does not play a significant role in protecting mice from aflatoxin B₁–induced carcinogenicity *in vivo* (83). Not all conjugates are transported by or interact with MRP1/Mrp1 such as nicotine glucuronide metabolites (84). Some glucuronide metabolites require the presence of GSH to be transported including the glucuronide metabolite of the lung carcinogen found in cigarette smoke, NNAL-O-glucuronide, whose transport by MRP1 (but not MRP2) is only detectable in the presence of GSH ($K_m$ 37 µM) (85). Certain unmodified toxins and drugs have also been shown to be transported by MRPI but in most cases (but not all) their transport is stimulated by GSH (in the absence of conjugation) (55; 59).

The toxicity of the heavy metals antimony (Sb) and arsenic (As) has been attributed (partially) to their ability to modify mitochondrial enzymes which impairs tissue respiration (86). In addition to conferring resistance to many natural product drugs, MRPI also has the ability to protect cells against the toxicity of Sb and As oxyanions such as sodium arsenite, sodium arsenate, and antimony potassium tartrate (12; 63). The protection against these toxic metal oxyanions has been shown to be associated with GSH efflux from the cell (87; 88). Recent work has demonstrated that detoxification of arsenic is in the form of arsenic–GSH conjugates such as As$^{III}$,(GS)$_3$, monomethylarsenic glutathione (MMA$^{III}$,(GS)$_2$) and dimethylarsenic glutathione (DMA$^{III}$,(GS)) (89). The protective role of MRPI from heavy metals, at least in certain forms, is further supported from the findings that heavy metal-selected tumour cell lines have been shown to overexpress MRPI (90).
In addition to protecting cells against exogenous toxicants, MRP1 can also protect some organs from endogenous toxic compounds. MRP1 mediates the ATP-dependent cellular export of unconjugated bilirubin (UCB), as mentioned earlier (91). UCB is toxic to the central nervous system and can cause encephalopathy in severely jaundiced newborns. In cultured mouse astrocytes, Mrp1 has been reported to be localized to the Golgi apparatus; low concentrations of UCB (40 nM) rapidly upregulate Mrp1 expression and promote its translocation to the plasma membrane (29). This rapid translocation of Mrp1 has been shown to be associated with protection of astrocytes from cytotoxicity induced by UCB (29). A recent study showed that mouse embryo fibroblasts (MEF) isolated from Abcc1(−/−) mice exposed to 40-140 nM [3H]bilirubin accumulated twice as much [3H]bilirubin compared to wild-type cells, confirming that Mrp1 limits intracellular accumulation of UCB and thus decreases its cytotoxicity (92).

It is also interesting to note that MRP1 protein expression can be modulated by certain exogenous chemicals. For instance, the hepatotoxin carbon tetrachloride has been shown to increase Mrp1 protein levels in mouse liver in a dose-dependent fashion, whereas tert-butyl hydroquinone and the flavonoid quercetin have been shown to induce MRP1 protein expression in a human breast cancer cell line (93; 94). Although several other chemicals appear to alter the expression patterns of MRP1/Mrp1, the mechanism(s) by which they do so is not fully understood (95; 96).

1.2.6 MRP1 and drug resistance in cancer chemotherapy

Increased levels of MRP1 expression have been found in a wide range of hematological and solid tumours. Intrinsically multidrug resistant tumour types such as non-SCLC ((NSCLC) (97-99) or chronic lymphoblastic leukemia (100; 101) exhibit a high level of MRP1 expression whereas others such as SCLC (102), gastric carcinoma (103; 104), and retinoblastoma (105)
exhibit high MRP1 expression with a lower frequency. In certain tumour types such as acute myeloblastic leukemia (AML), myelodysplastic syndromes (MDS) (106), and prostate cancer (107; 108), a correlation between MRP1 expression and the stage of the tumour has also been reported. High MRP1 expression was also found to be associated with a higher grade of tumour differentiation in digestive tract carcinomas (109), endometrial carcinomas (110), and various subtypes of NSCLC, such as lung adenocarcinoma and squamous cell carcinoma (98; 99).

Neuroblastoma, perhaps, provides the most convincing example of MRP1’s role in drug resistance in cancer (111; 112). In a mouse model of primary neuroblastoma, genetic modification including homozygous deletion of the Abcc1 gene increased the sensitivity of the tumours to MRP1 substrate drugs (vincristine, etoposide, doxorubicin) (61). It has also been demonstrated that there is a strong correlation of ABCC1 gene expression with the expression of the MYCN oncogene in primary neuroblastoma tumours which is strongly associated with poor prognosis outcome (111; 112). It has been shown that MYCN can up-regulate ABCC1 expression by interacting with E-box elements within the ABCC1 promoter (111). In a recent study using a transgenic mouse model of neuroblastoma, a new class of MRP1 inhibitor, reversan, used in combination with vincristine increased the survival of treated mice 2-fold compared to using vincristine alone (61). A significant potentiation was also observed in mice treated with a combination of etoposide with reversan versus etoposide alone (61). Thus, both in vivo animal studies and clinical data have shown that inhibiting MRP1 may be a reasonable approach to increasing the sensitivity of neuroblastoma tumours to drugs that are MRP1 substrates.

1.3 Role of polar amino acids in MRP1 substrate specificity, function and expression

In a mammalian plasma membrane with a typical thickness of 30Å (113), TM α-helices of single span membrane proteins typically consist of ~21 amino acids, frequently with the core
region abundant in aliphatic residues and short border regions enriched with polar aromatic Trp and Tyr residues (114; 115). TM α-helices of polytopic membrane proteins are more likely to have more amino acids due to ‘tilt’ caused by interactions between adjacent TMs. In both cases, however, positively charged amino acids are often preferentially located near the membrane interfaces where they can interact with the negatively charged head groups of the membrane phospholipids, and predominantly positioned at the cytoplasmic (or luminal) face rather than the extracellular face of the membrane (referred to as the “positive-outside” rule) (115; 116). Thus, ionizable residues (which are normally energetically unfavorable in the lipid bilayer) (117) can influence the membrane topology and anchoring of a protein in the plasma membrane. It has been shown that a single membrane-buried hydrogen bond between two proximal amino acid polar side chains may contribute as much stabilization energy as that provided by the sum of the van der Waals contacts along the full lengths of two interacting TM helices (118; 119). As such, these residues potentially represent a driving force in the folding of membrane proteins by mediating and stabilizing inter- and intra-helical interactions (117). Substitutions of polar residues, often conserved, in TM helices have been shown to adversely affect the function and stable expression of a many membrane proteins, indicating a role in maintaining protein conformation and functional integrity (120-122).

1.3.1 Prediction of transmembrane helices in polytopic proteins

Site-directed mutagenesis and subsequent functional analyses have demonstrated that a significant number of amino acids located in TM4, 6-8, 9, 10-11, 14, 16-17 or in close proximity to the membrane/cytosol interface of MRP1 are important for binding and/or transport of MRP1 substrates, as well as expression of the protein at the plasma membrane (26; 123-135). Most of the amino acids identified to be essential for substrate specificity (recognition and/or transport),
catalytic activity and/or expression are polar residues (Trp, Tyr, Arg, Asp, Glu, Lys, Thr, Ser) with some exceptions (Phe, Pro, Gly).

The boundaries of the TM helices of MRP1 cannot rigidly or precisely be defined, since the MRP1 transporter (like other ABC transporters) is dynamic and known to undergo conformational changes during its transport cycle, and thus its exact position in the lipid bilayer changes during substrate binding, translocation and release. The existing atomic homology models of MRP1 are all based on a nucleotide-bound outwardly facing crystal structure of a bacterial transporter, Sav1866, and thus can only provide limited information for reasons discussed in Section 1.3, as well as due to the differences between mammalian and bacterial membrane composition, thickness and fluidity. The topologies of MRP1 predicted by a variety of algorithms developed for this purpose are quite diverse (Table 1.3). At least part of this diversity, however, is likely due to the different assumptions made in the development of the algorithms which may or may not hold true with a complex polytopic 5-domain protein like MRP1 with 17 TMs and greater than average abundance of polar amino acids and Trp residues in its TM helices.

The boundaries of the 17 TMs in MRP1 predicted by six different algorithms, including SOSUI (116), TMPred9 http://www.ch.embnet.org/software/TMPRED_form.html, TMHMMv.2 (136), HMMTOPv.2 (137), TopPred (138) and PredictProtein-PHDhtm (139), and the results are summarized in Table 1.3. All six algorithms concur on the existence of five TMs in MSD0 which also agrees with the predictions based on a series of site-directed mutagenesis-based and epitope-insertion based studies (17; 140). However, not all the algorithms correctly predict the orientation of the TMs in MSD0. For example, it has been well established by the N-glycosylation of Asn\textsuperscript{19} and Asn\textsuperscript{23} as well as epitope-insertion studies that the NH\textsubscript{2}-terminus of MRP1 locates extracellularly (17; 140). Consistent with this, SOSUI, TMPred and TMHMMv.2
Table 1.3. Transmembrane (TM) helices of MRP1 predicted by six algorithms developed for protein topology determination

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<td>A1218</td>
<td>I1220</td>
<td>F1128</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>A1227</td>
<td>L1225</td>
<td>L1225</td>
<td>L1203&lt;sup&gt;(16)&lt;/sup&gt;</td>
<td>L1203&lt;sup&gt;(16)&lt;/sup&gt;</td>
<td>H1223</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R1249</td>
<td>L1244</td>
<td>L1247</td>
<td>S1221</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>: TM boundaries predicted by the algorithms which largely deviate from the prediction based on biochemical data but span other putative TMs, are in italic face and the respective putative TMs are superscripted in parentheses; otherwise they are in bold, italic face.
all predict that an extracellular NH$_2$-terminus of MRP1. Three other algorithms (HMMTOPv.2, TopPred and PredictProtein-PHDhtm) incorrectly locate the NH$_2$-terminus intracellularly, leading to further errors in assigning the orientations of the subsequent TM helices. Most of the algorithms identify 12 TM helices in the core region of MRP1 (except for the PredictProtein-PHDhtm which only identifies 9 TM helices for MRP1 with the reliability of topology prediction scored at 2/9, with 9 being the most reliable score); however, the precise boundaries of the TM helices differ with each algorithm (Table 1.3). For TM16, three out of six programs (SOSUI, TMPred, TMHMMv.2) predict that TM16 is located between residues 1197 to 1221 (Fig. 1.4A). This is in agreement with the prediction used to define the location (i.e. TM16) of amino acids assessed by a series of site-directed mutagenesis studies (17; 123; 128; 130; 133; 142; 143).

Previous study has shown that insertion of 10 amino acids corresponding to the hemagglutinin (HA) epitope (YPYDVPDYAS) at position 1222 locates to the extracellular side of the plasma membrane (142), indicating that TM16 and TM17 are connected by a very small extracellular hairpin segment. An antibody based approach confirmed that NBD2 is located intracellularly, as expected (15). Consistent with the biochemical data, all three programs also correctly predict the orientations of TM16 and TM17 and cytoplasmic location of NBD2.

In contrast to the above, the HMMTOPv.2, TopPred and PredictProtein-PHDhtm algorithms predict that TM16 is comprised of amino acids 1097 to 1128 and TM17 of amino acids 1203 to 1223, and the two TMs are connected by an intracellular segment (Table 1.3 and Fig. 1.4A). The TM16 and TM17 sequences predicted by these three programs partially overlap with what is predicted to be TM14/15 and TM16, respectively, by the other three algorithms and the biochemical data (Table 1.3). These predictions also inaccurately locate regions after residue 1223 (i.e. TM17 and NBD2) extracellularly.
Figure 1.4. Sequence alignments of MRP1-TM16 with comparable region in other ABCC subfamily proteins and location of TM16 in MRP1. A, human MRP1 residues 1191 – 1223 have been aligned with the corresponding sequences in its ABCC homologs and orthologs using ClustalW. Polar residues are in red and those ones have been described in Section 1.4 are in red boldface type, whereas (*) indicates identity; (:) strongly similar and (.) weakly similar. SUR, sulfonylurea receptor; Hum, human; Mus, mouse; Mon, monkey; Can, canine; Tau, bovine. Sequences were extracted from the SWISS-PROT protein sequence data bank. Swiss-Prot accession numbers: human ABCC1, P33527; mouse ABCC1, O35379; rat ABCC1, Q8CG09; macaque ABCC1, Q864R9; canine ABCC1, Q95M36; bovine ABCC1, Q8HXQ5. B, the location of TM16 varies according to the membrane protein topology algorithm used. Underlined resides indicate the TM helix as predicted by the six algorithms.
1.3.2 Role of polar amino acids in TM16 and proximal regions of MRP1

In the past decade or so, the structural and functional contributions of polar residues (Lys, Arg, Asp, Glu, His, Thr, Ser, Asn, Gln, Tyr, Cys, Trp) in putative TM16 and its proximal regions have been studied by site-directed mutagenesis (Fig. 1.4A and Table 1.4). In the region predicted to encompass TM16 (residues 1191 to 1223) (Fig. 1.4), the functional roles of several polar amino acids (Trp\textsuperscript{1198}, Cys\textsuperscript{1205}, Asn\textsuperscript{1208} and Cys\textsuperscript{1209}) as well as four ionizable residues (Arg\textsuperscript{1197}, Arg\textsuperscript{1202}, Glu\textsuperscript{1204} and Arg\textsuperscript{1222}) have been characterized in detail (128; 130; 144), and the findings are summarized below.

\textit{TM16-Arg}\textsuperscript{1197}

Arg\textsuperscript{1197} is well conserved among all MRP1 mammalian orthologs and ABCC family members (Fig. 1.4B). Opposite as well as same charge substitutions of Arg\textsuperscript{1197} (R1197E/K) did not affect the levels of MRP1 in HEK293 cells; however, the mutants displayed a substantial loss of E\textsubscript{2}17βG, LTC\textsubscript{4}, MTX and GSH-stimulated E\textsubscript{1}3SO\textsubscript{4} transport (to <10-25% of wild-type transport activity) (130). These results indicate that neither the basic character nor the size of the Arg\textsuperscript{1197} side chain is important for proper synthesis of the protein, but both properties are critical for the organic anion transport activities of MRP1. The lack of LTC\textsubscript{4} photolabeling indicates that the reduced LTC\textsubscript{4} transport activity (possibly other anions) of these mutants was associated with decreased substrate binding and not with changes in interactions of the transporter with ATP (130). Thus, Arg\textsuperscript{1197} functions more than just a topological determinant (145) and appears to be crucial for maintaining the architecture of the substrate binding site(s) of MRP1 (130). Although a Lys substitution maintains the basic character of Arg\textsuperscript{1197}, it was suggested that the bulkier, less ionizable Lys side chain in the R1197K mutant hinders the formation of the normal interhelical and/or intrahelical interactions which are established by Arg\textsuperscript{1197} in wild-type MRP1 (130). Thus,
<table>
<thead>
<tr>
<th>Location</th>
<th>Mutation</th>
<th>Protein levels in HEK 293T cells</th>
<th>Vesicular Transport Activity in HEK 293T cells</th>
<th>Drug Resistance in intact cells</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal (NH₃)</td>
<td>P1191A</td>
<td>Normal</td>
<td>Substrate-selective reduction (E₂SO₄ by 40%)</td>
<td>ND</td>
<td>(129)</td>
</tr>
<tr>
<td>TM</td>
<td>R1197E</td>
<td>Normal</td>
<td>Global loss (E₂17βG, LTC₄, MTX, E₁SO₄)</td>
<td>ND</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td>R1197K</td>
<td>Normal</td>
<td>Global loss (E₂17βG, LTC₄, MTX, E₃SO₄)</td>
<td>ND</td>
<td>(130)</td>
</tr>
<tr>
<td>TM</td>
<td>W1198A</td>
<td>Reduced (by 60%)</td>
<td>Global loss (GSH, E₂17βG, MTX, E₃SO₄) except LTC₄ (by 40%)</td>
<td>ND</td>
<td>(128)</td>
</tr>
<tr>
<td></td>
<td>W1198F</td>
<td>Reduced (by 60%)</td>
<td>Substrate-selective reduction (E₂17βG, MTX by 50%)</td>
<td>ND</td>
<td>(128)</td>
</tr>
<tr>
<td></td>
<td>W1198Y</td>
<td>Reduced (by 60%)</td>
<td>Substrate-selective reduction (MTX by 50%)</td>
<td>ND</td>
<td>(128)</td>
</tr>
<tr>
<td>TM</td>
<td>R1202D</td>
<td>Reduced (by ≥80%)</td>
<td>ND</td>
<td>ND</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td>R1202L</td>
<td>Normal</td>
<td>Substrate-selective reduction (E₃SO₄ by 50%)</td>
<td>ND</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td>R1202G</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>(130; 146; 147)</td>
</tr>
<tr>
<td>TM</td>
<td>E1204K</td>
<td>Reduced (by &gt;70%)</td>
<td>ND</td>
<td>ND</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td>E1204L</td>
<td>Normal</td>
<td>Global loss (E₂17βG, E₃SO₄, GSH; LTC₄ (by 50%)) except MTX</td>
<td>ND</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td>E1204D</td>
<td>Normal</td>
<td>Substrate-selective reduction (GSH by 75%)</td>
<td>ND</td>
<td>(130)</td>
</tr>
<tr>
<td>TM</td>
<td>S1205A</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>N1208A</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td>(135)</td>
</tr>
<tr>
<td>TM</td>
<td>S1209A</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Proximal (COOH)</td>
<td>R1222M</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined; Normal, wild-type level
the disruption of native inter- or intra-helical interactions may explain the loss of transport function of the R1197K mutant.

\[ \text{TM16-Trp}^{1198} \]

Trp\(^{1198}\), like Arg\(^{1197}\), is well conserved among all MRP1 mammalian orthologs and ABCC family members (Fig. 1.4B). It is also predicted to be located at the NH\(_2\) end (membrane/cytosol interface) of TM16 (Fig. 1.4A) (128). Mutants in which Trp\(^{1198}\) was substituted with a non-aromatic and non-polar Ala (which has a minimal side chain volume) or more conservative aromatic substitutions (with a non-polar Phe or a polar Tyr) were expressed at levels that were only 30-40% of those of wild-type MRP1 even though the expressed mutant proteins were localized at the plasma membrane. It was suggested that the bulky indole side chain of Trp at this position is critical to MRP1 stability at the plasma membrane (128). Transport of most organic anion substrates (\(E_217\beta G\), GSH, MTX and \(E_13\)SO\(_4\)) by the W1198A mutant was substantially reduced (by >80%) except for LTC\(_4\) transport which was only moderately reduced (by ~65%) (128), indicating that Trp\(^{1198}\) is crucial to the overall organic anion transport activity of MRP1. Phe and Tyr substitutions of Trp\(^{1198}\) maintained transport activities for LTC\(_4\), GSH, and E\(_13\)SO\(_4\) similar to (W1198F), or above (W1198Y), those of wild-type MRP1, whereas the transport of \(E_217\beta G\) and MTX by the W1198F and W1198Y mutants was ~50–75% that of wild-type MRP1. Thus, the retention of transport activity was greater for the Tyr mutant than the Phe mutant for all substrates (128). It was suggested that the steric bulk and aromaticity and, to a certain extent, the polarity of the indole side chain of Trp\(^{1198}\) contribute to tertiary interactions that determine the transport activity and substrate specificity of MRP1 (128).

The tendency of Trp residues in peptides to cluster at the hydrophobic interior of the lipid bilayer (observed in \(E. coli\) membrane mimicking lipid system) suggests that substituting them
with an amino acid with a small side chain volume can potentially reduce the efficiency of proper membrane anchoring of the protein and thus affect TM helix packing (148). It appears that the indole side chain of Trp\textsuperscript{1198} positioned at the TM16 membrane/cytosol interface is critical to the stable expression of MRP1. It was postulated that substitution of Trp\textsuperscript{1198} with any non-aromatic residue might disrupt a stabilizing π-cation interaction between the indole ring of Trp\textsuperscript{1198} and the basic side chain of Arg\textsuperscript{1202} that is found within one turn of the TM16 α-helix (128).

\textit{TM16-Arg^{1202}, Glu^{1204}}

All the opposite charge substitutions in TM helices of MRP1 described thus far result in mutant proteins that can be expressed at levels comparable to wild-type MRP1 levels in human embryonic kidney cells (HEK293) (Table 1.4). However, opposite charge substitution of the highly conserved Arg\textsuperscript{1202} and Glu\textsuperscript{1204} (Fig. 1.4), predicted to be embedded in the lipid bilayer within TM16 helix (defined as 1191-1223), reduced protein expression by >80% in HEK293 cells which precluded their functional characterization (130). R1202D and E1204K mRNA levels were determined to be comparable to wild-type MRP1 mRNA levels (130) suggesting that the stability of mutant mRNAs is not affected. On the other hand, levels of the neutral (R1202G, R1202L, E1204L) and same charge (R1202K and E1204D) mutants were comparable to wild-type MRP1 (130). This suggested that the introduction of an opposite charge at either position 1202 or 1204 caused misfolding of MRP1 during its biosynthesis and/or assembly, which presumably then targeted the mutant transporters for degradation by one of the quality control systems in the cell. It was previously suggested that the poor expression of these oppositely substituted MRP1 mutants was likely related to the fact that replacing Arg\textsuperscript{1202} with an Asp (or Glu\textsuperscript{1204} with a Lys) results in a net gain of two charges in the cytoplasmic half of TM16 which may affect its geometry in the membrane and its packing with other TM helices (130). It was
also noted that these substitutions bring two same-charge residues in close proximity to one another which also could be destabilizing due to the forces of electrostatic repulsion (130).

