

ID: DMD/2007/015479 - REVISED

**MUTATIONAL ANALYSIS OF A HIGHLY CONSERVED PROLINE RESIDUE IN
MRP1, MRP2 AND MRP3 REVEALS A PARTIALLY CONSERVED FUNCTION**

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Running title: Comparative analysis of a conserved Pro residue in MRP1, MRP2 and MRP3

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Number of text pages: 31

Number of tables: 0

Number of figures: 6

Number of references: 40

Number of words in the *Abstract*: 230

Number of words in the *Introduction*: 792

Number of words in the *Discussion*: 1620

ABBREVIATIONS: MRP, multidrug resistance protein; ABC, ATP-binding cassette, MSD, membrane spanning domain; NBD, nucleotide binding domain; LTC₄, leukotriene C₄; E₂17βG, 17β-estradiol 17β-D-glucuronide; E₁3SO₄, estrone sulfate; CL, cytoplasmic loop; MTX, methotrexate; HEK, human embryonic kidney; TM, transmembrane; TSB, Tris-sucrose buffer; GSH, glutathione; MAb, monoclonal antibody.

ABSTRACT:

The ATP-binding cassette multidrug resistance protein 1 MRP1 (ABCC1) mediates the cellular efflux of organic anions including conjugated metabolites, chemotherapeutic agents and toxicants. We previously described a mutation in cytoplasmic loop 7 (CL7) of MRP1, Pro1150Ala, which reduced leukotriene C₄ (LTC₄) transport but increased estradiol glucuronide (E₂17βG) and methotrexate (MTX) transport. Vanadate-induced trapping of [α ³²P]8N₃ADP by the Pro1150Ala mutant in the absence of substrate was also greatly reduced compared to wild-type MRP1 suggesting an uncoupling of ATP hydrolysis and transport activity. To determine if the functional importance of MRP1-Pro¹¹⁵⁰ is conserved, the analogous Pro¹¹⁵⁸ and Pro¹¹⁴⁷ residues in the MRP2 and MRP3 transporters, respectively, were mutated to Ala. Expression levels of the three mutants were unaffected; however, the vesicular transport activity of at least one organic anion substrate was significantly altered. As observed for MRP1-Pro1150Ala, LTC₄ transport by MRP2-Pro1158Ala was decreased. However, E₂17βG and MTX transport was comparable to wild-type MRP2, rather than increased as observed for MRP1-Pro1150Ala. In the case of MRP3-Pro1147Ala, LTC₄ transport was increased, while E₂17βG transport was unaffected. MTX transport by MRP3-Pro1147Ala was also increased, but to a lesser extent than for MRP1-Pro1150Ala. In contrast, all three mutants showed a marked reduction in levels of vanadate-induced trapped [α ³²P]8N₃ADP. We conclude that MRP1-Pro¹¹⁵⁰, MRP2-Pro¹¹⁵⁸ and MRP3-Pro¹¹⁴⁷ in CL7 differ in their influence on substrate specificity but share a common role in the nucleotide interactions of these transporters.

The multidrug resistance protein 1 (MRP1), MRP2 and MRP3 are members of the ATP-binding cassette (ABC) superfamily of transmembrane proteins, subfamily C (Leslie et al., 2005; Deeley et al., 2006). These proteins mediate transport of their substrates across the plasma membrane using ATP binding and hydrolysis as an energy source. All three transporters are composed of three membrane spanning domains (MSDs) containing 5, 6 and 6 transmembrane (TM) α -helices, respectively, and two functionally non-equivalent nucleotide binding domains (NBDs). We initially identified MRP1 as a transporter capable of conferring tumor cell resistance to chemotherapeutic agents including doxorubicin, etoposide and vincristine. Subsequently, it was determined that glutathione (GSH) is required for MRP1 to transport at least some of these unconjugated drugs (Loe et al., 1996b; Leslie et al., 2005).

The related transporter, MRP2, was first characterized as a canalicular multispecific organic anion transporter (cMOAT) responsible for biliary excretion of conjugated organic anions (Paulusma et al., 1996). A third homolog, MRP3, was identified through screening of a database of human expressed sequence tags (dEST), based on its homology with MRP1 (Kool et al., 1997). Although MRP1, MRP2 and MRP3 share 48-58% sequence identity, they each have their own distinct, yet overlapping, physiological function, tissue distribution and substrate specificity (Deeley et al., 2006).