Although the neutrally and same-charge substituted Arg\(^{1202}\) and Glu\(^{1204}\) mutants were readily detected in membrane vesicles, the functional properties of the two sets of mutants differed substantially (Table 1.4A) (130). Thus, the R1202G and R1202L mutants exhibited transport activities that were, in the case of most substrates (LTC\(_4\), E\(_2\)17βG, E\(_1\)3SO\(_4\), MTX), similar to wild-type MRP1 or only moderately reduced. In contrast, transport of LTC\(_4\), E\(_2\)17βG, and GSH by the E1204L mutant was substantially reduced (>50%) or not detected; only the MTX transport activity of E1204L remained comparable to wild-type MRP1. Furthermore, while the same-charge E1204D mutant exhibited transport activities that were similar to those of wild-type MRP1, apigenin-stimulated GSH transport was reduced by 75%. Thus, a neutral substitution of Glu\(^{1204}\) has a much greater effect on MRP1 transport activity than do neutral substitutions of Arg\(^{1202}\).

Further studies of the transport-compromised E1204L mutant showed that although its LTC\(_4\) transport activity was reduced, labeling of the mutant MRP1 with this photoactive substrate was not affected (Fig. 1.5A), indicating that Glu\(^{1204}\) is not an essential component of the LTC\(_4\) binding site on MRP1 (130). On the other hand, photolabeling studies with \(^{32}\)P-labeled azido derivatives of ATP suggested that the ATPase activity (often referred to as the catalytic activity) of E1204L differed substantially from wild-type MRP1. Photolabeling studies with \(^{32}\)P-labeled 8-azido derivative of ATP at 4°C (which minimizes ATP hydrolysis) showed binding of the ATP analog to E1204L comparable to wild-type MRP1 (Fig 1.5B); however, at 37°C in the presence of sodium vanadate which traps azido-ADP after azido-ATP hydrolysis, vanadate-induced trapping of azido-ADP (4-fold), particularly in NBD2, was substantially increased in E1204L compared to
Figure 1.5. Photolabeling of wild-type MRP1 and E1204L mutant proteins with [³H]LTC₄ and 8-azido-[α-³²P]ATP. A, the upper panel shows the [³H]LTC₄ photolabeling of membrane vesicles prepared from cells overexpressing wild-type MRP1 and E1204L mutant proteins (values uncorrected for MRP1 levels in the membrane vesicles are shown below the blot) and the lower panel shows the relative levels of MRP1 protein in the membrane vesicles. B, 8-N₃-[α-³²P]ATP photolabeling of wild-type MRP1 and E1204L at 4 °C. C, vanadate-induced 8-N₃-[α-³²P]ADP trapping of wild-type MRP1 and E1204L at 37°C; film exposures are 24 h (left) and 36 h (right). N-half and C-half with asterisks indicate the positions of the ³²P-labeled NH₂- and COOH-proximal halves of wild-type or mutant MRP1 proteins generated by mild autolysis. E indicates 32P-labeled endogenous proteins. This figure and data are extracted and adapted from Situ et al (130).
The wild-type protein (Fig. 1.5C) (130). These observations suggested that E1204L does not necessarily affect substrate binding but has a profound impact on the subsequent interactions with ATP/ADP particularly at NBD2 which can affect substrate translocation (149). Consequently, it was proposed that the E1204L mutation was somehow disrupting the catalytic activity of MRP1 in a way that affected the transport of some substrates but not others (130). Taken together, the distinctly different properties of the neutrally substituted Arg1202 and Glu1204 mutants support the idea that while both amino acids influence the stable membrane expression and transport activity of MRP1, the mechanisms by which they do so differ. The exploration of ways to rescue the expression of these intriguing TM16 mutants forms the main subject (or focus) of this thesis (see below, Section 1.5).

**TM16-Arg1222**

This ionizable basic amino acid, which is poorly conserved among ABCC family members, is predicted to be located at COOH-proximal end of TM16 in the outer leaflet of the lipid bilayer (Fig. 1.4). Replacing Arg1222 with Met generated a mutant protein similar to wild-type MRP1; it displayed wild-type levels of LTC4 transport and GSH-stimulated binding of the MRP1 inhibitor [125I] azido agosterol A (144). The protein levels, LTC4 transport activity, as well as photolabeling by LTC4 were all comparable to that of wild-type MRP1 (144), suggesting that Arg1222 located at the TM16 membrane/extracellular interface does not play an indispensable role in MRP1 function and expression.

In conclusion, the findings described above (Table 1.4) suggest that polar residues with side chain hydrogen-bonding and salt bridge potential, which cluster in the inner leaflet of the membrane or in the region proximal to the NH2-terminus of TM16, are critical for protein
expression and also likely contribute to the architecture of the substrate binding site(s) of MRP1 (133).

1.3.3 Role of polar amino acids in TM8 and cytoplasmic loop 5 of MRP1

Amino acids in TMs other than TM16 and TM17 are also important for MRP1 expression and function. A number of polar residues in putative TM6 (125), TM8 (126), TM10 (128; 135), TM11 (26), and TM14 (132) have been demonstrated by mutagenesis to be important for MRP1 expression and/or function and are summarized in Table 1.5 (and references therein). The properties of some of the mutants which affect MRP1 expression are described in detail below.

When Asp\(^{430}\) was replaced with an oppositely charged Lys, the resulting D430K mutant protein did not express well in HEK293 cells (126). The levels of D430K mutant MRP1 mRNA and wild-type MRP1 mRNA were comparable, indicating a post-transcriptional event was probably involved (126). Opposite and same charge mutants of Asp\(^{436}\) (D436K or D436E) were expressed at wild-type levels of MRP1 in HEK293 cells and localized properly to the cell membrane (126). However, the D436K mutant protein showed a significant decrease in overall organic anion transport activity (by 50-80% for LTC\(_4\), E\(_2\)17βG, E\(_1\)3SO\(_4\), GSH and MTX) (126). Wild-type levels of transport and kinetic parameters (\(K_m\) and \(V_{max}\)) of transport for LTC\(_4\), and E\(_2\)17βG were observed in the same charge mutant D436E (126). These data suggest that the charge rather than the volume of the Asp\(^{436}\) side chain is crucial for MRP1 transport activity (126).

Site-directed mutagenesis studies have shown that Ala substitution of four charged residues (Lys\(^{513}\), Lys\(^{516}\), Glu\(^{521}\), Glu\(^{535}\)) in cytoplasmic loop 5 (CL5) (Fig. 1.2), which are fairly conserved among other ABCC family proteins, caused a reduction in protein levels to \(\leq 30\%\) that of wild-type MRP1 (150). Homology models of the core structure of nucleotide-bound human
Table 1.5. Summary of the effects of polar amino acid mutations in TMs other than in or proximal to TM16 in MSDs 1 and 2 of MRP1

<table>
<thead>
<tr>
<th>Location</th>
<th>Mutation</th>
<th>Protein Levels</th>
<th>Vesicular Transport Activity</th>
<th>Drug Resistance in Intact Cells</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM6</td>
<td>K319D/L</td>
<td>Normal</td>
<td>Substrate-selective reduction (GSH by 50%)</td>
<td>ND</td>
<td>(126)</td>
</tr>
<tr>
<td>TM6</td>
<td>P323A</td>
<td>Normal</td>
<td>Substrate-selective reduction (GSH by 60%)</td>
<td>ND</td>
<td>(129)</td>
</tr>
<tr>
<td>TM6</td>
<td>K332D/L</td>
<td>Normal</td>
<td>Substrate-selective loss (LTC₄, GSH)</td>
<td>ND</td>
<td>(125)</td>
</tr>
<tr>
<td>TM6</td>
<td>H335E,L,Q</td>
<td>Normal</td>
<td>Substrate-selective loss (GSH, LTC₄)</td>
<td>ND</td>
<td>(125)</td>
</tr>
<tr>
<td>TM6</td>
<td>D336/K/ER/L</td>
<td>Normal</td>
<td>Global loss (E₂17βG, E₁3SO₄, GSH; LTC₄) except MTX (by 50%)</td>
<td>ND</td>
<td>(125;126)</td>
</tr>
<tr>
<td>TM8</td>
<td>D436K</td>
<td>Normal</td>
<td>Global reduction (LTC₄, GSH, E₂17βG, MTX, E₁3SO₄ by 50-80%)</td>
<td>ND</td>
<td>(126)</td>
</tr>
<tr>
<td>TM8</td>
<td>D436E</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td>(126)</td>
</tr>
<tr>
<td>TM8</td>
<td>W445A</td>
<td>Reduced (by 40%)</td>
<td>Global reduction (E₂17βG, LTC₄, GSH, MTX, E₁3SO₄ by &gt;75%)</td>
<td>ND</td>
<td>(128)</td>
</tr>
<tr>
<td>TM8</td>
<td>W445F</td>
<td>Reduced (by 30%)</td>
<td>Global reduction (GSH, E₂17βG, MTX, E₁3SO₄, LTC₄ by 50-90%)</td>
<td>ND</td>
<td>(135)</td>
</tr>
<tr>
<td>TM8</td>
<td>W445Y</td>
<td>Reduced (by 70%)</td>
<td>Substrate-selective reduction (E₂17βG, MTX, GSH by 40-60%)</td>
<td>ND</td>
<td>(135)</td>
</tr>
<tr>
<td>TM8</td>
<td>P448A</td>
<td>Normal</td>
<td>Global reduction (LTC₄ by 40%, GSH by 15%, E₂17βG by 25%, MTX by 20%, E₁3SO₄ by 60%)</td>
<td>ND</td>
<td>(129)</td>
</tr>
<tr>
<td>TM8</td>
<td>W459A</td>
<td>Normal</td>
<td>Substrate-selective reduction (GSH by 50%)</td>
<td>ND</td>
<td>(128)</td>
</tr>
<tr>
<td>TM10</td>
<td>T550A</td>
<td>Normal</td>
<td>Normal</td>
<td>Increased (VCR by 3-fold)</td>
<td>(135)</td>
</tr>
<tr>
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<td>----------------</td>
<td></td>
</tr>
<tr>
<td>TM10</td>
<td>T552A</td>
<td>Reduced</td>
<td>Normal</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(by 50%)</td>
<td></td>
<td>(135)</td>
<td></td>
</tr>
<tr>
<td>TM10</td>
<td>W553A/Y</td>
<td>Normal</td>
<td>Global loss (GSH, E$<em>{217}^\beta$G, MTX, E$</em>{3}$SO$<em>{4}$) except LTC$</em>{4}$ (by 50%)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(135)</td>
<td></td>
</tr>
<tr>
<td>TM10</td>
<td>W553F</td>
<td>Normal</td>
<td>Substrate-selective loss (GSH, MTX, E$<em>{3}$SO$</em>{4}$) except E$<em>{217}^\beta$G, LTC$</em>{4}$ (by 40%)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(135)</td>
<td></td>
</tr>
<tr>
<td>TM10</td>
<td>T556A</td>
<td>Reduced</td>
<td>Normal</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(by 40%)</td>
<td></td>
<td>(135)</td>
<td></td>
</tr>
<tr>
<td>TM10</td>
<td>P557A</td>
<td>Normal</td>
<td>Global loss (GSH, E$<em>{217}^\beta$G, MTX, E$</em>{3}$SO$<em>{4}$, LTC$</em>{4}$)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(135)</td>
<td></td>
</tr>
<tr>
<td>TM10</td>
<td>T564A</td>
<td>Reduced</td>
<td>Normal</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(by 40%)</td>
<td></td>
<td>(135)</td>
<td></td>
</tr>
<tr>
<td>TM10</td>
<td>Y568S</td>
<td>Reduced</td>
<td>Substrate-selective loss (E$_{217}^\beta$G by 60%)</td>
<td>Global reduction (vincristine, doxorubicin and VP16 by 2-fold)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(by 50%)</td>
<td></td>
<td>(135)</td>
<td></td>
</tr>
<tr>
<td>TM10</td>
<td>Y568A/F</td>
<td>Normal</td>
<td>Substrate-selective loss (E$_{217}^\beta$G by 50%) in Y568A</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>/W</td>
<td></td>
<td>(135)</td>
<td></td>
</tr>
<tr>
<td>TM11</td>
<td>N590A</td>
<td>Normal</td>
<td>Reduced (LTC$<em>{4}$, E$</em>{217}^\beta$G, GSH by 50 - 75%)</td>
<td>Global reduction (VCR, doxorubicin and VP16 by 2-fold)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(26)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N590Q/D</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(26)</td>
<td></td>
</tr>
<tr>
<td>TM11</td>
<td>R593E/D</td>
<td>Normal</td>
<td>Global loss or substantial reduction (E$<em>{217}^\beta$G, E$</em>{3}$SO$<em>{4}$, GSH; LTC$</em>{4}$, MTX)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>/L</td>
<td></td>
<td></td>
<td>(126)</td>
<td></td>
</tr>
<tr>
<td>TM11</td>
<td>R593K</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(130)</td>
<td></td>
</tr>
<tr>
<td>TM11</td>
<td>P595A</td>
<td>Normal</td>
<td>Global loss (E$<em>{217}^\beta$G, E$</em>{3}$SO$<em>{4}$, GSH; LTC$</em>{4}$, MTX)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(129)</td>
<td></td>
</tr>
<tr>
<td>TM11</td>
<td>N597A</td>
<td>Normal</td>
<td>Normal</td>
<td>Increased (VCR by 3-4-fold); Reduced (VP-16 by 3-fold)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(26)</td>
<td></td>
</tr>
<tr>
<td>TM14</td>
<td>K1092M/A/E/R/A</td>
<td>Normal</td>
<td>Normal</td>
<td>Substrate selective increase (doxorubicin, VCR by 2-3-fold)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(126)</td>
<td></td>
</tr>
<tr>
<td>TM14</td>
<td>S1097A</td>
<td>Normal</td>
<td>Substrate-selective loss (E$_2$17βG by 80%)</td>
<td>Global increase (doxorubicin, VCR, VP-16 by 1.5-3-fold)</td>
<td></td>
</tr>
<tr>
<td>------</td>
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<td>-------------------------------------------</td>
<td>----------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>TM17</td>
<td>S1233A</td>
<td>Normal</td>
<td>Substrate-selective reduction (E$_1$3SO$_4$ by 40%)</td>
<td>Normal (151)</td>
<td></td>
</tr>
<tr>
<td>TM17</td>
<td>S1235A</td>
<td>Reduced (by 60%)</td>
<td>Global loss (E$_2$17βG, LTC$_4$, MTX, E$_1$3SO$_4$)</td>
<td>Normal (151)</td>
<td></td>
</tr>
<tr>
<td>TM17</td>
<td>Y1236F</td>
<td>Normal</td>
<td>Global loss (E$_2$17βG, LTC$_4$, MTX, E$_1$3SO$_4$)</td>
<td>Substrate-selective reduction (VCR by 2 to 3-fold) (133)</td>
<td></td>
</tr>
<tr>
<td>TM17</td>
<td>S1237A</td>
<td>Normal</td>
<td>Global loss (GSH, E$_2$17βG, MTX, E$_1$3SO$_4$ except LTC$_4$ (by 40%))</td>
<td>Normal (133; 151)</td>
<td></td>
</tr>
<tr>
<td>TM17</td>
<td>Q1239A</td>
<td>Normal</td>
<td>Substrate-selective reduction (E$_2$17βG, MTX by 50%)</td>
<td>Normal (133)</td>
<td></td>
</tr>
<tr>
<td>TM17</td>
<td>T1241A</td>
<td>Normal</td>
<td>Substrate-selective reduction (MTX by 50%)</td>
<td>Substrate-selective reduction (VCR by 2-3-fold) (133; 151)</td>
<td></td>
</tr>
<tr>
<td>TM17</td>
<td>T1242A</td>
<td>Normal</td>
<td>Substrate-selective reduction (E$_2$17βG by 60%)</td>
<td>Global reduction (VCR, VP16 by 2-fold and anthracyclines by 3-fold) (123; 133)</td>
<td></td>
</tr>
<tr>
<td>T1242S</td>
<td>Normal</td>
<td>Substrate-selective reduction (E$_2$17βG by 60%)</td>
<td>Global reduction (VCR, VP16 by 2-fold and anthracyclines by 3-fold) (123)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1242C</td>
<td>Normal</td>
<td>Substrate-selective reduction (E$_2$17βG by 70%)</td>
<td>Global reduction (VCR, VP16 by 2-fold and anthracyclines by 3-fold) (123)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1242L</td>
<td>Normal</td>
<td>Substrate-selective reduction (E$_2$17βG by 50%)</td>
<td>Global reduction (VCR, VP16 by 2-fold and anthracyclines by 3-fold) (123)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1242D</td>
<td>Normal</td>
<td>Substrate-selective loss (E$_2$17βG) and reduction (LTC$_4$ by 30%)</td>
<td>Global reduction (VCR, VP16 by 2-fold and anthracyclines by 3-fold) (123)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TM17</td>
<td>Proximal (COOH)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reduced (by 60%)</td>
<td>Substrate-selective reduction (E$_2$17βG by 80%)</td>
<td>Global reduction (VCR, VP16 and anthracyclines by 2-fold)</td>
</tr>
<tr>
<td>T1242K</td>
<td>Reduced (by 60%)</td>
<td>Substrate-selective reduction (E$_2$17βG by 80%)</td>
<td>Global reduction (VCR, VP16 and anthracyclines by 2-fold)</td>
<td>(123)</td>
<td></td>
</tr>
<tr>
<td>TM17</td>
<td>Y1243F</td>
<td>Normal</td>
<td>Reduced (E$_2$17βG by 80%; LTC$_4$ by 30%)</td>
<td>Global reduction (VCR, VP16 and anthracyclines by 2-3-fold)</td>
<td>(133)</td>
</tr>
<tr>
<td>TM17</td>
<td>N1245A</td>
<td>Normal</td>
<td>Substrate-selective loss (E$_2$17βG)</td>
<td>Substrate-selective reduction (VP16 and anthracyclines by 2-3-fold) and increase (VCR by 2-fold)</td>
<td>(133)</td>
</tr>
<tr>
<td>TM17</td>
<td>W1246A</td>
<td>Normal</td>
<td>Substrate-selective loss (E$_2$17βG)</td>
<td>Global loss (VCR, vincristine, VP16, anthracyclines)</td>
<td>(152)</td>
</tr>
<tr>
<td></td>
<td>W1246C</td>
<td>Reduced (by 30%)</td>
<td>Substrate-selective loss (E$_2$17βG)</td>
<td>Global loss (VCR, vincristine, VP16, anthracyclines)</td>
<td>(152)</td>
</tr>
<tr>
<td></td>
<td>W1246F</td>
<td>Normal</td>
<td>Substrate-selective loss (E$_2$17βG)</td>
<td>Global loss (VCR, vincristine, VP16, anthracyclines)</td>
<td>(152)</td>
</tr>
<tr>
<td></td>
<td>W1246Y</td>
<td>Normal</td>
<td>Substrate-selective loss (E$_2$17βG)</td>
<td>Global loss (VCR, vincristine, VP16, anthracyclines)</td>
<td>(152)</td>
</tr>
<tr>
<td></td>
<td>R1249A</td>
<td>Slightly increased</td>
<td>Reduced (LTC$_4$ by 75%)</td>
<td>Loss (VCR, vincristine)</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td>R1249K</td>
<td>Normal</td>
<td>Global loss (LTC$_4$, E$_2$17βG, MTX, E$_1$3SO$_4$)</td>
<td>ND</td>
<td>(130)</td>
</tr>
<tr>
<td></td>
<td>R1249D</td>
<td>Normal</td>
<td>Global loss (LTC$_4$, E$_2$17βG, MTX, E$_1$3SO$_4$)</td>
<td>ND</td>
<td>(130)</td>
</tr>
<tr>
<td>TM17</td>
<td>E1253A</td>
<td>Normal</td>
<td>Reduced (E$_2$17βG by 75%; LTC$_4$ by 30%)</td>
<td>ND</td>
<td>(153)</td>
</tr>
<tr>
<td></td>
<td>E1253D</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td>(153)</td>
</tr>
<tr>
<td></td>
<td>E1253K</td>
<td>Normal</td>
<td>ND</td>
<td>ND</td>
<td>(153)</td>
</tr>
<tr>
<td></td>
<td>E1255A</td>
<td>Normal</td>
<td>Normal</td>
<td>ND</td>
<td>(153)</td>
</tr>
<tr>
<td>Proximal</td>
<td></td>
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<tr>
<td>(COOH)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

ND, not determined; Normal, wild-type level
MRP1 (154) predict that 3 of 4 of these residues are at the interface of CL5 (Glu\textsuperscript{535} excepted) with NBD2. Mutation of His\textsuperscript{1364} and Arg\textsuperscript{1367} in NBD2 predicted to be at the CL5 interface also resulted in reduced MRP1 levels. These observations suggested that maintaining the integrity of CL5 and its contact with NBD2 is important for the proper assembly and expression of MRP1 at plasma membrane in mammalian cells (150).

Further study of these CL5 ionizable residues showed that their expression could be rescued by exposing cells to 4-phenylbutyric acid (4-PBA) (155), a low molecular weight fatty acid derivative frequently used to rescue expression and membrane trafficking of processing mutants (as a “chemical chaperone” protecting them from the cellular quality control systems) (156; 157). Characterization of the 4-PBA-rescued mutant proteins demonstrated that the K513A mutant had organic anion transport activity (LTC\textsubscript{4} and E\textsubscript{2}17βG) comparable to wild-type MRP1 whereas the E521A and E535A mutants showed decreased uptake levels of these substrates. The mechanisms underlying the reduced activity of the two mutants were different. In the case of the 4-PBA-rescued E521A mutant, the apparent affinities (\(K_m\)) for E\textsubscript{2}17βG and LTC\textsubscript{4} were not significantly different from wild-type MRP1, whereas the \(V_{\text{max}}\) values for this mutant were decreased by 2 to 3-fold (155). In contrast, the rescued E535A mutant exhibited a 3 to 6-fold increase in \(K_m\) for E\textsubscript{2}17βG and LTC\textsubscript{4} relative to wild-type MRP1, whereas the \(V_{\text{max}}\) values were similar to wild-type MRP1 (155). Further, E535A displayed reduced LTC\textsubscript{4} photolabeling compared to wild-type MRP1 whereas E521A was similar to that of wild-type MRP1, suggesting that the E535A mutant caused a decrease in substrate binding (155). On the other hand, E521A decreased azidoATP photolabeling at 4 °C and vanadate-induced azidoADP trapping at 37 °C compared to wild-type MRP1, indicating that this mutant may affect ATP binding and/or hydrolysis (155). These differences in kinetic and photolabeling parameters suggest that the
mechanisms responsible for the reduced transport activities of the 4-PBA-rescued E521A and E535A mutants are different (155). In addition, limited trypsin digestions followed by immunoblotting with site-specific monoclonal antibodies (MAbs), showed that these two mutations affected the trypsin susceptibility and therefore, conformation of MRP1 in distinct manners, consistent with their distinctive phenotypes (105). In contrast to E521A, E535A, and K513A, 4-PBA treatment had little or no effect on the levels of K516A in HEK cells.

1.4 Misfolding of ABC Proteins

1.4.1 Biosynthesis of polytopic integral membrane proteins

In eukaryotes, synthesis of membrane proteins takes place on endoplasmic reticulum (ER)-bound ribosomes, where proteins are co-translationally translocated into the ER for folding into their native conformations (158). ER-resident proteins, such as foldases, molecular chaperones and N-linked oligosaccharide processing enzymes, prevent aggregation of nascent polypeptide chains and ensure their folding into a stable and energy-minimized state (159). Presently, only limited knowledge is available about the folding of polytopic membrane proteins. The most widely accepted model is a two-stage model (145; 160; 161). The first stage (which sets the difference between a polytopic membrane protein and a soluble protein) includes the formation and insertion of TM helices in the lipid bilayer of the ER as well as establishing the general topology. The second stage (which generally follows the folding rules of soluble proteins) includes the localization and folding of intracellular and extracellular domains (161; 162). In the case of multidomain membrane proteins, it requires quaternary organization of all the domains, and cooperativity in domain folding and assembly may also exist (163).

In most cases, the initial insertion which determines the topology of a membrane protein is assisted by a translocon complex (Fig. 1.6) (162). The best understood translocon is the Sec61
Figure 1.6. Co-translational biogenesis of a polytopic membrane protein. The elongating nascent polypeptide chain transfers from the ribosome to the Sec61 translocon complex at the ER membrane. Signal sequences (orange cylinders) trigger ribosome binding and open the gate of the translocon pore (blue disc) to initiate peptide chain moving into the ER lumen. Stop-transfer sequences (green cylinders) stop translocation and direct the elongating polypeptide chain beneath the ribosome and into the cytosol. Thus, transmembrane topology is established from the amino to the carboxyl terminus. Taken, with permission, from Skach (161).
translocon in the rough ER which forms a protein-conducting channel across the ER membrane and allows nascent polypeptide chains to pass across or integrate into lipid membranes (145). The major advantage of this mechanism is that it restricts the number of conformations available to the emerging polypeptide chain from the ribosome and provides a protected environment to the nascent chain (159). The structural and mechanistic details of this opening are still unknown. Rapoport and colleagues (164) proposed the idea that the translocon catalyzes partitioning of the nascent polypeptide segment in the translocon channel between the aqueous and membrane phases (164). Thus, the insertion probability increases with an increase in hydrophobicity of the polypeptide chain (165). Because the membrane is not a homogeneous environment, insertion probability also depends on the location of individual residues within the TM segment, especially for polar residues such that they are increasingly favorable as they approach the interfacial region (162). After membrane insertion, the protein or protein domain starts to search for its native conformation. Little is known about the membrane-inserted unfolded state or the pathway to the folded state in bilayers (162). A major component involved is the packing of the stably inserted helices as well as topology rearrangements (161; 166). In general, folding of membrane proteins or domains probably proceeds down a funnel-shaped energy landscape to an energy minimum which is determined by the net energetics of the interactions of the peptide chains with water, with each other, with the lipid bilayer (hydrocarbon core and interface) as well as with cofactors present in the cellular environment (166) (Fig. 1.7). However, unlike soluble proteins, the starting point for the polytopic membrane proteins or protein domains is much more constrained because much of the secondary structure and topology are set by the initial insertion process (166). Thus, the unfolded protein is probably much further down the energy funnel and closer to the folded state than that of soluble proteins (166). Also, the dominant factor in the hydrocarbon
Figure 1.7. Energy funnel representing chaperone-mediated protein folding events in the cell. A large ensemble of random conformations is produced after ribosomal biosynthesis from which partially folded intermediate states are formed. Interactions of the intermediate states with chaperones lead to folding into a native structure. Misfolding and aggregation pathways are also indicated (left) and upon chaperone association-dissociation interactions, the free energy of these states is raised, enabling escape from wrong folding pathways to the productive folding pathway (right). Mutations or adverse physiological conditions may trap destabilized/misfolded conformations either at low free energy depressions or at a point from which aggregates could also be formed. Taken, with permission, from Gregersen et al. (167).
core region of the bilayer is van der Waals forces rather than a hydrophobic effect observed in soluble proteins (162; 166; 168).

To facilitate functional diversification of a common protein or domain architecture (e.g. the core MSD of ABC proteins which consists of 6 TM helices (9)), one important feature of helix packing is that TM helices contain local distortions such as kinks to create a deviation of the helix axis and/or a shift in the regularity of side-chain positions (162). Such helical distortions or kinks may provide weak points in the helices to facilitate movements needed for protein function. For example, it has been shown that five mutants containing substitutions of Pro residues in or proximal to the TM helices of MSD1 (TM6-Pro\textsuperscript{343}, TM8-Pro\textsuperscript{448}, TM10-Pro\textsuperscript{557}, and TM11-Pro\textsuperscript{595}) and MSD2 (TM14-Pro\textsuperscript{1088}) significantly reduced organic anion transport by MRP1 (129). It has also been found that certain membrane-embedded Pro residues promoted the formation of native α-helices by keeping apart regions of sequence populated by β-sheet favoring residues during CFTR folding, ensuring native folding of the protein (169).

1.4.2 Diseases associated with loss of function of ABC proteins due to misfolding

ER quality control is required to differentiate between proteins on a productive folding pathway and those that cannot achieve the stability required to progress through the secretory pathway to their final destination (170). Selective destruction of misfolded or damaged polypeptides by the large ATP-dependent proteolytic machinery, the 26S proteasome, and/or other degradation machineries, such as lysosomes, prevents the accumulation of non-functional, potentially toxic proteins (171; 172) (Fig. 1.8). Defective folding and trafficking of ABC proteins to the cell surface that normally reside at the plasma membrane can lead to serious health problems as discussed below.

Cystic fibrosis (ABCC7/CFTR)
Figure 1.8. Cellular degradation pathways of misfolded proteins. The proteosomal degradation of misfolded protein in the ER involves several steps. 1. Recognition: ubiquitin ligases embedded in the ER membrane cooperate with accessory recognition factors to recognize misfolded proteins. Substrates are then extracted into the cytosol via an unknown channel. 2. Ubiquitination: following recognition, the ubiquitination machinery is recruited to the misfolded protein, either directly within the membrane or by interactions with cytoplasmic chaperones. The substrate is ubiquitylated by an E3 ligase. 3. Retranslocation: for polytopic membrane proteins, retrotranslocation may occur by removal of the protein through a channel and/or by removal of the protein and the surrounding membrane. 4. Degradation: the substrate is degraded by the 26S proteasome. Misfolded proteins can also be degraded by lysosomal machinery. Figure taken, with permission, from Guerriero and Brodsky (Figure 2) (173).
CF is the most common lethal inherited disorder in Caucasians (1 in 25 individuals is a carrier and it affects 1 in 2500 live births) and is caused by mutations in the gene coding for the CFTR/ABCC7 (174; 175). CF often leads to lung failure and premature death due to chronic bacterial infections and inflammation (176; 177).

Other effects include chronic fibrosis of the pancreas and approximately 98% of men with CF are infertile due to the lack of vas deferens (178). In the apical membrane of normal epithelial cells that line the airways, CFTR helps to modulate hydration of the mucus layer by functioning as a cAMP-dependent chloride channel that regulates salt and fluid transport (179). Deletion of Phe508 (ΔF508) in NBD1 is the most common mutation in CF patients, and it is found on at least one chromosome in 90% of affected individuals. The ΔF508 mutation causes misfolding of CFTR such that the protein is retained in the ER and rapidly degraded (180; 181).

In 2012, the FDA approved a CFTR potentiator, Ivacaftor®, for the treatment of CF patients harbouring a G551D mutation in the CFTR gene. Although this treatment alone does not appear to be effective in treating patients with homozygous ΔF508 deletion, it is being tested in phase II and III clinical trials in combination therapy with VX661 and VX809, respectively (182).

Persistent hyperinsulinemic hypoglycemia of infancy (ABCC8/SUR1)

There are over 100 SUR1/ABCC8 mutations identified in patients with PHHI that lead to the loss of the ATP-sensitive potassium channel (K_{ATP}) function by impairing the Mg^{2+}-ADP-dependent stimulation of K_{ATP} to release intracellular potassium (183; 184). PHHI is a disorder of pancreatic β-cell function characterized by excess insulin secretion and hypoglycemia (185). The loss or disruption of SUR1 in PHHI abolishes the ability of pancreatic β-cells to hyperpolarize when glucose is reduced and, thus, resulting in excess insulin release that produces hypoglycemia (186).
**Dubin-Johnson syndrome (ABCC2/MRP2)**

DJS is a rare, autosomal recessive disorder characterized by a defect in the transfer of certain endogenous and exogenous anionic conjugates from hepatocytes into the bile with mild symptoms (5). Mutations in ABCC2 causing a loss of a functionally active MRP2 protein at the bile canalicular membrane have been identified as the molecular basis of this disorder (5). MRP2 is normally localized at the apical membrane of bile canalicus where it functions as an ATP-dependent export pump for both conjugated and unconjugated amphiphilic anions (5). MRP2 (which is >50% identical to MRP1) is mostly located in the liver which explains its primarily hepatic effects (84). At least 25 mutations in ABCC2 have been reported in DJS patients of which 9 are missense mutations (187; 188). Several studies have reported evidence that some DJS-associated missense mutations in ABCC2 impair maturation of MRP2 (such as W709R in the Walker A motif and R768W in the ABC signature motif of NBD1 (187-189), I1173F in CL7 (190)), resulting in rapid degradation of the mutant proteins and failure to traffic to the plasma membrane. Further, missense mutations of two basic residues found in DJS patients (R393W in CL4 (191); R1150H in CL7 (190)) display wild-type levels of expression and localization to the plasma membrane; however, they impair the transport activity of MRP2 in HEK293 cells expressing the mutant proteins.

**Pseudoxanthoma elasticum (PXE) (ABCC6/MRP6)**

PXE is a heritable disorder of the connective tissue characterized by visual field loss and skin lesions, and occasionally cardiovascular complications and is caused by mutations in ABCC6/MRP6 (192). Its physiological substrate remains unknown although in vitro MRP6 is capable of organic anion transport (2; 193). More than 180 mutations in ABCC6 have been reported in humans with PXE and these mutations cluster within exons encoding CL7 and in the
COOH-terminal NBD2 (192; 194). It has been shown that many of these mutations or deletions interfere with the proper folding of the protein or disrupt its processing or integration into the plasma membrane (195; 196). Many of the missense mutations occur in ionizable residues, some of which are opposite charge substitutions such as R1314E and E1400K (194).

Progressive familial intrahepatic cholestasis type 2 (PFIC2), benign recurrent intrahepatic cholestasis type 2 (BRIC2), and intrahepatic cholestasis of pregnancy (ICP) (ABCB11/BSEP)

The Bile Salt Export Pump (BSEP) is a liver-specific ABC protein encoded by ABCB11 that functions at the apical surface as an ATP-dependent bile salt export pump to transport monovalent bile salts from the hepatocyte into the bile canaliculus (197). Mutations in ABCB11 (BSEP) have been associated with three clinical forms of liver disease, PFIC2, BRIC2, and ICP. Functional studies suggest that the clinical phenotypes of PFIC2, BRIC2, and ICP may directly correlate with the amount of mature (glycosylated) BSEP protein that is expressed at the canilicular cell surface and that strategies to stabilize the cell surface mutant protein may be of therapeutic benefit (198). Most BSEP mutants accumulate in perinuclear aggresome-like structures in the presence of the proteasome inhibitor MG-132, suggesting that mutations cause protein instability and ubiquitin-dependent degradation (199).

1.5 Experimental approaches to restoring levels of misfolded ABC proteins

After ribosomal biosynthesis, the fact that a polypeptide chain can be converted into many different types of non-native structures exposes the prospect of several protein folding defects. Further, genetic or sporadic mutations can also cause other non-native destabilized conformational states. These events can result in dead-end conformations or non-productive pathways to be favoured. As described above, several ABC family proteins have been implicated in genetic diseases and in many cases, protein misfolding has been shown to be responsible.
Because of its relevance to ABC-related and other human diseases that involve misfolded membrane proteins, many studies have described physical, chemical and pharmacological approaches to rescue the levels and function of misfolded proteins.

1.5.1 **Biosynthesis at subphysiological temperatures**

Multiple studies have demonstrated that reducing the temperature of the growth environment below 37 °C can promote the expression of misfolded proteins both *in vivo* and *in vitro* (200-202). Biophysical evidence suggests that certain mutations in proteins can act by altering the thermal stability of structured folding intermediates causing the protein to misfold (203). At lower temperatures, the kinetics of folding and assembly can be altered to favour native folding, thus causing the misfolded proteins to fold and function properly.

One well-studied example of a temperature-sensitive misfolded mutant ABC protein is the ΔF508 deletion mutant of CFTR that fails to fold properly, is mostly degraded, and does not traffic appropriately to the plasma membrane (200). Lowering the growth temperature (to 30 °C or 27 °C) of mammalian cells expressing the protein causes a portion of the mutant protein to mature into fully glycosylated proteins and properly localize at the plasma membrane with a half life comparable to that of wild-type CFTR (204; 205). At lower temperatures, mutant CFTR expressing cells are competent for cAMP-stimulated chloride flow (the single channel currents of wild-type and ΔF508 CFTR are comparable under similar conditions), suggesting functional rescue of the CFTR protein (205-207). Reduced temperatures also enhanced the expression of PFIC2-causing misfolded D482G mutant of BSEP/ABCB11 (199). This strategy of restoring or improving levels (and function) of misfolded membrane proteins is further discussed in Chapter 2.
1.5.2 Biosynthesis in the presence of chemical chaperones

Chemical chaperones are a family of structurally unrelated classes of small molecules that help mediate (or facilitate) the correct assembly of polypeptides, but are not themselves components of the final functional structures (208). Osmolytes can act as protein stabilizers including glycerol, sorbitol, sugars, methylamines, glycine, betaine and glycerophosphorylcholine or free amino acids (e.g. glycine, taurine, proline) or low molecular weight compounds such as deuterium oxide (D$_2$O), dimethyl sulfoxide (DMSO) and 4-PBA have been associated with chaperone-like function to rescue misfolded proteins in vitro (209). The mechanism by which these compounds improve protein folding and increase thermodynamic stability is not fully understood (209). It has been speculated that these compounds do not bind directly to proteins and their mode of action is unspecific resulting from a hydration effect, which promotes the ability of water molecules to establish favorable interactions with polar groups in the protein backbone, thus increasing protein compactness (Fig. 1.9A) (209; 210). This effect limits the population of protein conformations close to the native state configuration, by favoring folding-promoting contacts (209). Further, these compounds minimize the formation of intermediate states that might lead to folding dead-ends (209). However, it is clear that the effect of these compounds on misfolded proteins varies and they are definitely not interchangeable.

Several chemical chaperones have been shown to rescue the membrane expression and function of misfolded ΔF508 CFTR protein, at least in part. This was achieved using natural osmolytes (glycerol, betaine, taurine and trimethylamine N-oxide (TMAO)) (211-213). Our lab recently demonstrated that the levels of several MRP1 mutant proteins (K513A, K516A, E521A, E535A) in the CL5 region could be rescued by exposing transfected cells to 4-PBA and to a lesser extent, glycerol and DMSO (155). In addition, as described in Section 1.3.3, functional
Figure 1.9. Schematic diagrams illustrating the effects of chemical or pharmacological chaperones on protein stability. (A) The unfavourable interaction of the polypeptide with osmolytes (closed circle) promotes preferential protein hydration by water molecules (open circles) resulting in an increase of the free energy of both the native and the unfolded state. As indicated, the energy rise is more significant in the unfolded state, as a result of the increased protein surface area exposed upon unfolding. (B) When a pharmacological cofactor or ligand binds to a destabilized protein variant, it acts as a folding nucleation point, yielding a lower energy conformation. Taken, with permission, from Leandro and Gomes (209).
characterization of the 4-PBA-rescued K513A CL5 mutant showed wild-type levels of organic anion transport activity. In contrast, although levels of the E521A and E535A proteins could be improved by exposure of transfected HEK293 cells to 4-PBA, the ‘rescued’ mutants were functionally deficient (Section 1.3.3), and although stable, the conformations of the mutant proteins differed from wild-type MRP1 (155).

Several chemical chaperones (4-PBA, glycerol, DMSO, TMAO) have been shown to have a chaperoning effect on membrane proteins other than ABC proteins, including disease-causing mutants of Aquaporin 2, the Low-Density Lipoprotein (LDL) receptor, V2 vasopressin receptor, α1-antitrypsin, organic cation/carnitine transporter (OCTN2) (214-216).

1.5.3 Biosynthesis in the presence of pharmacological chaperones

It has been shown that low molecular weight exogenous compounds, classified as pharmacological chaperones and that bind reversibly to a specific protein, are also able to restore, at least in part, folding and function of mutant proteins (217; 218). Unlike the chemical chaperones which do not appear to bind directly to the protein, these compounds (e.g. diazoxide, VPA-985) can either bind weakly to a specific target protein, or act as competitive inhibitors, agonists/antagonists, or protein cofactors (209). Usually, these compounds either induce refolding or stabilization of the mutant protein, or contribute to structuring a particular region or domain within a protein (209). A chaperoning effect exerted by the agent is considered effective when the pharmacological ligand or cofactor binds to a target protein resulting in an energetic contribution favouring the native state (Fig. 1.9B). The energetically favourable contribution may directly result from a gain in enthalpy arising from the new interactions, or from a change in quaternary structure (209). Several high throughput drug screening studies have shown that expression, membrane localization, and activity of the ΔF508 and certain other processing
mutants of CFTR could be enhanced by expressing the mutant protein in the presence of small molecules called correctors (219; 220) which can act as pharmacological chaperones to correct defective CFTR maturation (221). A recent study using differential scanning fluorimetry identified a CFTR corrector, RDR1, which binds directly to the first NBD1 of CFTR, the location of the ΔF508 mutation in CF, and partially rescues its function both in cells and in an ΔF508-CFTR mouse model (222; 223). Thus far, correctors have shown limited ability to improve ΔF508-CFTR trafficking. Even after corrector treatment, ΔF508-CFTR has <15% the activity of wild-type CFTR, and the corrector efficacy may vary depending on the cell type examined (224; 225). Inconsistencies have been reported regarding the amount of rescued, functional CFTR required to restore normal function of the lung; it is believed that at least 10-35% of total CFTR function is required to prevent significant pulmonary morbidity (226). Recently, it has been shown that a combination of correctors produced additive or synergistic effects, improving the expression of mutant CFTR at the cell surface up to 9-fold over a single compound treatment suggesting that the development of combination therapies to correct CFTR trafficking may be required to obtain clinical efficacy (225).

1.5.4 Introduction of compensation “second-site rescue” mutations

Identification and characterization of second-site “rescue” mutations (an approach to compensate or offset the disruptive effect of a first mutation in a protein by introducing one or more additional mutations in the protein) can also be a useful experimental strategy to rescue misfolded proteins. For example, a V510D suppressor mutation in NBD1 of CFTR has been shown to promote maturation of the ΔF508 CFTR protein. In a 3-dimensional model of CFTR, Val510 is predicted to reside on the NBD1 surface and in close proximity to Phe508 (227). Further, a negative charge at position 510 is essential for this ‘rescue’ effect since mutation of Val510 to a
neutral or polar amino acid (Cys, Gly, Ala, Ser, Asn, Pro, Thr, Tyr or Lys) did not have the same effect (228; 229). Recent atomic modeling studies have proposed that the V510D mutation in NBD1 promotes maturation and stabilizes ΔF508-CFTR at the membrane through formation of a salt bridge with Arg\textsuperscript{1070} in CL4 of MSD2 (229). Several other NBD1 ‘suppressor’ mutations in CFTR (I539T, G550E, R553M, R555K in NBD1) which can rescue expression of the ΔF508 CFTR protein have also been described (230). Further, Arg suppressor mutations (i.e. substituting residues with Arg as second-site “rescue” mutations) L65R (TM1), T199R (TM3), I306R (TM5), or F343R (TM6) also promoted maturation of P-glycoprotein (ABCB1) processing mutants (231). It has been suggested that Arg residues may rescue defective folding of P-glycoprotein by forming new hydrogen bond interactions and/or by mimicking the effect of substrate binding which may promote/stabilize packing of the TM segments (232).

It should be mentioned that additional approaches to treating diseases caused by protein misfolding mutations, such as CFTR-ΔF508, include the use of one or more gene repair strategies. In the last two decades, single-and double-stranded DNA fragments, small single-stranded oligonucleotides, RNA-DNA chimeras and triple-helix forming oligonucleotides have been developed for their potential to correct mutations and thus serve as therapeutic agents (233; 234). These nucleic acid-based repair elements can be introduced by non-viral (usually lipid-based) delivery methods or using recombinant adeno-associated virus or other viral vectors. However, safety issues concerning adeno-associated virus-based viral delivery and random integrations still remain to be solved (233).

Most of these strategies to rescue folding defects described above have been demonstrated through \textit{in vitro} studies. With respect to the approaches described in Sections 1.6.1-3, it is, unfortunately, not practical (let alone safe) to cool CF patients to subphysiological
temperatures for any significant period of time or to infuse them with high concentrations of osmolytes required to rescue misfolded proteins. The recent success with Ivacaftor® in CF suggests that pharmacological chaperones with appreciable specificity (182) are therefore the most likely avenue for therapeutic interventions, despite the prohibitive costs (~$300,000 per year per patient currently).