MRP1 is expressed ubiquitously throughout the body, except in the liver where it is usually not detectable, and is mainly found in the basolateral membranes of polarized epithelial cells (Leslie et al., 2005). Studies of *Mrp1*($-/-$) mice showed that this transporter mediates the release of leukotriene C₄ (LTC₄) during inflammatory responses, and protects several normal tissues from cytotoxic agents (Wijnholds et al., 1997; Wijnholds et al., 1998). *In vitro*, MRP1 transports a wide variety of endogenous and exogenous compounds, many of which are conjugated to

glucuronide, GSH or sulfate (Leslie et al., 2005). For example, in addition to LTC₄, the conjugated steroids estradiol 17 β -glucuronide (E₂17 β G) and estrone sulfate (E₁3SO₄) have been identified as MRP1 substrates (Leier et al., 1994; Loe et al., 1996a; Qian et al., 2001).

As alluded to above, MRP2 and MRP1 transport a similar spectrum of substrates but they differ in their affinity for many of these. In addition, MRP2 is expressed in a more limited number of tissues than MRP1, being found primarily in liver, intestine, lung and kidney. Furthermore, MRP2 is found in the apical membrane of polarized cells in these tissues in contrast to the basolateral localization of MRP1 (Leslie et al., 2005).

Although MRP1 and MRP3 have the highest sequence similarity among the ABCC transporters, the two proteins differ markedly with respect to their affinities for several common substrates. Most strikingly, MRP3 has a very low affinity and capacity to transport GSH conjugates and, unlike MRP1 and MRP2, does not efflux GSH nor require this tripeptide for drug transport (Zeng et al., 2000; Zelcer et al., 2001; Oleschuk et al., 2003). Like MRP1, however, MRP3 is expressed on the basolateral membrane of polarized cells but in a more limited number of tissues (Leslie et al., 2005).

We previously described a functionally complex MRP1 mutant in which a conserved Pro residue located at the beginning of a cytoplasmic loop (CL7) that connects TM15 to TM16, was replaced with Ala (Koike et al., 2004). Compared to wild-type MRP1, MRP1-Pro1150Ala displayed decreased levels of LTC₄, E₁3SO₄ and GSH transport, but substantially increased levels of E₂17 β G and methotrexate (MTX) transport. Accompanying the altered transport profile of this mutant, substantial changes in the interaction of MRP1 with nucleotide were observed. Although no difference in ATP binding was detected, the ability of MRP1-Pro1150Ala to trap 8N₃ADP under hydrolytic conditions in the presence of sodium orthovanadate (but in the absence

of substrate) was severely diminished. Since the ability to detect vanadate-inducible trapped [α - 32 P]8N₃ADP complexes is often considered an indicator of the ATPase activity of a protein (Urbatsch et al., 1995), our observations suggested that either ATP hydrolysis by the MRP1-Pro1150Ala mutant was reduced or that the release of ADP may be enhanced (Koike et al., 2004).

It was also observed that while the apparent K_m (ATP) for both wild-type MRP1 and MRP1-Pro1150Ala was the same during LTC₄ transport, the K_m (ATP) for the mutant transporter was reduced more than 4-fold during E₂17 β G transport. This change in ATP dependence suggests that E₂17 β G, but not LTC₄, interacts with the MRP1-Pro1150Ala mutant in a way that increases its apparent affinity for ATP. The complexity of the MRP1-Pro1150Ala mutant phenotype, together with the high conservation of this Pro residue among ABCC family members (Fig. 1), has prompted us to investigate whether the corresponding residues in the MRP1 homologs, MRP2 and MRP3, are also functionally important. We have therefore determined the consequences of Ala substitution of MRP2-Pro¹¹⁵⁸ and MRP3-Pro¹¹⁴⁷ on the transport properties and nucleotide interactions of these two transporters.

Materials and methods

Materials. [14,15,19,20-³H]LTC₄ (194.6 Ci mmol⁻¹) and [6,7-³H]E₂17 β G (53 Ci mmol⁻¹) were purchased from Perkin Elmer Life Sciences (Woodbridge, ON, Canada). [3',5',7'-³H (n)]MTX sodium salt (49.6 Ci mmol⁻¹) was from Moravek Inc. (Brea, CA). [α - 32 P]8N₃ATP (12.0 Ci mmol⁻¹) and [γ - 32 P]8N₃ATP (10.6 Ci mmol⁻¹) were purchased from Affinity Labeling Technologies, Inc. (Lexington, KY). LTC₄ was purchased from CalBiochem (San Diego, CA). AMP, ATP and E₂17 β G were purchased from Sigma Chemical Co. (St. Louis, MO). Creatine

kinase and creatine phosphate were obtained from Roche Diagnostic (Laval, QC, Canada). MTX sodium salt was purchased from Faulding (Vaudreuil, QC, Canada). Monoclonal antibodies M₂I-4 and M₃II-9 specific for MRP2 and MRP3, respectively, were purchased from Alexis (San Diego, CA).