1.6 Rationale, hypotheses and objectives

As reviewed in this Chapter, previous site-directed mutagenesis studies of MRP1 have identified many polar residues within the TMs of MSD1 and MSD2 to play crucial roles in structure and function of MRP1. They may contribute directly or indirectly to one or more of the substrate binding sites of MRP1, or its proper folding and/or stable expression at the membrane, and/or its transport function as described in Section 1.3. TM16 of MRP1, which may contain up to 15% ionizable residues, has been identified by various mutagenesis and photolabeling studies as particularly important for substrate recognition and is linked by a hairpin loop to the equally important TM17 (123; 126; 128; 130; 133; 143; 152).

As mentioned previously, opposite charge substitutions of two of these ionizable residues in TM16 of MRP1 which are highly conserved (Arg$^{1202}$ and Glu$^{1204}$) reduce levels of MRP1 protein by >80% in transfected HEK cells (130). Neutral and same charge substitutions at these two sites do not affect MRP1 protein levels in HEK293 cells although they affect the transport function of MRP1 by substantially different mechanisms (130). However, although Arg$^{1202}$ and Glu$^{1204}$ are important for both the expression and function of MRP1, the roles of each amino acid are distinct. Most notably, as discussed in Section 1.3, a neutral substitution of Glu$^{1204}$ has a much greater effect on the transport activity of MRP1 than do neutral substitutions of Arg$^{1202}$ and further, has a profound effect on the properties of MRP1 to interact with ATP/ADP particularly at
NBD2. The major goal of this thesis research was to understand more precisely how Arg$^{1202}$ and Glu$^{1204}$ are involved in MRP1 expression and its localization to the plasma membrane of HEK293 cells. To achieve this goal, a variety of experimental strategies were explored in an effort to rescue the misfolded MRP1 R1202D and E1204K. The results of these studies provide insight into the folding mechanisms of MRP1 which may be relevant to other ABC membrane proteins (e.g. CFTR, BSEP etc) involved in human health and disease.

Accordingly, based on the suggestion that the failure of MRP1 R1202D and E1204K to be expressed at significant levels in transfected HEK293 cells is due to misfolding and subsequent degradation of the mutant proteins (130), it was hypothesized that levels of these mutant proteins would be enhanced by incubation of cells harbouring these mutants at subphysiological temperatures, or by incubation in the presence of chemical or pharmacological chaperones, or a combination thereof. It was further hypothesized that, based on studies of several other misfolded mutant ABC proteins (229-232), introduction of strategically chosen ‘second-site’ amino acid substitutions into MRP1 R1202D and E1204K would improve levels of the mutant protein in mammalian cells by providing stabilizing interhelical side-chain interactions. To test these hypotheses, experimental approaches used previously to rescue expression of misfolded mutants of related ABC proteins were employed to increase levels of MRP1 R1202D and E1204K in HEK293 cells. The first two objectives were to determine the effects of incubating transfected cells (i) at subphysiological temperatures (27 °C or 30 °C versus 37 °C) (Chapter 2) or (ii) in the presence of the chemical ‘chaperones’ DMSO, glycerol and 4-PBA (Chapter 3) on plasma membrane levels of R1202D and E1204K. The third objective was to employ in silico analyses of a homology model of the core structure of MRP1 to first identify putative second-site stabilizing amino acid substitutions and then introduce these substitutions
into of the R1202D and E1204K mutants by site-directed mutagenesis followed by measurement of mutant protein levels (Chapter 4). A general discussion of the thesis research is provided in Chapter 5 along with proposals for future research directions.
Chapter 2  Effect of Subphysiological Temperatures on Levels of Misfolded TM16 Mutants R1202D and E1204K in Mammalian HEK Cells

2.1  Introduction

As reviewed in Chapter 1, the TMs of MRP1 contain a significant number of ionizable amino acids (125; 126; 130; 144). This is unusual because the large desolvation penalty for burying polar groups makes strongly polar/charged residues energetically unfavorable choices in the lipid environment of the plasma membrane (235). Two of these ionizable amino acids, Arg\textsuperscript{1202} and Glu\textsuperscript{1204}, are located in TM16 in the inner leaflet of the membrane (Fig. 2.1) and have previously been shown to be important for normal expression of MRP1 at the plasma membrane (130). Thus, oppositely charged mutants R1202D and E1204K are expressed at levels ≤20% of wild-type MRP1 levels in HEK293T cells (130). On the other hand, levels of the neutral (R1202G, R1202L, E1204L) and same charge (R1202K and E1204D) mutants are comparable to wild-type MRP1 (130). This suggests that the introduction of an opposite charge at either position 1202 or 1204 leads to misfolding of MRP1 during its biosynthesis and assembly, which presumably then targets the mutant transporters for degradation by one of the quality control systems in the cell. As discussed in Section 1.3.2, it was previously suggested that the poor expression of these oppositely substituted MRP1 mutants was likely related to the fact that replacing Arg\textsuperscript{1202} with an Asp (or Glu\textsuperscript{1204} with a Lys) results in a net gain of two charges in the cytoplasmic half of TM16 which may affect its geometry in the membrane and its packing with other TM helices (130). It was also noted that these substitutions bring two same-charge residues in close proximity to one another which could be destabilizing due to the forces of electrostatic repulsion (130). Although the neutrally and same-charge substituted Arg\textsuperscript{1202} and Glu\textsuperscript{1204} mutants were readily detected in membrane vesicles, the properties of the two sets of mutants differed
Figure 2.1. Location of Arg\textsuperscript{1202} and Glu\textsuperscript{1204} in human MRP1/ABCC1. A, A secondary structure of MRP1 showing the predicted location of R1202 and E1204 in TM16 adapted from (2). B, Homology model of the core structure (MSD1, NBD1, MSD2, NBD2) of human MRP1 (154) based on the crystal structure of the ABC transporter Sav1866 from \textit{Staphylococcus aureus} with TM16 shown in blue, and 3-D space-filling views of R1202 in purple and E1204 in red. C, A zoom-in view of the R1202 side chain shown as a stick. D, A zoom-in view of the E1204 side chain shown as a stick. Fig. 2.1B-D were generated in Pymol. TM, transmembrane helix; MSD, membrane spanning domain; NBD, nucleotide binding domain; A, Walker A; B, Walker B; C, ABC signature sequence.
substantially (Table 2.1 and Section 1.3.2) (130). In general, a neutral substitution of Glu\textsuperscript{1204} had a much greater effect on MRP1 transport activity than did neutral substitutions of Arg\textsuperscript{1202} (Section 1.3.2 and Fig. 1.5). The distinctly different properties of the neutral substituted Arg\textsuperscript{1202} and Glu\textsuperscript{1204} mutants support the idea that while both amino acids influence the folding, assembly and stable membrane expression and transport activity of MRP1, the mechanisms by which they do so are different.

The reasons for the failure of the R1202D and E1204K mutants to be expressed at the plasma membrane in mammalian cells are not known. It appears likely that the mutations lead to the production of misfolded MRP1 which fails to acquire or maintain its native three-dimensional structure which in turn impairs its ability to reach the plasma membrane (236). Thus, while stringent quality control systems in the ER execute multiple attempts to ensure correct protein folding, failure of these attempts lead to proteolysis of misfolded proteins via the ubiquitin-proteasome pathway, autophagy-lysosome system and/or calpain Ca\textsuperscript{2+}-activated cysteine proteases (236).

Misfolded mutant proteins that fail to traffic post-translationally along the early secretory pathway compartments (ER, ER-Golgi interface and trans-Golgi network) to their destined subcellular locations are often referred to as “processing” mutants (236). In some cases, the “processing” mutants result in serious human diseases, such as CF and PHHI, as reviewed in Section 1.4.2. For example, disease-causing mutations that disrupt protein trafficking are found in the TM helices of MSD0 of \textit{ABCC8}/SUR1 (A116P and V187D) which result in PHHI (237). They are also found in NBD1 of CFTR/\textit{ABCC7} as well as in CL4 of \textit{ABCB1}/BSEP (G238V) also causing PFIC2 (238). In all of these examples, plasma membrane trafficking of the mutant ABC proteins could be fully or partially rescued/restored \textit{in vitro} by lowering the temperature
Table 2.1. Summary of organic anion transport activities of MRP1 mutants with neutral or same-charge substitutions of R1202 and E1204 in TM16 of MSD2.

<table>
<thead>
<tr>
<th>MRP1 Mutant</th>
<th>Organic anion substrate</th>
<th>% Wild-type MRP1 transport activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E&lt;sub&gt;2&lt;/sub&gt;17βG</td>
<td>LTC&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>R1202G</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td>R1202L</td>
<td>115</td>
<td>120</td>
</tr>
<tr>
<td>E1204L</td>
<td>&lt;10</td>
<td>50</td>
</tr>
<tr>
<td>E1204D</td>
<td>100</td>
<td>115</td>
</tr>
</tbody>
</table>

<sup>a</sup> The values shown are taken from Situ et al (130).

<sup>b</sup> ND, not done.
(≤30 °C) at which mammalian cells expressing these mutants were incubated for 24 – 48 h. In all cases, protein function was also restored. Subphysiological temperatures (30 °C) have also been reported to increase the maturation efficiency of a recombinant Cys-less P-gp mutant, in which the seven Cys residues in TMs 2, 7 and 11 as well as NBD1 and NBD2 were changed to Ala (231). Thus, restoration of stable expression and membrane localization of various ABC proteins by subphysiological temperature appears to be independent of the location of the mutation within the protein.

The broad and apparently non-selective ability of reduced temperatures to restore plasma membrane expression and function of ABC protein processing mutants made it very appealing to determine if this experimental approach would also be successful in restoring membrane expression of the MRP1 processing mutants R1202D and E1204K. If so, then it might be possible to obtain sufficient protein to allow the transport activities of these mutant transporters to determine whether the mutations affect substrate binding and/or transport.

2.2 Materials and methods

2.2.1 Materials

Lipofectamine™ 2000, Alexa Fluor®488 conjugated F(ab’)2 fragment of goat anti-mouse IgG (H+L) and Alexa Fluor®546 conjugated F(ab’)2 fragment of goat anti-rat IgG (H+L) were from Invitrogen (Carlsbad, CA). Triton X-100, phenylmethylsulfonyl fluoride (PMSF), DNaseI, RNase A, dithiothreitol (DTT), propidium iodide and 4’,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical Co. (St. Louis, MO). Protease inhibitor cocktail was purchased from Roche Diagnostics (Indianapolis, IN). Bovine serum albumin (BSA) was from ICN Biomedicals (Aurora, OH). MRP1-specific mouse MAb QCRL-1 and QCRL-3 were produced in house (239). MRP1-specific rat MAb MRPr1 was a kind gift from Drs. R.J. Scheper
Mouse MAb against calnexin (sc-23954) was from Santa Cruz Biotechnology (Paso Robles, CA). Western Lightning™ chemiluminescence reagent Plus was from Perkin Elmer Life Science (Waltham, MA).

2.2.2 Cell culture and transfection of MRP1 expression vectors in HEK293T cells

SV40-transformed human embryonic kidney (HEK293T) cells were maintained in 175 cm² flasks in Dulbecco's modified Eagle’s medium (DMEM) supplemented with 4 mM L-glutamine and 7.5% fetal bovine serum (FBS) (Gibco) in 5% CO₂ at 37 °C. Wild-type or mutant pcDNA3.1(-) MRP1 expression vectors (130) were transfected into HEK293T cells. Approximately 18 x 10⁶ cells were plated per 150 mm dish and then cultured for 24 h in complete medium prior to transfection (~90% confluence) with 40 µg DNA per 75 µl Lipofectamine™ 2000 mixture, according to the manufacturer’s instructions. Transfected cells were grown at 37 °C for 12 h after addition of the Lipofectamine/DNA mixtures. Cells from each 150 mm dish were then subcultured into two (Fig. 2.2A) or three (Fig. 2.2B) 6-well plates, and incubated at the desired temperature (37 °C, 30 °C or 27 °C) and time (48, 72 or 120 h). Untransfected HEK293T cells were included in all experiments as a negative control.

2.2.3 Preparation of whole cell lysates and immunoblotting

Whole cell lysates were prepared by collecting the transfected HEK293T cells by centrifugation (1,200 rpm, 10 min, 4 °C), washing with cold phosphate-buffered saline (PBS) and resuspending in lysis buffer containing protease inhibitors, 50 mM Tris-HCl (pH 7.5), 250 µg/ml DNaseI, 10 mM MgCl₂ and 0.25% sodium dodecyl sulfate (SDS) for ~20 min at 4 °C. Insoluble material was removed from the lysates by centrifugation (15,000 rpm, 10 min, 4 °C). Protein
Figure 2.2. Schematic illustrating the protocol followed for incubation of transfected HEK293T cells at subphysiological temperatures. A, Transfected cells from each 150 mm dish were subcultured into two 6-well plates and incubated at 37 °C or 30 °C for 48 h. B, Transfected cells from each 150 mm dish were subcultured into three 6-well plates and incubated at the temperature and time indicated.
concentrations in the cell lysates were determined using BioRad Dc Protein Assay kit (BioRad, Mississauga, ON) using BSA as standard.

Levels of wild-type and mutant MRP1 proteins were determined by immunoblotting (130; 239). Briefly, proteins (5 or 10 µg per lane) were resolved on a 7% polyacrylamide gel and electrotransferred to BioTrace™ polyvinylidene fluoride (PVDF) membranes (0.45 µm pore size) (Pall Corporation, Pensacola, FL) for 90 min at 100 V at 4 °C. Membranes were blocked in 4% (w/v) skim milk in TBS-Tween (TBS-T) solution (150 mM NaCl and 0.5% (v/v) Tween 20 in 10 mM Tris, pH 7.5) at room temperature for 1 h followed by overnight incubation with the human MRP1-specific mouse MAb QCRL-1 (diluted 1:5,000 in blocking solution) at 4 °C (130; 239). After washing with TBS-T, immunoblots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (diluted 1:10,000 in blocking solution) (Pierce, Edmonton, AB) at room temperature for 1 h. Chemiluminescence reagent (PerkinElmer) as used to detect binding of the conjugates and the light emitted was captured on film (Ultident, St. Laurent, QC) (241). Relative levels of MRP1 protein were estimated by densitometry of the films using Image J software (http://rsb.info.nih.gov/ij/). The PVDF membranes were stained with a solution of Amido Black to confirm equal loading of total proteins in the lanes. Statistical analysis of the differences in MRP1 protein expression among various treatment and control groups was performed using one-way analysis of variance (ANOVA) with a Dunnett post-hoc test. P values < 0.05 were considered statistically significant.

2.2.4 Indirect immunofluorescence analyses

HEK293T cells were seeded at 0.75 x 10⁶, 1 x 10⁶ and 1.5 x 10⁶ cells per well in 6-well plates on coverslips coated with 0.1% gelatin for incubation at 37 °C, 30 °C and 27 °C, respectively. After 24 h, cells were transfected with either wild-type or mutant MRP1 expression
vectors as described above in Section 2.3.2. After 48 h at the desired temperature, the coverslips were washed with PBS and immunofluorescence measured according to one or both protocols described below.

*Indirect immunofluorescent detection of MRP1 proteins*

Transfected cells were fixed with 4% paraformaldehyde at room temperature for 10 min followed by washing with PBS. Cells were then permeabilized by adding 0.2% Triton X-100 in PBS for 5 min. After washing with PBS, cells were blocked with 1% BSA in PBS followed by incubation with MRP1-specific mouse MAb QCRL-3 (diluted 1:2,500 in 1% BSA and 0.1% Triton X-100 in PBS) for 1 h at room temperature (239). After washing with PBS, the coverslips were incubated with Alexa Fluor®488 conjugated F(ab’)2 fragment of goat anti-mouse IgG (H+L) (diluted 1:1,000 in 1% BSA and 0.1% Triton X-100 in PBS containing 10 µg/ml RNase A) in the dark for 30 min at room temperature. Coverslips were then washed and incubated with 2 µg/ml propidium iodide in PBS to counterstain cell nuclei, followed by mounting on the slides containing SlowFade® antifade solution (Molecular Probes Inc., Eugene, OR). Slides were examined using a Leica TCS SP2 MS multiphoton system confocal microscope (Heidelberg, Germany) in the Queen’s University Cancer Research Institute Cytometry and Imaging Facilities (http://qcri.queensu.ca/Cytometry_and_Imaging_5021.html).

*Indirect immunofluorescent detection of MRP1 and calnexin.*

Cells were fixed in 100% cold methanol for 20 min on ice followed by washing and incubation in 0.1% Triton X-100 at room temperature for 10 min. After washing, cells were blocked in 3% BSA in PBS for 45 min at room temperature. Cells were then incubated with MRP1-specific rat MAb MPRr1 (diluted 1:1,800) (240) and mouse MAb against calnexin (diluted 1:50) in 1% BSA in PBS for 90 min at 37 ºC. After washing with PBS, the coverslips
were incubated with Alexa Fluor®546 conjugated F(ab’)2 fragment of goat anti-rat IgG (H+L) (diluted 1:1,200) and Alexa Fluor®488 conjugated F(ab’)2 fragment of goat anti-mouse IgG (H+L) (diluted 1:400) in 1% BSA in PBS in the dark for 90 min at 37 °C. Coverslips were washed and mounted on the slides containing SlowFade® antifade solution as before. A small amount of DAPI solution (<1 µl) was added to the antifade solution to counterstain cell nuclei. Slides were examined by confocal microscopy as above.

2.3 Results

2.3.1 Effect of temperature on levels of wild-type and mutant MRP1 proteins

As described earlier, MRP1 mutants R1202D and E1204K are reported to be poorly expressed in HEK293T cells at 37 °C (130). These findings were first confirmed by immunoblotting of whole cell lysates of transfected HEK293T cells incubated at 37 °C using MAb QCRL-1. As expected (130), levels of the R1202D and E1204K mutant proteins at 37 °C were <10% and ~10% of wild-type MRP1, respectively (Fig. 2.3). Similar results were obtained in >15 independent experiments.

To determine if incubating cells at subphysiological temperatures could rescue expression of the R1202D and E1204K mutants, HEK293T cells transfected with either wild-type or mutant expression vectors were grown in a 30 °C incubator for 48 h (Fig. 2.2A). Whole cell lysates were prepared, total protein determined and relative MRP1 levels determined by immunoblotting. As shown in Fig. 2.4, incubation of cells at 30 °C increased levels of both the wild-type (~1.5 fold) and R1202D (>10 fold) and E1204K (~4 fold) mutant MRP1 proteins; in addition, a mixture of fully glycosylated MRP1 (190 kDa) and underglycosylated immature MRP1 (170 kDa) was observed (Fig. 2.4A). However, at 30 °C the levels of both mutant proteins remained low at just
Figure 2.3. Levels of wild-type and R1202D and E1204K mutant MRP1 proteins in HEK293T cells after incubation at 37 °C for 48 h. Cells were transfected with wild-type or mutant pcDNA3.1(-) MRP1 expression vectors. Whole cell lysates were prepared after 48 h and MRP1 levels determined by immunoblotting with MAb QC RL. Relative levels of MRP1 protein were determined by densitometry and are indicated below the blot. HEK, control whole cell lysates prepared from untransfected HEK293T cells.
Figure 2.4. Levels of wild-type and R1202D and E1204K mutant MRP1 proteins in HEK293T cells after incubation at 30 °C for 48 h. A, Shown is a representative immunoblot of MRP1 protein levels. Cells were transfected with wild-type or mutant pcDNA3.1(−) MRP1 cDNA expression vectors and after 48 h, whole cell lysates were prepared and MRP1 levels determined by immunoblotting (10 µg protein per lane) with MAb QCRL-1. The 190 and 170 kDa bands represent fully glycosylated and underglycosylated immature MRP1 proteins, respectively. Total relative levels of MRP1 (190 + 170 kDa) were determined by densitometry and are indicated below the blot. HEK, control whole cell lysates prepared from untransfected HEK293T cells. B, Summary of densitometry results for MRP1 expression levels obtained in 4 independent transfections. * denotes statistically significant difference between wild-type and mutant MRP1 levels in cells incubated at 30 °C and wild-type MRP1 levels in cells incubated at 37 °C (p < 0.05).
30-40% of wild-type MRP1 at 37 °C (Fig. 2.4B). Thus, reducing temperature was only partially effective at restoring expression of these mutants.

Cells were also incubated at 27 °C for 48 h, 72 h or 120 h as well as at 30 °C for 72 h or 120 h (Fig. 2.2B) to explore if any of these conditions could further increase levels of these mutant proteins. Incubation of cells at 30 °C for 48 h resulted in the maximal increase in wild-type (~1.3-fold) and R1202D (>10 fold) and E1204K (~3-fold) mutant MRP1 proteins (Fig. 2.5), which were comparable to the increases in levels observed previously at 30 °C (Fig. 2.4). The other conditions tested either decreased or had little effect on levels of the MRP1 proteins (Fig. 2.5).

**2.3.2 Effect of temperature on localization of wild-type and R1202D and E1204K mutant MRP1 proteins**

The cellular localization of the R1202D and E1204K mutant proteins was determined by examining transfected cells by indirect immunofluorescence and confocal microscopy using MAb QCRL-3 after incubation of cells at 37 °C, 30 °C or 27 °C for 48 h. Fig. 2.6 (A-C) shows that at 37 °C, cells expressing the R1202D and E1204K mutants displayed a fluorescence pattern similar to that of cells expressing wild-type MRP1 and in all cases, the MRP1 proteins are largely localized to the plasma membrane. After incubation at 30 or 27 °C, however, a significant portion of the MRP1 proteins were localized inside the cell, close to the nucleus in most cases (Figs. 2.6D - I). When cells incubated at 30 °C were stained with antibodies against MRP1 and calnexin (an ER marker), the yellow colour in the merged images suggested colocalization of these two proteins in the ER (Fig. 2.7D-F). These results indicate that the cellular localization of the poorly expressed TM16 mutants R1202D and E1204K at physiological and reduced temperatures is similar to that of wild-type MRP1.
Figure 2.5. Levels of wild-type and R1202D and E1204K mutant MRP1 proteins in transfected HEK293T cells after prolonged incubation (72 h, 120 h) at 30 °C and 27 °C.

Cells were transfected with A, wild-type or B, R1202D or C, E1204K pcDNA3.1(−) MRP1 expression vectors and then grown at 37, 30 or 27 °C for the times indicated above the blots.

Whole cell lysates were prepared and MRP1 levels determined by immunoblotting (10 µg protein per lane) with MAb QCRL-1. Relative levels of MRP1 protein were determined by densitometry and are indicated below the blot. HEK refers to control whole cell lysates prepared from untransfected HEK293T cells.
Figure 2.6. Immunolocalization of wild-type and R1202D and E1204K mutant MRP1 proteins in HEK293T cells after incubation at various temperatures for 48 h. Cells were seeded in 6-well plates and transfected with either wild-type (A, D, G), R1202D (B, E, H), or E1204K (C, F, I) mutant MRP1 cDNA expression vectors. Cells were then incubated at 37 °C (A-C), 30 °C (D-F) or 27 °C (G-I) for 48 h and MRP1 immunofluorescence detected as described in Section 2.3.4.i. Cellular DNA was stained with propidium iodide (red) and MRP1 was detected with MAb QCRL-3 and Alexa Fluor® 488 conjugated secondary antibody (green).
Figure 2.7. Immunolocalization of calnexin, and wild-type and R1202D and E1204K mutant MRP1 proteins in HEK293T cells after incubation at 37 °C and 30 °C for 48 h. Cells were seeded in 6-well plates on coverslips, and transfected with either wild-type (A, D) or R1202D (B, E) or E1204K (C, F) mutant MRP1 cDNA expression vectors. Cells were then incubated at 37 °C or 30 °C for 48 h, and MRP1 and calnexin detected by immunofluorescence as described in Section 2.3.4.ii. Confocal images of wild-type and mutant MRP1 proteins in cells incubated at 37 °C (A – C) and 30 °C (D – F). Cellular DNA was stained with DAPI (blue). MRP1 was detected with MAb MRPr1 and Alexa Fluor® 546 conjugated secondary antibody (red); calnexin was detected with anti-calnexin MAb and Alexa Fluor® 488 conjugated secondary antibody (green). Colocalization of MRP1 and calnexin is indicated by a yellow colour in the merged images.
2.3 Discussion

Incubation of cells at subphysiological temperatures in vitro has been demonstrated to restore functional expression of processing mutants of several ABC proteins at the plasma membrane of mammalian cells which are otherwise recognized as misfolded proteins and targeted for degradation (199; 237; 238; 242). In the present study, we have shown that incubation of cells expressing the MRP1 processing mutants R1202D and E1204K at 30 °C can increase the levels of these mutants. Whether or not function is restored is not yet known. Maximal levels (30-40% of wild-type levels at 37 °C) were achieved by incubating transfected cells at 30 °C for 48 h at which the cell density and morphology of the transfected cells appeared to be most comparable to that at 37 °C.

The magnitude of the increase achieved is similar to that reported for ΔF508 CFTR where plasma membrane expression of this chloride channel could be restored from barely detectable levels to 25 – 40% of wild-type levels (205; 224). Whole cell currents from ΔF508-CFTR expressing cells incubated at 30 °C were increased >7-fold, suggesting that not only was the rescued channel at least partially expressed at the plasma membrane, it was also active (205). Single-channel current measurements showed that the properties of the rescued ΔF508 CFTR were comparable to those of wild-type CFTR except the mutant protein had a lower probability of being in the open state (205). Thus, the rescued ΔF508 CFTR, like the R1202L/G and E1204D MRP1 mutants which exhibited mostly wild-type MRP1 transport activities (Table 2.1), displayed channel properties similar to that of wild-type CFTR.

In contrast to CFTR, however, MRP1 transports multiple substrates instead of simply conducting chloride or other ion (e.g. bicarbonate (243)) currents (though it has been demonstrated that CFTR is capable to directly mediate GSH efflux (244)). Thus, it is possible
that the rescued R1202D and E1204K proteins might display substrate selective changes in patterns of substrate recognition and/or transport. Whether the rescued R1202D and E1204K mutant proteins behave more like functional R1202L/G and E1204D mutants or the transport-compromised E1204L mutant (Table 2.1) could not be reliably determined in this study because the levels of expression were too low. For patients suffering from CF, it is believed that rescuing membrane expression of mutant CFTR to just 6-10% of normal CFTR activity might be sufficient to alleviate CF symptoms (224).

It is worth noting that when expressed, the R1202D and E1204K proteins were fully glycosylated (190 kDa) (Fig. 2.3) and localized at the plasma membrane (Fig. 2.6) at 37 °C in a manner indistinguishable from wild-type MRP1. However, at the reduced temperatures (30 °C, 27 °C), substantial amounts of wild-type and both mutant MRP1 proteins were underglycosylated (170 kDa) (Figs. 2.4A & 2.5B, C) and localized intracellularly (Fig. 2.6). These features of the R1202D and E1204K mutants suggest that they are different from some of the extensively studied processing mutants of other ABCC proteins such as ΔF508 CFTR (205) and the V187D mutant of SUR1 (237) which appear almost exclusively as underglycosylated proteins at 37 °C.

Incubating cells expressing these mutants at reduced temperatures seems to mainly increase the amount of fully glycosylated proteins (205; 224; 237). Similar to MRP1 mutants R1202D and E1204K, the D482G mutant of ABCB11/BSEP, which is present at levels ~2.5% of wild-type levels, are predominantly fully glycosylated at 37 °C (199). Incubating cells expressing ABCB11/BSEP D482G at reduced temperatures also increases a heterogeneous population of both fully glycosylated and underglycosylated mutant proteins (199) just like the R1202D and E1204K mutants described here.
Co-localization confocal microscopy studies of MRP1 and calnexin suggest that the intracellular wild-type and mutant MRP1 proteins in cells incubated at subphysiological temperatures reside in the ER (Fig. 2.7). Because the turnover rate of protein biosynthesis is often slowed down by reduced temperature (245), some newly synthesized MRP1 proteins might reside in the ER for a prolonged period of time before being directed to the plasma membrane. It has been reported in both transfected HeLa and drug-selected H69AR cells that at least half of the newly synthesized MRP1 is rapidly degraded (14). Based on a comparable localization pattern of the wild-type and mutant MRP1 proteins at all temperatures, it seems that introducing an opposite charge mutation at either position 1202 or 1204 reduces MRP1 levels but does not substantially impair its processing and ability to insert into the plasma membrane. Since protein folding is known to be a dynamic process of trial and error (246), it may be that a small portion of the R1202D and E1204K proteins “escapes” the destabilizing effect of the mutations. However, it seems likely that the majority of the mutant MRP1s cannot attain properly folded tertiary structure to pass sequential checkpoints in the quality control system along the early secretory pathway (ER, ER-Golgi interface and trans-Golgi network) (158). This disruption in the folding process of the MRP1 mutants may be partly alleviated, but may not be fully compensated for, by incubating cells expressing the mutants at subphysiological temperatures.

In contrast to the cellular responses to elevated temperatures (e.g. induction of heat-shock proteins), the cellular responses to subphysiological temperatures are poorly understood at the molecular level. However, similar to heat stress, the unfolded protein response (UPR) is thought to be induced in conditions of cold stress (245; 247). UPR is a complex molecular pathway activated in response to cellular stresses and mainly characterized by up-regulation of ER molecular chaperones to improve cell survival by facilitating correct folding or assembly of
proteins and preventing protein aggregation (247). Activation of UPR seems critical to generating a favourable cellular environment to promote proper folding of ΔF508 CFTR at subphysiological temperature (242). Up-regulation and/or activation of molecular chaperones such as heat-shock proteins to assist in protein synthesis, unfolding/refolding, protein maturation and trafficking have been reported in cells in response to cold stress (242; 245). Also, cold-shock proteins (CSPs) have been identified in association with the induction of UPR (242; 245). For example, upregulation of cold stress protein Csdel (a ribonucleoprotein involved in the regulation of transcription of molecular chaperones involved in UPR e.g. Hsp90) has been reported when incubating ΔF508 CFTR expressing cells at 26 °C for 48 h (242). Whether or not the increased amounts of wild-type and R1202D and E1204K MRP1 proteins observed after incubation of cells at 30 °C result from a similar mechanism remains to be determined.

Culture of mammalian cells at subphysiological temperatures has also been well documented to reduce cellular metabolism which is characterized by reduced energy consumption, reduced protease activities, cell cycle arrest at G1, and delayed apoptosis (245; 248). For example, Moore and colleagues (249) reported that up to 87% of CHO cells arrest at G1 phase when the incubation temperature was decreased from 37 °C to 30 °C. G1 phase is when the majority of protein synthesis occurs which may counterbalance the decreased rate of cell growth at sub-physiological temperature (245). The consequences of a prolonged but mild “cold shock” have been exploited as a means to increase the yield of recombinant proteins expressed in mammalian cells (248; 250), which may (partially) account for the observed increases in both wild-type and mutant MRP1 protein levels in HEK293T cells at 30 °C.

Finally, it is interesting to note that rescue of ΔF508-CFTR levels by reduced temperature (30 °C) does not occur in all cell types (238). Thus, ΔF508-CFTR export requires a local
biological folding environment that is sensitive to stress-inducible factors found in some cell types, which is (in part) distinct from the wild-type pathway (238). Thus, it is possible that the HEK293T cell line is not the ideal cell model to investigate the effect of reduced temperatures on levels of the R1202D and E1204K mutants. The effect of reduced temperature appears to help stabilize the mutant proteins; however, it is not sufficient to overcome the phenotype of the R1202D and E1204K mutants. The cellular microenvironment around the newly synthesized mutant proteins may be required to initiate an appropriate response to temperature reduction in order to reverse the folding hurdle generated by the R1202D and E1204K mutations.

In conclusion, although some increases in protein levels were observed using the HEK293T expression system by incubation at subphysiological temperatures, they were insufficient to allow functional characterization of the R1202D and E1204K mutants. For this reason, other methods for restoring expression of these processing mutants were explored and are described in Chapter 3.
Chapter 3 Chemical Approach to Rescuing TM16 Processing Mutants of MRP1(ABCC1)

3.1 Introduction

As discussed in Chapters 1 and 2, misfolded proteins in mammalian cells are often recognized by the ER quality control machinery and subjected to degradation (251). It is well established that membrane spanning proteins are first transported into the ER in an unfolded state, where folding of nascent polypeptides is assisted by ER-resident chaperones such as binding immunoglobulin protein (BiP) and hypoxia responsive domain-1 (Hrd1) (161). These molecular chaperones can discriminate between native and non-native polypeptides and selectively transport the former to their final destinations e.g. plasma membrane (251).

Previous studies have identified a number of low molecular weight molecules permeable to the cell membrane, such as glycerol, DMSO and 4-PBA, that help misfolded mutant proteins to escape the ER quality control systems, and thus restore their subcellular localization and often function (156; 217; 252). These relatively non-specific acting small molecules are widely used in experimental systems and are named chemical chaperones because they appear to mimic the effects of in vivo or natural physiological chaperones by stabilizing the mutant proteins and facilitating their folding (156; 253).

To date, the molecular actions of the chaperoning activities associated with these agents are not well defined. Glycerol and DMSO (Fig. 3.1) are osmotically active and are believed to exert their effects by increasing the difference in free energy between partially folded/unfolded intermediates and the native state, which drives the equilibrium towards the native state (254). They may also reduce the free movement of proteins and thus reduce the frequency of non-productive folding (156). In contrast, 4-PBA (Fig. 3.1) is a hydrophobic molecule and is thought
to bind to the hydrophobic segments in partially folded or unfolded intermediates, protecting them from the quality control systems (156; 157). 4-PBA may also influence many levels of gene regulation via its ability to inhibit histone deacetylases which decreases the ability of the histones to bind to DNA, resulting in chromatin expansion and genetic transcription (255). For example, 4-PBA has been reported to induce the mRNA levels of physiological chaperones (e.g. heat shock proteins) in the IB3-1 human bronchial epithelial cell line derived from a CF patient heterozygous for the CFTR mutation ΔF508 (256).

Regardless of their mechanisms of action, incubation of cells with glycerol, DMSO and 4-PBA has been reported to restore plasma membrane expression of several processing mutants that result in serious human diseases. The trafficking-defective mutation which results in cystic fibrosis is found in the NBD1 of ABCC7/CFTR (ΔF508) mentioned previously (211; 212; 257). Additionally, glycerol and DMSO have been shown to enhance human P-gp expression in the yeast Saccharomyces cerevisiae (258) as well as in human HL60 leukemia cells (259).

The objective of the studies described in this chapter was to determine the extent to which the chemical chaperones DMSO, 4-PBA and glycerol could restore the plasma membrane expression of the MRP1 processing mutants R1202D and E1204K in HEK293T cells.

3.2  Materials and methods

3.2.1  Materials

Reagents for transfections, whole cell lysate preparations, and immunoblotting were obtained from the sources as described in Section 2.2.1. 4-PBA and anti-α-tubulin mouse MAb were purchased from Sigma Chemical Co. (St. Louis, MO). Glycerol and DMSO were purchased from Fisher Scientific (Ottawa, ON, Canada).
Figure 3.1. Structures of chemical chaperones. Shown are the chemical structures of the chemical chaperones used in attempts to rescue protein expression of MRP1 processing mutants R1202D and E1204K in HEK293T cells.
3.2.2  **Cell viability assay**

HEK293T cells were maintained at 37 °C as described in Section 2.2.2. The effect of various concentrations of glycerol and DMSO on cell viability and density was determined using a trypan blue dye exclusion assay. Untransfected HEK293T cells were seeded in a 6-well plate at 1 x 10⁶ cells per well overnight and then grown in the absence and presence of glycerol (5 and 8%) and DMSO (1.5 and 2%). After 72 h, cells were collected and diluted in a ratio of 1:2. Equal amounts of a 0.4% trypan blue solution and cell suspension were combined, and then one drop of the mixture was applied to a hemacytometer, and cell viability and density determined by examining and counting cells under a microscope.

3.2.3  **Transfection of MRP1 expression vectors in HEK293T cells and exposure to chemical chaperones**

Wild-type or R1202D, E1204K and K513A mutant pcDNA3.1(−) MRP1 DNA expression vectors were transfected into HEK293T cells (130). K513 is located in CL5 (between TM7 and TM8) of MRP1, and introducing an Ala mutation generates a MRP1 processing mutant that is expressed at 30-40% of the levels of wild-type MRP1 (150). Approximately 1.5 x 10⁶ cells were seeded per well in a 6-well plate and then cultured for 24 h in complete medium prior to transfection (~90% confluence) with 4 µg DNA per 10 µl Lipofectamine™ 2000, according to the manufacturer’s instructions.

Transfected cells were incubated with the Lipofectamine/DNA mixtures for 6 h. Medium was then replaced by fresh medium with or without glycerol (1, 3, 5%), DMSO (0.5, 1, 2%) or 4-PBA (5 mM). Untransfected HEK293T cells grown in medium with or without glycerol, DMSO or 4-PBA were included in all experiments as negative controls. After 48 h, cells were collected.
In some experiments, transfected cells were incubated at 30 °C in the presence of glycerol (3%), DMSO (1%) or 4-PBA (5 mM) for 48 h prior to being collected.

3.2.4 Preparation of whole cell lysates and immunoblotting

Whole cell lysates were prepared and levels of the wild-type and mutant MRP1 proteins determined by immunoblotting as described in Section 2.2.3. To confirm equal loading of proteins in the lanes, the membranes were also probed with anti-α-tubulin mouse MAb (diluted 1:10,000 in blocking solution) at room temperature for 1 h, followed by incubation with HRP-conjugated goat anti-mouse secondary antibody (diluted 1:10,000 in blocking solution) (Pierce, Edmonton, AB) for 1 h at room temperature. Relative levels of MRP1 protein expression were estimated by densitometry of the films using Image J software (http://rsb.info.nih.gov/ij/).

3.2.5 Statistical analysis

Statistical analysis of the differences in MRP1 protein expression among various treatment and control groups was performed using one-way analysis of variance (ANOVA) with a Dunnett post-hoc test. P values < 0.05 were considered statistically significant.

3.3 Results

3.3.1 Effect of glycerol, DMSO and 4-PBA on cell viability and density of HEK293T cells

To begin to investigate whether treatment with glycerol, DMSO and 4-PBA could improve the levels of the R1202D and E1204K MRP1 processing mutants, the maximal tolerated concentrations of these agents on HEK293T cells were first determined using a trypan blue exclusion assay (260). Two concentrations of glycerol (5 and 8%) and DMSO (1.5 and 2%) were selected for testing based on concentrations reported in the literature to be effective in rescuing other misfolded membrane proteins (211; 216; 261; 262). As shown in Fig. 3.2, glycerol (5 and 8%) and DMSO (1.5 and 2%) had little or no effect on the viability of HEK293T cells. The
density of cells, however, was decreased by ~75% in the presence of 8% glycerol and ~25% in the presence of 2% DMSO (Fig. 3.2). 4-PBA was also tested by Dr. S. Iram (Cole lab) and concentrations up to 5 mM were determined to have no effect on HEK293T cell viability, density or morphology. Based on the above results, maximal concentrations of glycerol (5%), DMSO (2%) and 4-PBA (5 mM) were used in all subsequent experiments.

3.3.2 Effect of glycerol on levels of wild-type and mutant MRP1 proteins.

To determine if glycerol exposure could rescue plasma membrane expression of the R1202D and E1204K mutants, HEK293T cells transfected with either wild-type or mutant expression vectors were grown in the presence of 1, 3 or 5% glycerol for 48 h. Whole cell lysates were prepared, total protein was quantified and relative MRP1 levels determined by immunoblotting as before. As shown in Fig. 3.3, exposure of cells to glycerol did not change the levels of the mutant proteins relative to wild-type MRP1. Wild-type MRP1 levels were unaffected by 3 and 5% glycerol; however, a small (10%) but statistically significant decrease ($P<0.05$) was observed in cells exposed to 1% glycerol (Fig. 3.3B, left). Longer exposure (60 s) of film allowed the effect of glycerol on the mutant protein levels to be determined without reference to wild-type MRP1 (overexposed) (Fig. 3.3A, middle). A significant 20% ± 6% increase in R1202D levels was observed in cells exposed to 5% glycerol ($P < 0.05$). Exposure to 1% and 3% glycerol caused a 50% ± 20% decrease and 20% ± 10% increase in R1202D levels, respectively, but neither effect was statistically significant ($P>0.05$ (Fig. 3.3B)). E1204K levels decreased by 30% ± 20% ($P>0.05$) and increased by 30% ± 10% and 40% ± 20% (by1%, 3% and 5% glycerol, respectively, but none of the effects were statistically significant ($P>0.05$) (Fig. 3.3B).
Figure 3.2. Effect of glycerol and DMSO on cell growth and viability of HEK293T cells. Untransfected HEK293T cells were seeded at 1 x 10^6 cells per well in a 6-well plate and then exposed to glycerol and DMSO at the indicated concentrations for 72 h. Trypan blue exclusion assay was performed to determine A, cell density (expressed as % of untreated controls) and B, viability (expressed as % of total number of cells). Results shown are from a single experiment and similar results were obtained in one additional independent experiment.
**A**

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Relative expression:

*MRP1 (5 s exp)*

*corrected (1)*

*MRP1 (60 s exp)*

*corrected (2)*

*α-tubulin (5 s exp)*

*(1)*

*(2)*

**B**

5 s exp

![Graph showing relative MRP1 levels for WT, R1202D, and E1204K at 5 and 60 s expression times.]

60 s exp

![Graph showing relative MRP1 levels for WT, R1202D, and E1204K at 5 and 60 s expression times.]

% glycerol
Figure 3.3. Levels of wild-type and R1202D and E1204K mutant MRP1 proteins in transfected HEK293T cells after 48 h exposure to glycerol. A, Cells were transfected with wild-type or mutant pcDNA3.1(-) MRP1 expression vectors and then grown for 48 h in the presence of glycerol for the concentrations indicated above the representative blot shown. Whole cell lysates were prepared and MRP1 levels determined by immunoblotting (10 µg protein per lane) with MAb QCRL-1. Top, 5 s film exposure. Middle, 60 s film exposure. Bottom, 5 s film exposure of α-tubulin loading control. Relative levels of MRP1 and α-tubulin were determined by densitometry and are indicated below the blots. MRP1 protein expression levels were corrected based on α-tubulin expression. HEK refers to control whole cell lysates prepared from untransfected HEK293T cells. B, Bar graph representation of relative levels of wild-type and mutant MRP1 proteins obtained in 3-5 independent experiments. Bars represent means (± SD). * denotes statistical difference ($P < 0.05$) compared to the untreated controls.
3.3.3 **Effect of DMSO on levels of wild-type and mutant MRP1 proteins**

To determine if exposure to DMSO could rescue plasma membrane expression of the R1202D and E1204K mutants, HEK293T cells transfected with either wild-type or mutant expression vectors were grown in the presence of 0.5%, 1% or 2% DMSO for 48 h. Whole cell lysates were prepared, total protein quantified and relative MRP1 levels determined by immunoblotting. As shown in Fig. 3.4A, exposure of cells to DMSO either had no effect or caused a 10-20% decrease in levels of the wild-type and mutant MRP1 proteins. The decrease (20% ± 0.3%) in expression was only significant ($P<0.05$) in the case of wild-type MRP1 expressing cells treated with 0.5% DMSO relative to untreated cells (Fig. 3.4B, left).

Longer exposure (30 s) of film was used to allow the effect of DMSO on the mutant protein levels to be determined without reference to wild-type MRP1 (overexposed) (Figs. 3.4A, middle & 3.4B, right). The changes observed in E1204K levels, between cells grown in the presence or absence of DMSO, were not statistically significant although considerable variability was noted (Fig. 3.4B, right). Levels of the R1202D mutant were significantly lower ($P<0.05$) in the presence of 0.5% and 1% DMSO (50% ± 6% and 60% ± 3%, respectively) but unchanged in the presence of 2% DMSO (Fig. 3.4B, right).

3.3.4 **Effect of 4-PBA on levels of wild-type and mutant MRP1 proteins.**

To determine if exposure of cells to 4-PBA could rescue plasma membrane expression of the R1202D and E1204K mutants, HEK293T cells transfected with either wild-type or mutant expression vectors were grown in the presence of 5 mM 4-PBA for 48 h. Whole cell lysates were prepared, total protein was quantified and relative MRP1 levels determined by immunoblotting as before. As shown in Fig. 3.5, exposure to 4-PBA did not change the levels of the mutant proteins relative to untreated cells expressing wild-type MRP1. However, 4-PBA exposure increased
wild-type MRP1 levels by 30% ± 7% (P < 0.05) compared to untreated cells (Fig. 3.5B, left).

Longer exposure (> 2 min) of film was used to allow the effect of 4-PBA on the mutant protein levels to be determined without reference to wild-type levels (overexposed) (Fig. 3.5A, middle). A statistically significant 25% ± 3% decrease in both R1202D and E1204K levels was observed in transfected cells exposed to 4-PBA (Fig. 3.5B, right).

3.3.5 Effect of glycerol, DMSO and 4-PBA on levels of MRP1 mutant K513A

The relative ineffectiveness of glycerol, DMSO and 4-PBA to increase levels of the R1202D and E1204K mutants was somewhat unexpected. For this reason, a poorly expressing misfolded MRP1 mutant that is known to be rescuable by exposure of cells to these chemical agents, K513A (150), was tested as a positive control under the same experimental conditions as described above for R1202D and E1204K. As shown in Fig. 3.6, 5% glycerol (panel A), 1.5% DMSO (panel B) and 5 mM 4-PBA (panel C) increased levels of the K513A mutant from ~40% to 70%, 80% and 110% of wild-type MRP1 levels, respectively, as expected.

3.3.6 Effect of DMSO, glycerol and 4-PBA on levels of wild-type and mutant MRP1 proteins in cells incubated at 30 °C.

In a final series of experiments, a combination of a chemical chaperone and reduced temperature was tested to determine if this would be more effective in rescuing membrane expression of the R1202D and E1204K mutants than the individual modalities alone. Thus, HEK293T cells transfected with either wild-type or mutant expression vectors were grown in the presence of 3% glycerol, 1% DMSO or 5 mM 4-PBA for 48 h at 30 °C. Whole cell lysates were prepared, total protein quantified and relative MRP1 levels determined by immunoblotting as before. The effect of 3% glycerol in combination with reduced temperature could not be reliably evaluated because of excessive cell death caused by the cell culture conditions. Decreased cell
density was also noted in DMSO and 4-PBA treated cells incubated at reduced temperature. Exposure of cells to 30 °C and DMSO (1%) or 30 °C and 4-PBA (5 mM) had little effect on levels of the mutant proteins relative to wild-type MRP1 expressed in the untreated cells incubated at 30 °C (Fig. 3.7, top). However, exposure of cells to DMSO (1%) or 4-PBA (5 mM) at 30 °C decreased wild-type MRP1 levels by ~20% and ~60%, respectively (Fig. 3.7, top). In addition, similar to what was observed at 30 °C alone, a mixture of fully glycosylated MRP1 (190 kDa) and underglycosylated MRP1 (170 kDa) was observed. Longer exposure (60 s) of film was used to allow the effect of the combined treatment on levels of the mutant proteins to be determined without reference to wild-type MRP1 levels (overexposed) (Fig. 3.7, middle). A 60% decrease in R1202D levels was observed in cells exposed to 5 mM 4-PBA at 30 °C whereas a 30% increase was observed in exposure to 1% DMSO at 30 °C (Fig. 3.7, middle). On the other hand, 2.4-fold and 3.6-fold increases in E1204K levels were observed between cells grown in the presence or absence of 1% DMSO or 5 mM 4-PBA at 30 °C, respectively (Fig. 3.7, middle).

### 3.4 Discussion

In this study, the ability of chemical chaperones to increase levels of the MRP1 processing mutants R1202D and E1204K was investigated. Osmolytes glycerol and DMSO as well as the hydrophobic compound 4-PBA are among the most common chemical chaperones used to restore expression of misfolded proteins (156). A variety of different conditions were tested but the maximal effects observed were a 30% increase in R1202D levels and a 3.6-fold increase in E1204K levels which were achieved by exposing transfected cells to 1% DMSO at 30 °C and 5 mM 4-PBA at 30 °C, respectively. However, levels of R1202D and E1204K in the treated cells still remained the same relative to wild-type MRP1 levels in untreated cells (≤ 20%).
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Relative expression:

- MRP1 (15 s exp): corrected (1)
- MRP1 (30 s exp): corrected (2)
- α-tubulin (5 s exp): (1) (2)

B

15 s exp

1.5

Relative MRP1 levels

0.0

0.1

0.5

1.0

1.5

0.0 0.5 1 2

0.0 0.5 1 2

0.0 0.5 1 2

1.0 1.5 2.0

0.0 0.5 1 2

15 s exp

30 s exp

WT

R1202D

E1204K

R1202D

E1204K

*
Figure 3.4. Levels of wild-type and R1202D and E1204K mutant MRP1 proteins in transfected HEK293T cells after 48 h exposure to DMSO. A, Cells were transfected with wild-type or mutant pcDNA3.1(-) MRP1 expression vectors and then grown for 48 h in the presence of DMSO for the concentrations indicated above the representative blot shown. Whole cell lysates were prepared and MRP1 levels determined by immunoblotting (10 µg protein per lane) with MAb QCRL-1. Top, 15 s film exposure. Middle, 30 s film exposure. Bottom, 5 s film exposure of α-tubulin loading control. Relative levels of MRP1 and α-tubulin were determined by densitometry and are indicated below the blots. MRP1 protein expression levels were corrected based on α-tubulin expression. B, Bar graph representation of relative levels of wild-type and mutant MRP1 proteins obtained in 3-5 independent experiments. (B) Bars represent means (± SD). * denotes statistical difference (P < 0.05) compared to the untreated controls.
Figure 3.5. Levels of wild-type and R1202D and E1204K mutant MRP1 proteins in transfected HEK293T cells after 48 h exposure to 4-PBA. A, Cells were transfected with wild-type or mutant pcDNA3.1(−) MRP1 expression vectors and then grown in the presence of 5 mM 4-PBA for 48 h. Whole cell lysates were prepared and MRP1 levels determined by immunoblotting (10 µg protein per lane) with MAb QCRL-1. Top, 15 s film exposure. Middle, > 2 min film exposure. Bottom, 10 s film exposure of α-tubulin loading control. Relative levels of MRP1 and α-tubulin were determined by densitometry and are indicated below the blots. MRP1 protein expression levels were corrected based on α-tubulin expression. B, Bar graph representation of relative levels of wild-type and mutant MRP1 proteins obtained in 4 independent experiments. Bars represent means (± SD). * denotes statistical difference (P < 0.05) compared to the untreated controls.
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Relative expression:

- MRP1 (15 s exp) corrected
- α-tubulin (15 s exp)

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Relative expression:

- MRP1 (15 s exp) corrected
- α-tubulin (10 s exp)

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Relative expression:

- MRP1 (15 s exp) corrected
- α-tubulin (10 s exp)
Figure 3.6. Levels of wild-type and K513A mutant MRP1 proteins in transfected HEK293T cells after 48 h exposure to chemical chaperones. Cells were transfected with wild-type or mutant pcDNA3.1(-) MRP1 expression vectors and then grown in the presence of 5% glycerol, 1.5% DMSO or 5 mM 4-PBA for 48 h. Whole cell lysates were prepared and MRP1 levels determined by immunoblotting (10 µg protein per lane) with MAb QCRL-1. Levels of mutant MRP1 proteins compared to untreated wild-type levels after exposure to A, 5% glycerol; B, 1.5% DMSO; C, 5 mM 4-PBA. Relative levels of MRP1 and α-tubulin were determined by densitometry and are indicated below the blots. MRP1 protein expression levels were corrected based on α-tubulin expression. Results shown are from a single experiment and similar results were obtained in 1 additional independent experiment.
Figure 3.7. Levels of wild-type and R1202D and E1204K mutant MRP1 proteins in transfected HEK293T cells after 48 h exposure to DMSO or 4-PBA at 30 °C.  A, Cells were transfected with wild-type or mutant pcDNA3.1(-) MRP1 expression vectors and then grown in the presence of 1% DMSO or 5 mM 4-PBA for 48 h at 30 °C. Whole cell lysates were prepared and MRP1 levels determined by immunoblotting (10 µg protein per lane) with MAb QCRL-1. 

Top, 10 s film exposure. Middle, 60 s film exposure. Bottom, 5 s film exposure of α-tubulin loading control. D, DMSO (1%); P, 4-PBA (5 mM); -, no chemical added. HEK refers to control whole cell lysates prepared from untransfected HEK293T cells grown at 30 °C. Relative levels of MRP1 and α-tubulin were determined by densitometry and are indicated below the blots. MRP1 protein expression levels were corrected based on α-tubulin expression. B, Bar graph representation of the representative blot shown. Results shown are from a single experiment and similar results were obtained in 1 additional independent experiment.
The osmolytes glycerol and DMSO have long been used to stabilize proteins during purification and storage as well as to protect them from environmental stresses such as high salt concentration, desiccation and freezing (254). It is not clear how exactly osmolytes can stabilize proteins and, in the case of mutant proteins, stabilize and/or promote folding of otherwise misfolded mutants to native or native-like conformations during protein biosynthesis. It is generally thought that osmolytes such as glycerol and DMSO stabilize proteins by increasing their hydration (254) and/or limiting their free movement (156). Glycerol and DMSO apparently do not directly interact with proteins but rather they are excluded from the protein surface (263; 264). Preferential exclusion of glycerol and DMSO results in preferential hydration of the protein, which leads to an increase in free energy of both native and partially folded or unfolded states (254). Partially folded and unfolded states/intermediates of a protein have more surface area than its more compact native state. Consequently, as mentioned earlier, this favors the equilibrium towards the native or native-like state (254). In addition, osmolytes like glycerol and DMSO may reduce the free movement of proteins by elevating the density of the folding environment, thus preventing aggregation of partially folded or unfolded intermediates, and reducing the frequency of “folding-detours” to non-productive folding pathways (156).

Levels of the MRP1 R1202D and E1204K mutants in cells treated with glycerol did not significantly increase in comparison to wild-type MRP1 levels in untreated cells. Only 5% glycerol significantly increased levels of the R1202D mutant but the increase was modest (20%) (Fig. 3.3A, middle and Fig. 3.3B, right). These results indicate that the ability of glycerol to increase the thermodynamic stability of native conformation of MRP1 (increase protein hydration) and/or improving the folding efficiency of MRP1 (increase density of folding environment) are not enough to overcome the “folding” hurdle generated by the R1202D and
E1204K mutations in TM16. This suggests that opposite charge substitution of these amino acids in TM16 introduces structural disruptions that are too severe to be rescued by a relatively nonspecific and indirect-acting agent like glycerol.

Similar to glycerol, neither concentration of DMSO tested significantly increased levels of the R1202D and E1204K mutant proteins (Fig. 3.4). These results further support the idea that the disruptions caused by R1202D and E1204K mutations are too severe to be corrected by nonspecific and indirect-acting agents.

Unexpectedly, exposure to 1% glycerol or 0.5% DMSO caused a significant 20% decrease in levels of wild-type MRP1 whereas higher concentrations of these agents had no effect. Similar decreases were also observed for the mutant proteins. The reasons for this decrease are not known. The cell morphology and density of HEK293T cells transfected with either wild-type or mutant vectors appeared comparable to untransfected controls, all grown in the presence of 1% glycerol or 0.5% DMSO. Since glycerol and DMSO are relatively nonspecific agents, they likely have multiple effects on cells. The “rescuing” effect of glycerol and DMSO on other misfolded mutant proteins has been reported to be optimal at ≥5% and ≥1%, respectively (211; 216; 261; 265), and the effects at very low concentrations have not been reported in detail. It has been observed that glycerol at concentrations of 0.1–1% inhibited DNA synthesis in keratinocytes (266). Further, DMSO (as low as 0.5%) has been shown to repress SV40 T oncoprotein expression in murine mesenchymal 3T3 T stem cells (CSV3 cells) by posttranslational mechanisms (267). These observations suggest that low concentrations of glycerol and DMSO can have detrimental effects on cells that may be relevant to the observed decrease in wild-type MRP1 levels after exposure of cells to 1% glycerol or 0.5% DMSO.
4-PBA is an approved drug used to treat elevated blood ammonia levels in urea cycle disorders by increasing waste nitrogen excretion in the form of phenylacetylglutamine (a metabolite of 4-PBA) (157). It also has been shown to functionally restore the plasma membrane expression of processing mutants of CFTR such as ΔF508 (257). The chaperone-like activity of PBA in correcting protein misfolding, mislocalization and aggregation has been demonstrated both in vitro and in vivo (199; 257; 268; 269). However, its mechanism of action appears complex and not well defined (270). First of all, 4-PBA can act as a histone deacetylase inhibitor which has been shown to modulate transcription of a variety of genes important in regulating cell development and proliferation through multiple signaling pathways in different cell types (271). For example, genomic and proteomic profiling of 4-PBA-treated IB3-1 CF bronchial epithelial cells demonstrated that molecular chaperones such as heat shock proteins as well as proteins involved in trafficking activity and ion transport were differentially expressed compared to untreated cells (256; 272). However, these effects are likely not solely attributable to the inhibition of histone deacetylases. Thus, 4-PBA has also been reported to decrease the stability of HSC70 mRNA (273) and thus decrease the levels of HSC70 protein (269; 274), a constitutively expressed homolog of stressed-induced HSP70 molecular chaperone which has been implicated in targeting the ΔF508-CFTR mutant protein to an ER-associated degradation pathway (253).

In addition to its activity as a histone deacetylase inhibitor, 4-PBA is considered as a chemical chaperone belonging to the class of hydrophobic compounds (156; 157). In contrast to the preferential exclusion/non-binding mechanism proposed for glycerol and DMSO, the suggested mechanism of action for hydrophobic compounds such as 4-PBA is their ability to bind directly to the exposed hydrophobic surfaces in unfolded or partially folded protein intermediates.
in order to protect them from aggregation or a “quality control check” and subsequent degradation (156; 157). Thus, the mechanism of action of 4-PBA appears multifaceted and may vary to the individual protein and its mutants.

In the current study, 4-PBA significantly increased wild-type MRP1 levels by 30% ± 7% but had little or no effect on the R1202D and E1204K mutants. Thus, like glycerol and DMSO, 4-PBA is relatively ineffective on the MRP1 mutant proteins. However, unlike 4-PBA, glycerol and DMSO decreased or had no effect on wild-type MRP1 levels. The increase in wild-type MRP1 is perhaps not surprising as 4-PBA has been shown to increase levels of wild-type proteins such as CFTR by enhancing the native/correct folding of wild-type proteins, presumably via similar pathways discussed above for mutant proteins (275). It has been reported in pulse-chase experiments with both transfected (HeLa) and drug-selected (H69AR) MRP1-overexpressing cells that at least half of the [35S]methionine incorporated into MRP1 was not chased into mature glycosylated protein, suggesting that a relatively large proportion of the protein was degraded rapidly in the ER (14). Although this phenomenon may be cell type-specific, it is not unreasonable to speculate that PBA treatment was able to enhance the folding efficiency of otherwise degraded wild-type MRP1 proteins, at least in my hands. The failure of PBA to increase levels of the R1202D and E1204K mutants suggests that increasing the general folding and trafficking capacity of ER and cytosol and/or binding to the exposed hydrophobic regions of MRP1 is not sufficient to reverse the severe disruptions to MRP1 structure generated by the R1202D and E1204K mutations.

When cells expressing the MRP1 mutants are grown in the presence of a chemical chaperone at reduced temperature, a synergistic effect could possibly be observed if reduced temperature would increase the concentration of the folded mutant protein in the ER for a
chemical chaperone to exert its effects. Reduced temperature (30 °C) increased levels of R1202D and E1204K to ~40% of wild-type levels although confocal microscopy indicated that a substantial amount of these rescued proteins were localized intracellularly, likely retained in the ER (Section 2.3). Regardless of their mechanisms of action, it appears most likely that chemical chaperones exert their effects through conversion of the unstable folding intermediates to stable ones (156). Unlike the R1202D and E1204K MRP1 mutants which localize at the plasma membrane if expressed at 37 °C, however, many disease-causing processing mutants are largely trapped in the ER and in some cases in the trans-Golgi network (276-283). Therefore, combining the effects of reduced temperature and a chemical chaperone may increase the accumulation of the MRP1 mutant proteins in the ER to engage the chemical chaperone to promote native-like folding.

In this study, combined treatments caused a decrease in wild-type MRP1 levels and had little effect on the levels of the mutant proteins relative to wild-type MRP1. Cell density decreased when the transfected cells were exposed to both 30 °C and a chemical chaperone which might have adversely affected the transfection efficiency of wild-type MRP1, resulting in decreased protein levels. These results indicate that DMSO and 4-PBA could not increase levels of the intracellularly trapped R1202D and E1204K mutants over that observed by reduced temperature alone. It is clear that these mutations severely impede the folding of MRP1 to such an extent that neither a chemical chaperone alone or in combination with reduced temperature, is sufficient to overcome the thermodynamic and/or kinetic barrier imposed by these mutations to achieve a level high enough to allow functional characterization of the mutant proteins.
Chapter 4  Effect of Structure-guided Second-site Mutations on Rescuing

Expression of MRP1 Processing Mutants R1202D and E1204K in Mammalian Cells

4.1  Introduction

Both physical (Chapter 2) and chemical (Chapter 3) approaches to restoring levels of the MRP1 mutants R1202D and E1204K in mammalian cells met with limited or no success. Because mRNA levels of these MRP1 mutants in HEK cells are similar to wild-type levels, it is reasonable to believe that these opposite charge mutations do not affect mRNA stability but rather, disrupt MRP1 folding and/or assembly during translation (130). The relative insensitivity of these mutants to rescue by physical and chemical means also suggests that the opposite charge substitutions of Arg^{1202} and Glu^{1204} cause severe changes in the structure of MRP1 which cannot be compensated by a non-specific rescue approach, at least in mammalian cells with intact quality control mechanisms. On the other hand, recent studies in the Cole laboratory have shown that the levels and, to a large degree, the function of these mutants can be restored if they are expressed in the more permissive quality control environment of insect cells (284). However, the kinetics of LTC_4 transport and the conformation of NBD2 (as shown by limited trypsin digests) are altered to moderate degree (unpublished data, Gao and Cole), indicating that the structures of the R1202D and E1204K mutants in insect cells differ from the structure of the wild-type protein expressed in HEK cells.

Charged residues are rare but usually quite conserved in TM helices of many polytopic membrane proteins because the desolvation penalty for burying polar groups is large (235; 285). The extent to which these polar residues maintain structural integrity crucial to the stability of a membrane protein is highly dependent on the protein and remains not well understood (122). Genetic and biophysical studies have identified polar, irreplaceable residues for functions...
potentially forming electrostatic interactions (235). For example, YgfU, a fourteen TM containing uric acid transporter from *E. coli* was shown to have five irreplaceable residues for its function, all of which are polar and reside within TM helices (286). Similarly, the *E. coli* lactose permease has only six polar, irreplaceable residues for its function, all residing within TM helices (287; 288).

To better understand the molecular mechanisms underlying the inability of R1202D and E1204K to be properly expressed in mammalian cells, or to be rescued by conventional physical methods (Chapters 2 and 3) it seemed important to answer the following questions: (1) do intra and/or interhelical interactions involving Arg\textsuperscript{1202} and Glu\textsuperscript{1204} that are important for proper protein folding and assembly exist in wild-type MRP1, and if so, (2) are some or all of these interactions lost in R1202D and/or E1204K such that they cause misfolding, or impaired assembly to a degree that the MRP1 protein is destabilized and subject to degradation by the proteosome.

To address the above questions, the existing Sav1866-based atomic homology models of the nucleotide-bound core structure of MRP1 (154) were energy minimized in the context of a prospective mutation and then utilized to postulate possible structural explanations for reduced protein levels of the R1202D and E1204K mutants observed in HEK cells. As expected, α-helical wheel projections of TM16 indicate that the two residues are on opposite faces of the α-helix. Consequently, it is reasonable to assume that opposite charge substitutions of Arg\textsuperscript{1202} or Glu\textsuperscript{1204} perturb intra- and/or inter-helical electrostatic interactions critical to folding and expression of MRP1 in different ways. In support of this assumption, substitution of either Arg\textsuperscript{1202} and Glu\textsuperscript{1204} with neutral amino acids was sufficient to restore MRP1 to wild-type levels in mammalian cells in both cases, but only R1202L function was fully restored (130). In contrast, the neutrally substituted E1204L exhibited substantially reduced transport function (except for MTX). Thus,
uptake levels of E$_2$17βG, E$_1$3SO$_4$, GSH and LTC$_4$ by mutant E1204L were <10%, 10%, <25% and 50% of wild-type MRP1 levels, respectively (130). Despite the reduced levels of LTC$_4$ transport, however, photolabeling of E1204L by [³H]LTC$_4$ was comparable to wild-type MRP1. On the other hand, while 8-azido[³²P]ATP photolabeling of E1204L under non-hydrolytic conditions (4°C) was comparable to wild-type MRP1, vanadate-induced trapping of azido-[³²P-ADP] at 37°C (permissive for hydrolysis) was markedly increased. This nucleotide-bound form of the mutant, like wild-type MRP1, could no longer be labeled with [³H]LTC$_4$. Together, these observations indicate that neutral substitution of Glu$^{1204}$ profoundly altered the post-hydrolysis nucleotide interactions of the transporter which could impair the translocation or release of the substrates, resulting in (partial) uncoupling of the transporter’s catalytic activity to transport substrates (130).

Previous studies have demonstrated that introducing second-site mutation(s) in cis with a mutation adversely affecting the levels of related ABC proteins, including CFTR (ABCC7) (229; 289-291) and Pgp (ABCB1) (292; 293), can produce a compensatory effect that results in increased levels of the mutant proteins. In studies of CFTR, the primary mutation has almost always been the disease-associated ∆F508 in NBD1. This deletion affects CFTR assembly by disrupting the interface between NBD1 and CL4 of MSD2 (227; 294). Most of the compensating mutations of CFTR-∆F508 were empirically discovered using unbiased large-scale mutagenesis experiments. The compensating mutations of Pgp-∆Y490 (a Pgp mutant lacking the residue equivalent to Phe$^{508}$ in CFTR) as well as other processing mutants found in the TM helices and NBDs were designed by empirically substituting residues predicted to line the drug-binding pocket with arginine, mimicking the rescuing effect of drug substrate interactions with Pgp (231; 232). However, for MRP1, we decided to take advantage of the availability of a homology model.
to predict potential networks of interactions that in turn might be used to guide the selection of second-site mutations anticipated to restore protein levels. In an earlier study, analysis of a homology model of MRP1 successfully identified a cluster of charged residues (Lys$^{513}$, Lys$^{516}$, Glu$^{521}$, His$^{1364}$, and Arg$^{1367}$) predicted to lie at the interface of CL5 with NBD2, and subsequent mutagenesis studies where each residue was replaced with Ala demonstrated that these residues play a critical role in the plasma membrane expression of human MRP1 (150). In the present study, a similar approach was taken in an attempt to rescue R1202D and E1204K levels in HEK cells.

4.2 Materials and Methods

4.2.1 Materials

Reagents for transfections, whole cell lysate preparations, and immunoblotting were obtained from the sources as described in Chapter 2, Section 2.2.1.

4.2.2 Molecular modeling and energy minimization

Three dimensional models of the nucleotide-bound 4-domain core of human MRP1 (amino acids 300–871, 971–1531) were generated based on the crystal structure of the AMP-PNP bound ABC transporter Sav1866 from Staphylococcus aureus (PDB ID: 2HYD) as described previously (154). Models of the MRP1 core with point mutations were generated and subjected to energy minimization using the SANDER module of AMBER 7.0 software (http://ambermd.org) to find the local energy minimum, i.e., the least energy occupied by the initial conformation. The minimization method involved three steps: i) fixing the protein and minimizing water molecules, ii) fixing the α-carbon backbone, and iii) minimizing the whole system. For calculations in each step, 500 cycles of steepest descent minimization were carried out, followed by 1500 steps of conjugate gradient minimization. The complete system, including
the box of water molecules, was used in the calculation. Figures of the 3D structures were generated in PyMOL molecular graphics system (version 1.2.9) (295).

4.2.3 Site-directed Mutagenesis

Generation of MRP1 mutants by site-directed mutagenesis has been described previously (150). Briefly, the mutagenesis template for generating the mutants was prepared by subcloning a 1986 bp Xmal fragment encoding amino acids 780-1440 from pcDNA3.1(−)-MRP1κ into pGEM-3z (Promega) to create pGEM3z-MRP1-Xmal (127). Single mutants Y1133A, Y1133F, Y1133H and V1248D and V1248E were created using pGEM3z-MRP1-Xmal as template, while double mutants R1202D/Y1133A, R1202D/Y1133F and R1202D/Y1133H were created using pGEM3z-MRP1-R1202D-Xmal as template, and pGEM3z-MRP1-E1204K-Xmal was used to create the double mutants E1204K/V1248D and E1204K/V1248E. Mutagenic oligonucleotide primers were obtained from IDT Inc. (Coralville, IA). Mutagenesis was performed using Pfu turbo DNA polymerase (Stratagene, La Jolla, CA) with the following sense primers (substituted nucleotides for amino acid mutation are underlined): Y1133A (5′-TTC GTC CAG AGG TTC GCC GTG GCT TCC TCC CG-3′); Y1133F (5′- AC TTC GTC CAG AGG TTC TTC GTG GCT TCC TCC CG-3′); Y1133H (5′- TC GTC CAG AGG TTC CAC GTG GCT TCC TCC CG-3′); V1248D (5′-G TAC TTG AAC TGG CTG GAT CGG ATG TCA TCT GAA A-3′) and V1248E (5′-G TAC TTG AAC TGG CTG GCT CGG ATG TCA TCT GAA A-3′). Subsequently, a BsmBI/ClaI fragment (~1.5 kb) containing the mutation was subcloned back into pcDNA3.1(−)-MRP1κ. The fidelity of the fragments was confirmed by sequencing (The Centre for Applied Genomics, Toronto, ON, Canada).
4.2.4 Cell culture and transfection

HEK293T cells were maintained at 37 °C as described in Chapter 2, Section 2.2.2. Wild-type or mutant pcDNA3.1(-) MRP1 expression vectors were transfected into HEK293T cells using standard protocols. Approximately 1 x 10^6 cells were seeded per well in a 6-well plate and then cultured for 24 h in complete medium (to ~90% confluence) prior to transfection with 4 µg DNA in 10 µl Lipofectamine™ 2000, according to the manufacturer’s instructions. Transfected cells were grown at 37 °C for 42 h after addition of the Lipofectamine/DNA mixtures. Untransfected HEK293T cells were included as a negative control.

4.2.5 Preparation of whole cell lysates and immunoblotting

Cells were collected; whole cell lysates were prepared; and levels of wild-type and mutant MRP1 proteins were determined by immunoblotting as described in Chapter 2, Section 2.2.3. Relative loading of proteins in the lanes was determined by probing with anti-α-tubulin mouse MAb and relative levels of MRP1 protein expression determined as described in Chapter 2, Section 2.2.4.

4.3 Results

4.3.1 Prediction of intra- and inter-helical interacting partners of residue Arg^{1202} based on a MRP1 homology model

Based on a MRP1 homology model derived from the crystal structure of nucleotide bound Sav1866 (154), Arg^{1202} lies in TM16 and is exposed on the surface of MRP1, i.e. facing the lipid bilayer, with its side chain extending towards TM15 (Fig. 4.1). The side chain of Arg^{1202} (NH₃⁺) is predicted to form ionic interactions with the -OH group on the side chain of Tyr^{1133} in TM15 (Fig. 4.1). The model suggests the possibility that this potential electrostatic interaction between Arg^{1202} and Tyr^{1133} may be further stabilized by Trp^{1198} (TM16) through a π-cation
interaction (between the \(\pi\)-electrons in the aromatic Trp side chain and the cationic side chain of \(\text{Arg}^{1202}\) (Fig. 4.1). Thus, the potential side chain interactions between \(\text{Arg}^{1202}\), \(\text{Tyr}^{1133}\) and \(\text{Trp}^{1198}\) may be described as tripartite and as such, are well positioned to contribute to inter-helical contact and promote stable interactions between TM15 and TM16.

### 4.3.2 Predicted interactions of MRP1 \(\text{Arg}^{1202}\) mutants with nearby amino acids

Based on the homology model, it is speculated that replacing \(\text{Arg}^{1202}\) with Asp causes intra and/or inter-helical instability due to electrostatic charge repulsion of the negative Asp residue with the \(\pi\)-electrons of \(\text{Trp}^{1198}\) in TM16 and the hydroxyl group of the \(\text{Tyr}^{1133}\) side chain in TM15, which potentially could disrupt protein folding and/or assembly (Fig. 4.2A). The predicted distance between \([-\text{NH}_3^+]\) of \(\text{Arg}^{1202}\) side chain and \([-\text{OH}]\) group of \(\text{Tyr}^{1133}\) side chain is \(~4.54\,\text{Å}\). It decreases to \(~2.66\,\text{Å}\) between \([-\text{O}^-]\) of \(\text{Asp}^{1202}\) side chain and \([-\text{OH}]\) of \(\text{Tyr}^{1133}\) side chain. By substituting \(\text{Arg}^{1202}\) with a smaller, non-polar residue (Leu) (Fig. 4.2B left), any interaction with \(\text{Tyr}^{1133}\) might be weakened; similarly, substitution of \(\text{Arg}^{1202}\) with Gly (Fig. 4.2B right) could cause a complete loss of interactions with all neighbouring residues due to the absence of a side chain. However, neither substitution would be expected to cause electrostatic repulsions with neighbouring residues. The model also suggests that substitution of \(\text{Arg}^{1202}\) with the smaller but similarly charged Lys (Fig. 4.2C) would maintain the interactions with the neighbouring residues as predicted for the wild-type MRP1.

### 4.3.3 Design of second-site rescue mutations for \(\text{R1202D}\)

Due to the predicted interaction between the side chains of \(\text{Arg}^{1202}\) and \(\text{Tyr}^{1133}\), it seemed reasonable to test the idea that a second-site substitution at \(\text{Tyr}^{1133}\) might be able to restore expression of the R1202D protein by relieving the electrostatic repulsion caused by an opposite charge substitution as in R1202D. Consequently, \(\text{Tyr}^{1133}\) was replaced by Ala (a smaller residue
Figure 4.1 MRP1 homology models showing location of Arg$^{1202}$ and predicted interactions with surrounding residues in proximity. Surface view (A) and Zoom-in view (B) of MRP1 homology model showing location of Arg$^{1202}$ and its putative hydrogen bond interaction with TM15-Tyr$^{1133}$ and its cation-π interaction with TM16-Trp$^{1198}$. All three residues are shown as sticks and appear to be exposed on the surface to the lipid bilayer. The respective TMs of these residues are labeled. TM, transmembrane helix.
Figure 4.2 MRP1 homology models showing differences in predicted interactions of Arg\textsuperscript{1202} mutants with surrounding residues in proximity. Surface view (left) and Zoom-in view (right) of MRP1 homology model showing (A) opposite charge substitution, R1202D, causing electrostatic charge repulsion/clash with TM15-Tyr\textsuperscript{1133} and TM16-Trp\textsuperscript{1198}; (B) neutral charge substitutions R1202L (left) and R1202G (right) do not cause electrostatic charge repulsion/clash with Tyr\textsuperscript{1133} and Trp\textsuperscript{1198}; (C) same charge substitution R1202K. All the models including the mutations are energy minimized as described in Section 4.2.2. Residues are shown as ball (A) or sticks (B-C). TM, transmembrane helix.
Figure 4.3 Second-site substitutions at Tyr^{1133} are predicted to rescue protein expression of the R1202D mutant. Zoom-in views showing second-site substitutions at Tyr^{1133} that are proposed to relieve some of the electrostatic charge repulsion introduced by Asp^{1202}: (A) Wild-type; (B) R1202D; (C) R1202D/Y1133A; (D) R1202D/Y1133F; (E) R1202D/Y1133H. The respective TM domains of these residues are also indicated.
to create a spatial cavity for conformational adaptation introduced by R1202D (Fig. 4.3A), Phe (a side chain that preserves aromaticity but lacks the H-bonding hydroxyl group of Tyr$^{1133}$ (Fig. 4.3B)) and His (to neutralize the negative charge of Asp$^{1202}$ (Fig. 4.3C)).

4.3.4 MRP1 protein expression of rescue mutations alone and in cis with R1202D

To investigate if mutating Tyr$^{1133}$ to Ala, Phe or His could improve levels of the R1202D mutant protein, MRP1 expression vectors containing the mutations were generated by site-directed mutagenesis (Section 4.2.3) and transfected into HEK 293T cells (Section 4.2.4). Whole cell lysates were prepared, total protein determined and relative MRP1 levels determined by immunoblotting. Substitution of Tyr$^{1133}$ to Ala, Phe or His caused a decrease in MRP1 levels by >20% relative to wild-type levels which is comparable to those of R1202D (Fig. 4.4A). This indicates that these three substitutions of Tyr$^{1133}$ substantially affect the folding /assembly of the transporter. In addition, substitution of Tyr$^{1133}$ to Ala, Phe or His in cis with the R1202D mutation resulted in a decrease in MRP1 protein levels <10% of wild-type levels expression (Fig. 4.4B). Thus, substituting Ala, Phe, or His for Tyr$^{1133}$ does not reverse the detrimental effect of the R1202D mutation on MRP1 protein expression levels in HEK cells.

4.3.5 Predicting the intra- and inter-helical interacting amino acids of Glu$^{1204}$ using a MRP1 homology model

Glu$^{1204}$ also lies in TM16 and the homology model suggests that its side chain may interact with the side chain of Asn$^{1208}$ (between the functional carboxyl group of Glu$^{1204}$ and the amido group of Asn$^{1208}$) within TM16 and the side chain of Asn$^{1245}$ in TM17 (between the carboxyl group of Glu$^{1204}$ and the amido group of Asn$^{1245}$) through side chain hydrogen bonding (Fig. 4.5). Glu$^{1204}$ may also interact with Leu$^{379}$ in TM7 (between the carboxyl group of Glu$^{1204}$ and the side chain alpha carbon of Leu$^{379}$) through weak van der Waals interactions (Fig. 4.5).
Figure 4.4 Levels of MRP1 mutants associated with the structure-guided rescue of the R1202D mutant in HEK293T cells. Cells were transfected with wild-type or mutant pcDNA3.1(-) MRP1 expression vectors. Whole cell lysates were prepared after 48 h at 37 °C and MRP1 levels determined by immunoblotting (10 μg protein per lane) with MAb QCRL-1. (A) Single mutants of Tyr1133: Y1133A/F/H. (B) Y1133A/F/H mutants in cis of R1202D. Relative levels of MRP1 and α-tubulin were determined by densitometry and are indicated below the blots. MRP1 protein expression levels were corrected based on α-tubulin protein expression. Φ refers to whole cell lysates prepared from untransfected HEK293T cells. Similar relative levels of mutant MRP1 proteins were obtained in 3 additional independent experiments.
Figure 4.5 MRP1 homology models showing location of Glu$^{1204}$ and its predicted interactions with surrounding proximal residues. Zoom-in view (left) and schematic diagram (right) of MRP1 homology model showing potential interactions of Glu$^{1204}$ with TM17-Asn$^{1245}$ (hydrogen bonding), TM16-Asn$^{1208}$ (hydrogen bonding) and TM7-Leu$^{379}$ (van der Waals forces) with the the distance between atoms indicated in Å. All residues in the cartoon view are shown as sticks with TM16, TM17 and TM7 shown in green. TM, transmembrane helix.
4.3.6 Predicted interactions of MRP1 Glu$^{1204}$ mutants with near-by amino acids.

The model predicts that mutating Glu$^{1204}$ to the oppositely charged Lys or Arg (Fig. 4.6A) would cause significant conformational changes of the amino acid side chains both at the site of mutation as well as at neighbouring sites. The E1204K or E1204R substitutions could not only introduce a positive charge at this site, but they could also result in the loss of H-bond interactions with Asn$^{1208}$ and Asn$^{1245}$. Together these changes could affect the stability and/or prevent proper folding of MRP1.

In contrast to a Lys substitution, the model suggests that substitution of Glu$^{1204}$ with smaller but similarly charged Asp (Fig. 4.6B) might not cause any loss of interaction with residues in TM17 (Asn$^{1245}$) or TM7 (Leu$^{379}$) and thus suggests that this mutation is less likely to adversely affect protein folding or expression levels. This prediction is in accordance with the experimental observation that the mutant E1204D protein is expressed at levels ~80-100% that of wild-type MRP1 (130). On the other hand, the model suggests that substitution of Glu$^{1204}$ with a smaller, non-polar Leu (Fig. 4.6C) might lead to weakening of the inter-helical interactions with Asn$^{1245}$ and Asn$^{1208}$ due to the absence of a charged side chain which in turn, may affect protein conformation. This may explain, at least in part, why the transport function of the E1204L mutant was substantially reduced.

4.3.7 Design of potential second-site rescue mutations for E1204K

It was predicted that substitution of Val$^{1248}$ (TM17) (Fig. 4.7A) with Asp or Glu (Fig. 4.7) could potentially re-establish the inter-helical interaction predicted to be lost between TM16 and TM17 in E1204K (Fig. 4.7B) and thus may rescue expression of the E1204K mutant in HEK cells.
Figure 4.6 MRP1 homology models showing differences in predicted interactions of Glu\textsuperscript{1204} mutants with surrounding proximal residues. Zoom-in view (top) and schematic diagram (bottom) of MRP1 homology model showing (A) opposite charge substitutions E1204K/R lose H-bond interactions with TM17-Asn\textsuperscript{1245} and TM16-Asn\textsuperscript{1208} and orientation of the Lys side chain at position 1204 is now closer to Val\textsuperscript{1248} than Asn\textsuperscript{1245}; (B) same charge substitution E1204D and (C) neutral charge substitution E1204L do not affect interactions with TM17-Asn\textsuperscript{1245} and TM16-Asn\textsuperscript{1208}. TM16, TM17 and TM7 in the schematic views are shown in blue, red and green, respectively. Solid lines, hydrogen bonding; dotted lines, van der Waals forces of interaction. Distances between atoms are indicated in Å. All the models are energy minimized as described in Section 4.3.2.
Figure 4.7 Schematic diagrams of MRP1 homology model showing strategic mutations at TM17-Val\textsuperscript{1248} to negatively charged amino acids may potentially enhance interactions with TM16. (A) Side chain of Asp\textsuperscript{1248} potentially forms van der Waals interactions with Leu\textsuperscript{1203} in TM16. Side chain of Glu\textsuperscript{1248} potentially forms van der Waals interactions with Ala\textsuperscript{1200} and Leu\textsuperscript{1203} in TM16. (B) Side chain of Glu\textsuperscript{1248} potentially forms H-bond with Lys\textsuperscript{1204} and van der Waals interactions with Ala\textsuperscript{1200} and Leu\textsuperscript{1203} in TM16. Distances between atoms are indicated in Å.
4.3.8 MRP1 protein expression of rescue mutations alone and in cis with E1204K

To investigate if mutating TM17-Val\textsuperscript{1248} to Asp or Glu is an effective means to improve the levels of the Glu\textsuperscript{1204} mutant, MRP1 expression vectors containing the mutations singly and in combination were generated by site-directed mutagenesis (Section 4.2.3) and transfected into HEK 293T cells (Section 4.2.4). Whole cell lysates were prepared, total protein determined and relative MRP1 levels determined by immunoblotting. As shown in Fig. 4.8, single substitution of Val\textsuperscript{1248} to Asp or Glu resulted in decreased MRP1 protein expression (≤30% of wild-type levels) (i.e. comparable to E1204K mutant levels) in both cases. This indicates that these substitutions substantially and adversely affect folding/assembly of the transporter. The V1248D and V1248E substitutions were also made in cis with the E1204K mutation. As shown in Fig. 4.8, neither substitution rescued or improved the levels of E1204K, in contrast to the effect predicted by the model. Indeed, it appeared that MRP1 levels were even further decreased (to <10% of wild-type levels in both cases).

4.4 Discussion

In the present study, an atomic homology model representing a nucleotide bound form of the core 4-domain structure of MRP1 has been used to derive possible molecular explanations for the low levels of TM16 processing mutants R1202D and E1204K in HEK cells reported earlier [6] and confirmed here (Fig. 4.4 and 4.8). This model was also used to design second-site mutations predicted to improve or rescue levels of the R1202D and E1204K processing mutants. This approach has been used successfully for rescuing processing mutants of several other ABC proteins including CFTR and P-glycoprotein (229; 231; 289-292) and recently, MRP1 (150). In CFTR, second-site mutations (I539T, G550E, R553M/Q, and R555K) in NBD1 were discovered using a chimeric construct of CFTR (generated by substituting mouse-NBD1 (mNBD1)
Figure 4.8 Levels of MRP1 mutants associated with the structure-guided rescue of the E1204K mutant in HEK293T cells. Cells were transfected with wild-type or mutant pcDNA3.1(-) MRP1 expression vectors. Whole cell lysates were prepared after 48 h at 37 °C and MRP1 levels determined by immunoblotting (10 μg of protein) with MAb QCRL-1. Single mutants of Val\textsuperscript{1248}: V1248D/E and V1248D/E mutants in cis of E1204K. Relative levels of MRP1 and α-tubulin were determined by densitometry and are indicated below the blots. MRP1 protein expression levels were corrected based on α-tubulin levels. Similar relative levels of mutant MRP1 proteins were obtained in 2 additional independent experiments.
into human CFTR) and were found to compensate for the effects of the ΔF508 mutation (296; 297). However, the mechanism of rescue is not completely understood. Recently, however, it was shown that two of the suppressor mutations (R553K, R560T) in NBD1 restored inter-domain interfaces between cytoplasmic loop 4 (CL4) and NBD1 that are disrupted by the ΔF508 mutation in CFTR (230). In another study, second-site mutations (F270S and R1168M at the cytoplasmic face of TM2 and TM12, respectively) in the yeast oligomycin resistance protein, Yor1p, partially corrected the improper function associated with the Yor1p-ΔF670 mutant (analogous to CFTR-ΔF508) and reinstated its membrane trafficking with a different mechanism (298). Neither of these rescue mutations is located in NBD1 (where ΔF670 is) or at its predicted inter-domain interactions (CL1 and CL4), suggesting that they might promote the general properties of Yor1p folding instead of influencing the specific lesion at NBD1 and its inter-domain interactions (298). The authors also suggested that these rescue mutations, located at the cytoplasmic face of TM helices, might modulate Yor1p folding by influencing the general interactions of TM helices with the lipid bilayer of ER during biogenesis (298). Although these second-site rescue mutations of Yor1p were identified after screening ~300,000 independent transformants (298), the crystal structure of CFTR-NBD1 was helpful in understanding the structural changes caused by these rescue mutations. Recently, analysis of a homology model of MRP1 predicted a cluster of charged residues (Lys513, Lys516, Glu521, His1364, and Arg1367) at the interface of CL5 with NBD2 (150). Substituting these residues with Ala revealed that they play a critical role in the plasma membrane expression of human MRP1, highlighting the importance of maintaining the integrity of this inter-domain interface to the stability of MRP1 protein during its biosynthesis (150).
As mentioned earlier, the model predicted that there are no apparent perturbations introduced by neutral or same charge substitutions of Arg\textsuperscript{1202}, suggesting that a compensating interaction(s) may be created upon the loss of the electrostatic interaction between Arg\textsuperscript{1202} and Tyr\textsuperscript{1133} by changing the interfacial coordinates. Thus, the model helps provide an explanation for why these mutations do not cause misfolding and degradation of MRP1 and/or alter transport activity (130). However, mutating positively charged Arg\textsuperscript{1202} to negatively charged Asp\textsuperscript{1202} may cause electrostatic repulsion with Tyr\textsuperscript{1133} and Trp\textsuperscript{1198}. Of these three residues, the side chain of Arg or Asp is the most mobile to form bonds while the Trp side chain is the least mobile due to the bulkiness of its indole ring (the side chain of Trp\textsuperscript{1198} has a predicted probability of >90% facing in the orientation appearing in the homology model). Thus Tyr\textsuperscript{1133} appeared to be a better site than Trp\textsuperscript{1198} to introduce a mutation (Ala, Phe or His) alone and \textit{in cis} with R1202D to relieve some of the electrostatic repulsion introduced by the negatively charged Asp\textsuperscript{1202} (Fig. 4.3).

The observation that the singly substituted Y1133A/F/H mutants were all expressed at levels ≤20% of wild-type MRP1 (Fig. 4.3A) indicates that Tyr\textsuperscript{1133} is critical for the proper folding and assembly of MRP1. It also might suggest that not only can Tyr\textsuperscript{1133} form interactions with Arg\textsuperscript{1202}, but also with Trp\textsuperscript{1198}. Mutating Tyr to Ala, Phe or His removes the -OH group predicted to form a hydrogen bond with the amino group of the indole side chain of Trp\textsuperscript{1198} (H-O---H-N at ~2.4 Å or O-H---N-H at ~2.5 Å based on model prediction). An earlier study from our laboratory suggests that mutating Trp\textsuperscript{1198} to Ala, Tyr or Phe also decreases MRP1 protein expression (128), consistent with the idea that Trp\textsuperscript{1198} forms bonds with neighbouring residue(s) that are important for proper folding, assembly or membrane trafficking (Fig. 4.9).

When the second-site Tyr\textsuperscript{1133} mutations were introduced into R1202D, protein levels were not rescued (Fig. 4.3B), and indeed, they appeared lower than the single R1202D and
Y1133X mutant levels. This suggests that relieving the hypothesized electrostatic repulsion between Tyr\textsuperscript{1133} and Asp\textsuperscript{1202} with Ala/Phe/His substitutions of Tyr\textsuperscript{1133} was not sufficient to restore proper folding of the mutant protein. Thus, it might be necessary to take into account the proposed tripartite interactions among Arg\textsuperscript{1202}, Tyr\textsuperscript{1133} and Trp\textsuperscript{1198} when designing rescue mutations instead of focusing on interactions between only two residues, in this case Arg\textsuperscript{1202} and Tyr\textsuperscript{1133} (Fig. 4.9).

The proposed interaction between Trp\textsuperscript{1198} and Tyr\textsuperscript{1133} or Arg\textsuperscript{1202} has been discussed earlier. Not only might R1202D disrupt the \(\pi\)-cation interaction between Arg\textsuperscript{1202} and Trp\textsuperscript{1198} and thus affect the secondary structure of TM16, but it could also perturb Trp\textsuperscript{1198} - Tyr\textsuperscript{1133} inter-helical interactions indirectly. Another possibility is that electrostatic attraction rather than repulsion may occur between Asp\textsuperscript{1202} and Tyr\textsuperscript{1133} (the predicted O---H distance is 1.66 Å) which may potentially distort the helices and perturb Trp\textsuperscript{1198} - Tyr\textsuperscript{1133} inter-helical interactions. Preliminary studies on wild-type and R1202D synthetic TM16 peptides using circular dichroism (CD) suggested differences in their secondary structures. In addition, differences in the electrophoretic mobilities of wild-type TM16 and R1202D TM16 peptides by (PAGE) analysis were noted (Appendix I). Although very preliminary, these findings suggest that the R1202D substitution could distort the physicochemical properties of TM16. Thus, the assumption that mutating Tyr\textsuperscript{1133} to Ala, Phe or His may produce a compensatory effect to overcome possible structural distortions caused by R1202D may be wrong and in fact, it could introduce steric hindrance with Trp\textsuperscript{1198} causing further destabilization of the protein.
Figure 4.9 MRP1 homology models showing predicted interactions of MRP1 Trp\textsuperscript{1198} and its mutants with Tyr\textsuperscript{1133} and Arg\textsuperscript{1202}. Zoom-in view of MRP1 homology model showing (A) location of Trp\textsuperscript{1198} and its putative interacting partners Tyr\textsuperscript{1133} and Arg\textsuperscript{1202}; (B) disruption of the putative tripartite network in mutants W1198A; (C) W1198F and (D) W1198Y. The respective TM domains of these residues are labeled.
The model predicted that substituting Glu\textsuperscript{1204} with opposite charge residues Lys or Arg disrupts intra- and/or inter-helical interactions (Fig. 4.6A). It is reasonable to speculate that such differences in electrostatic interactions could also result in a large change in free energy of the E1204K or E1204R mutants, thus destabilizing the mutant proteins. However, even if the model predicted that the conformation of E1204L differs from wild-type MRP1, the difference would appear to be insufficient to disrupt the assembly or significantly destabilize the protein. Thus, predictions made based on the homology model of MRP1 are reasonably consistent with the experimental observations already made thus far (130).

The approach to rescuing E1204K was to replace Val\textsubscript{1248} in TM17 with negatively charged amino acids in an attempt to restore the predicted stabilizing bonding interactions between the side chains of amino acids in TM16 and TM17 that are lost in E1204K. The observation that the singly substituted V1248D and V1248E mutants were very poorly expressed in HEK cells indicates that Val\textsubscript{1248} is critical to the proper folding and assembly of MRP1 (Fig 4.8). However, MRP1 levels remained very low when these Val\textsubscript{1248} substitutions were introduced in cis into E1204K, suggesting that the predicted bonding interactions between TM16 /TM17 either do not exist in the native protein or were not restored for reasons not yet known. It may be that replacing a neutral Val with a charged Asp or Glu introduces non-native electrostatic repulsion or attraction with other polar or charged residue(s) in the vicinity which adversely affects folding or assembly of the protein. As illustrated in Fig. 4.10, Asp\textsubscript{1248} and Glu\textsubscript{1248} may form non-native interactions with the -OH group of Tyr\textsuperscript{1126} in TM15 which can potentially distort helices and may in this way disrupt critical intra- and/or inter-helical interactions.

In summary, the homology model correctly predicted that Tyr\textsuperscript{1133} and Val\textsuperscript{1248} were important for expression of MRP1 in HEK cells; however, the model did not correctly predict...
Figure 4.10 MRP1 homology models showing potential electrostatic charge repulsion between TM17-V1248D and TM15-Tyr^{1126}. Zoom-in view of MRP1 homology model showing (A) TM17-V1248D may cause electrostatic charge repulsion/clashes with TM15-Tyr^{1126}; (B) V1248D mutation in cis with E1204K may also cause electrostatic charge repulsion/clashes with Tyr^{1126}. The respective TM domains of these residues are labeled.
second-site mutations to rescue the TM16 misprocessing mutants R1202D and E1204K. On this note, it should be remembered that the homology of MRP1-TM15/16/17 with the corresponding TM helices of Sav1866 on which the model was built is extremely low. The sequence similarity of TM15/16/17 between MRP1 and Sav1866 is just 25%, 24% and 22%, respectively.

Orientations of the side chains and the rotation flexibility of the key elements on the side chains are crucial to form interactions between residues, and the side chains in the model are essentially just “best guesses”. Asp, Arg and especially Lys, have highly mobile side chains (299) which may pose greater challenges in accurately predicting their interacting partners based on models derived from a crystallized snapshot of the native protein or, in this case, a very distantly related bacterial protein.

At a moderate resolution (e.g. 2 Å) of an X-ray crystal structure, the α-carbon back bone of the protein and most atoms are visible which allows the construction of 3-D model with limited accuracy (300). However, individual elements are not distinguishable and ambiguities exist in the atomic structure especially the orientation of side chains which render major uncertainties in predicting interacting partners, bond length and strength (300). Further, differences in membrane environment and physiological functions (e.g. undefined function and substrate transport profile of Sav1866) between a human and a bacterial protein also limit the predictive power of a Sav1866-based MRP1 atomic homology model (154). Of note, the crystal structure of Sav1866 is in an ATP-bound state and it is very likely that its overall conformation would be very different from the nucleotide-free form of the protein.

Based on the work described in this thesis, the MRP1 homology model has served as a useful tool to provide intriguing hypotheses and explanations for the biochemical observations such as reduced protein expression of MRP1 containing the TM16-R1202D, -E1204K, TM16-
Trp$^{1198}$, TM15-Tyr$^{1133}$ and TM17-Val$^{1248}$ mutations. However, the level of accuracy of such model in predicting interactions among residues in the region under study is expected to be low and thus approached with caution. Recently, an X-ray crystal structure of mouse P-glycoprotein in a nucleotide free state (high affinity state) was resolved at 3.8Å (301). Although this resolution is still too low to resolve the side chain orientations, it would be interesting to examine/validate these interactions in a MRP1 homology model derived from a mammalian ABC transporter. Nonetheless, the region in TM16 where Arg$^{1202}$ and Glu$^{1204}$ residues locate and the nearby regions in TM15 and TM17 appear to be critical to stable expression of MRP1 protein in mammalian cell system.
Chapter 5 Future Directions

5.1 Investigating the degradation pathways evoked in response to misprocessed MRP1 mutants in mammalian cells.

Protein misfolding typically elicits two major stress responses in the ER (302; 303). First, an unfolded protein response (UPR), is induced (unless there is a lesion(s) in the UPR machinery) (304) which transiently decreases the flux of nascent peptides into the ER to alleviate ER stress and increases levels of expression of various components of the protein-folding machinery, such as chaperone proteins (e.g. Hsp70), enzymes (e.g. UDP-glucose:glycoprotein glycotransferase), foldases, kinases (e.g. PERK (double-stranded RNA-activated protein kinase (PKR)–like ER kinase)) and transcription factors like activating transcription factor 6 (302-304). In the second response, a misfolded protein is subjected to ER-associated degradation (ERAD) if it is considered as “terminally misfolded” (302; 303). The process of identifying a folding intermediate as terminally misfolded is unclear (305). It is likely that some residues are exposed in the non-native structures which are recognized by molecular chaperones and E3 ubiquitin ligase complexes (305).

Misfolded soluble proteins typically display hydrophobic patches and thus can be recognized by molecular chaperones such as Hsp70 and Hsp90 (302; 303). However, the mechanism of recognition of misfolded transmembrane segments in the lipid bilayer of ER, where folding and packing of TM helices occur as discussed in Chapter 1, Section 1.4.1, is less clear. It is thought that a series of inter- and intra-helical hydrogen bonds and salt bridges ensures the proper folding and arrangement of TM helices (118). Thus, it has been proposed that a misfolded membrane protein may expose hydrogen bonding polar residues to the hydrophobic environment of the membrane where they can be potentially recognized by ER membrane-
associated molecular chaperones (306). For example, it has been demonstrated that degradation of a disease-causing CFTR mutant (G85E in MSD1) is dependent on a ER-membrane associated chaperone Derlin-1 which interacts with the ER transmembrane ubiquitin ligases RING Finger Protein 5 (RNF5) and Hrd1 (171; 307). In addition to membrane-associated quality control factors, cytosolic chaperones can potentially mediate the quality control pathways by recognizing the cytoplasmic domain(s) of a misfolded membrane protein (307). Several studies have demonstrated that misfolded NBD1-ΔF508 CFTR is recognized and targeted to ERAD by the cytosolic chaperone Hsc70 which recruits ubiquitin ligase C terminus of Hsc70-interacting protein (CHIP) (308; 309). Separate studies also showed that membrane-associated chaperones (e.g. DNAJB12) and ligase (RMA1) can independently recognize and ubiquitinate CFTR-ΔF508 (310; 311). Several other ER membrane-associated quality control factors have been proposed for their roles in recognizing misfolded transmembrane domains, such as translocating chain-associated membrane which is an essential component of the Sec61 translocon (312) and B-cell receptor-associated protein 29/31 (BCR-29/31) which are transmembrane proteins that localize in the ER (313; 314).

It would be very interesting to study which factors play a role in the recognition and degradation of the MRP1 mutants and determine whether they are the same or different for R1202D and E1204K. This will not only further our understanding on the biosynthesis of MRP1 protein and the importance of TM16 but may also extend to the regulation of biosynthesis of polytopic membrane proteins in general.
5.2 Investigating the structural changes caused by opposite charge mutations in TM16 using peptide mimetics.

As mentioned previously, strongly polar residues are relatively rare in TM helices of polytopic membrane proteins but are often highly conserved, suggesting molecular interactions that either functionally or structurally favour these residues (235). Due to the large desolvation penalty for burying polar groups, the extent to which these polar residues maintain structural integrity crucial to the membrane protein stability remains poorly understood (122; 315). It has been argued that such interactions contribute mainly to protein function (e.g. substrate binding/specificity) than to stability (316). In fact, the structure-function dependence often complicates the interpretation of structural contributions of polar interactions and the roles of individual polar residues can only be inferred from the activity of the whole protein (235). Furthermore, the extent to which these polar residues drive the arrangement/packing of TM helices and/or stabilize intra and interhelical interactions is unclear and likely varies among different protein classes.

Because the Arg$^{1202}$ to Asp and Glu$^{1204}$ to Lys mutations strongly affect MRP1 protein levels in mammalian cells which cannot be easily restored, it seems that they are important for MRP1 stability; even when rescued in more permissive insect cells, the mutant proteins are functionally impaired and/or misfolded. Structural studies on membrane proteins are challenging due to their high hydrophobicity and tendency to form aggregates (317). Also, it is often difficult to detect structural changes arising from the change of one amino acid in a full-length protein. To minimize these problems, peptides comprised of single TM segments or hairpin TM segments (two adjacent TM helices connected by a short (<15 amino acid) linker) in detergent micelles (e.g. SDS, L-α-lysophosphatidylcholine, and perfluorooctanoic acid) have been used as study tools (318; 319).
The use of helix-loop-helix TM segments is often based on the assumption that consecutive TM helices are always in contact with each other (likely scenario in TMs connected with shorter loops) which is often not known (320). Therefore, although not ideal, synthetic peptides corresponding to TM helices can be used as an imperfect surrogate to study changes in structure of membrane proteins at the micro-environmental level since such peptides would more readily lend themselves to biophysical analyses including CD, differential scanning calorimetry and nuclear magnetic resonance. Comparative PAGE analyses under native and reducing conditions can also be used to reveal potential differences in migration patterns between wild-type and mutant peptides. This approach has been used extensively by the Deber group in their studies of CFTR. They have used a hairpin peptide corresponding to the TM3/4 segment of CFTR with and without CF-phenotypic point mutation (V232D) and by gel migration patterns and CD spectra revealed non-native bonding between the adjacent TM helices (321). Their data suggest the V232D mutation in TM4 of CFTR results in the formation of a hydrogen bond with Gln\textsuperscript{207} in TM3 which explains the difference in mobility observed for these two TM hairpins under various electrophoretic gel conditions. TM hairpins that do not contain a hydrogen bond (wild-type or double mutants) exhibited in a slower electrophoretic mobility in SDS inferring a more elongated conformation in a slower electrophoretic mobility (321-325). It was postulated that such a putative hydrogen bond could alter the normal assembly and alignment of CFTR TM helices and/or impede their movement in response to ion flow (321-326).

The interactions of individual peptides corresponding to a modified (and short) TM16 (A\textsubscript{1195}NRWLAVRLECVGNCIVLFAALFAV\textsubscript{1219}) or TM17 (A\textsubscript{1227}GLVGLSVSYSQTYYLNWLVRMS\textsubscript{1251}) of MRP1 in detergent micelle as a membrane mimetic to determine the relative position of these TM helices in the membrane have been
examined by de Foresta and colleagues (327). By utilizing the intrinsic fluorescent properties of Trp residues in TM16 (Trp$^{1198}$) and TM17 (Trp$^{1246}$), they observed an increase in fluorescence intensity of both TM peptides indicating that the polarity of the solvent surrounding the Trp-containing TM16 or TM17 peptides decreases due to the binding of peptides to the detergent micelle (327). The authors concluded that these results suggest amphipathic properties and an interfacial location of TM16 and TM17. Unsurprisingly, this is consistent with the mutagenesis studies supporting a role for TM16 and TM17 in lining the hydrophilic substrate translocation pathway of MRP1 (327). Far UV CD spectra showed that the isolated TM16 peptide (26 aa) did not behave as a spontaneous stable α-helical segment in membrane mimics (327). It is likely that the secondary structures of TM16 and TM17 within native MRP1 are different from those of the short isolated TM peptides in this study. Furthermore, since the TM16 and TM17 helices extend well into the cytoplasm, it is plausible that important bonding interactions between these helices could continue well into the cytoplasm. Mutagenesis studies as well as homology model-based predictions suggest a high probability of interactions between TM16 and TM17, and they likely form a hairpin structure at the surface of the micelles (327). Unpublished work by A. Hernandez in the Cole lab has shown that mg quantities of an extended MRP1 hairpin TM16/17 fused to a His-thioredoxin or maltose binding protein tag can be obtained in E. coli -hairpin TM16/17 fusion protein and the cleaved TM16/17 moiety could both be labelled with the photoactive substrate LTC$_4$. Ongoing studies are aimed at a biophysical characterization of the hairpin TM16/17 fusion protein in a membrane mimetic environment. Specifically, it will be interesting to assess whether the TM16 non-expressing mutants (R1202D and E1204K) affect secondary structure, size, shape or gel migration patterns of these hairpin TM16/17 segments. The MRP1 hairpin TM16/17 polypeptide should prove to be a valuable tool to study mutant-mediated changes in secondary or
tertiary interhelical folding interactions and may provide structural insight into the whole MRP1 protein.
References


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Appendix I  Synthesis and Preliminary Characterization of Synthetic Peptides

Corresponding to Wild-type TM16 and R1202D and E1204K

This section contains detailed materials and methods relating to characterization of synthetic peptides.

Materials and methods

Materials

Crude synthetic peptides (<60% purity) corresponding to wild-type TM16 (defined as the 25 amino acids spanning Val$^{1194}$ to Ala$^{1218}$) as well as R1202D and E1204K mutant TM16 were purchased from Chi Scientific (Maynard, MA). Three Lys residues were added to the NH$_{2}$- and COOH-termini of the peptides to improve water solubility and facilitate peptide purification [346]. Additionally, Lys-tagged TM peptides are better able to insert into membrane mimetic environments such as SDS micelles, where they may adopt helical secondary structures with native-like tertiary contacts. The sequences of the peptides are as follows (Lys tags in boldface, mutated amino acid underlined): wild-type MRP1: H-KKK-V$^{1194}$ANRWLAVRLECVGNCIVLFAALFA$^{1218}$-KKK-OH (molecular weight 3518), R1202D mutant: H-KKK-V$^{1194}$ANRWLAVDLECVGNCIVLFAALFA$^{1218}$-KKK-OH (molecular weight 3477), and E1204K mutant: H-KKK-V$^{1194}$ANRWLAVRLKCVGNCIVLFAALFA$^{1218}$-KKK-OH (molecular weight 3517).

Peptides were purified by reverse phase HPLC using a C18 semi-preparative column (Peeke Scientific, Redwood City, CA) and acetonitrile (Sigma) as eluant. Fractions containing the correct product (>98% purity) were identified by MALDI-TOF mass spectrometry using a Voyager DE Pro (Applied Biosystems, Foster City, CA). Solvent was removed by lyophilization, and the purified peptides were stored at -20 °C.
Analysis of TM16 peptides by circular dichroism spectroscopy

CD was performed on the purified Lys-tagged TM16 peptides using a JASCO J-815 CD spectrophotometer (Harvard University, Cambridge, MA) at 25 °C. Peptides were dissolved at a final concentration of 20 µM in buffer containing 20 mM Tris pH 8.0 and 34 mM SDS. The pathlength used for far-UV CD analysis was 0.1 cm. An average of 10 scans was accumulated for each CD plot. Plots were baseline corrected for buffer. For all peptides, observed ellipticity [θ] was normalized for protein concentration using the equation [θ] = θ x100/ncl, where n is the number of amino acids in the protein, c is the molar protein concentration and l is the path length of the cell (cm). Percentage secondary structure was calculated using the program CDNN [347].

Analysis of TM16 peptide electrophoretic mobility by PAGE

Peptides in NuPAGE Sample Buffer with 10X NuPAGE Reducing Agent (Invitrogen) were subjected to SDS-PAGE using pre-cast NuPAGE 4-10% Bis-Tris gel (Invitrogen) according to the manufacturer’s specifications. For SDS-PAGE under non-reducing conditions, the NuPAGE reducing agent was excluded. Gels were stained with Coomassie blue according to standard protocols.
Appendix I. Biophysical characterization of MRP1 TM16 peptides. A, Far-UV CD spectra for purified peptides corresponding to TM16 of wild-type MRP1, mutants R1202D, and E1204K; buffer (control). CD plots correspond to an average of at least 10 accumulated scans. B, the MRP1 TM16 purified peptides were subjected to SDS-PAGE under non-reducing (no DTT) and reducing (+DTT) conditions as indicated. The molecular weight markers and pattern of migration of each of the peptides were revealed by staining with Coomassie blue.
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