Site-directed mutagenesis. Generation of the MRP1-Pro1150Ala mutant has been described previously (Koike et al., 2004). Mutations in MRP2 and MRP3 were similarly generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The template for generating the MRP2-Pro1158Ala mutant was created by subcloning a 2.2 kb ApaI/ClaI fragment into pGEM-7Zf(+) (Promega, Madison, WI). The mutagenesis template for the MRP3-Pro1147Ala was a pBluescript II KS(+) plasmid (Stratagene) containing the full length MRP3 cDNA as described previously (Oleschuk et al., 2003). Mutagenic oligonucleotide primers were obtained from IDT Inc. (Coralville, IA). Mutagenesis was performed according to the manufacturer's instructions with the following sense primers (substituted nucleotides are underlined, new or disrupted restriction sites are italicized, and the restriction enzyme used is indicated in parentheses): MRP2-Pro1158Ala (5'-C ACC AGG TCC GCG ATC TAC TCT C-3') (BstUI), MRP3-Pro1147Ala (5'-GTC AGC CGC TCC GCG ATC TAC TCC C-3') (BstUI). The MRP2-Pro1158Ala mutation was subcloned back into pcDNA3.1(-) MRP2 as a 1.75 kb Bsu36I/SfiI fragment and the MRP3-Pro1147Ala mutation was subcloned into pcDNA3.1(+) MRP3 as a 1.24 kb AgeI/SacII fragment (Oleschuk et al., 2003). The fidelity of the fragments was then verified by sequencing (ACGT Corp., Toronto, ON, Canada).

Transfection of MRP1, MRP2 and MRP3 expression vectors in HEKSV293T cells.

Mutant and wild-type pcDNA3.1(-)MRP1_k, pcDNA3.1(-)MRP2 and pcDNA3.1(+)MRP3 DNA expression vectors were transfected into SV40-transformed human embryonic kidney (HEK293T) cells. Cells were maintained in DMEM medium supplemented with 4 mM L-

glutamine and 7.5% fetal bovine serum. Approximately 18×10^6 cells were seeded per 150 mm plate and 24 h later, cells (50-75% confluency) were transfected with 20 μ g DNA using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 48 h at 37°C, the HEK293T cells were collected and stored as cell pellets at -70°C until needed.

Membrane vesicle preparation. Transfected cell pellets were thawed, disrupted by argon cavitation at 300 psi, and membrane vesicles were prepared as described previously (Loe et al., 1996b; Létourneau et al., 2005b). Membrane vesicles were aliquoted and stored at -70°C until required and used within 3 months of preparation. Protein concentrations were determined using a Bradford assay (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin as a standard.

Determination of MRP1, MRP2 and MRP3 protein levels in transfected cells. Levels of MRP1, MRP2 and MRP3 proteins in the membrane vesicles were determined by immunoblot analysis. Briefly, proteins were resolved on a 7% SDS-polyacrylamide gel and electrotransferred to a PVDF membrane (Pall Corporation, Pensacola, FL). Immunoblots were blocked with 4% (w/v) skimmed milk powder in TBS containing Tween 20 (TBS-T) for 1 h followed by incubation with the human MRP1-specific murine MAb QCRL-1 (1:10,000), the MRP2-specific murine MAb M₂I-4 (1:10,000) or the MRP3-specific murine MAb M₃II-9 (1:7,500) (Hipfner et al., 1994; Ito et al., 2001a; Oleschuk et al., 2003; Létourneau et al., 2005a) diluted in the blocking solution. After washing, immunoblots were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce, Edmonton, AB, Canada) followed by application of Western Lightning™ chemiluminescence blotting substrate (Perkin-Elmer Life Science, Woodbridge, ON, Canada) and exposed to film (Ultident, St. Laurent, QC, Canada). The films were analyzed by densitometry using Image J software (<http://rsb.info.nih.gov/ij/>).

MRP-mediated transport of ^3H -labeled organic anions by membrane vesicles. ATP-dependent uptake of ^3H -labeled organic anion substrates by the MRP1-, MRP2- and MRP3-enriched membrane vesicles was measured using a rapid filtration technique in a 96-well format as described previously (Tabas and Dantzig, 2002; Létourneau et al., 2005b). All reactions were carried out in a final reaction volume of 30 μl with 250 mM sucrose, 50 mM Tris-HCl (pH 7.5) buffer (TSB) containing AMP or ATP (2 mM), MgCl_2 (10 mM), creatine phosphate (10 mM) and creatine kinase (100 $\mu\text{g ml}^{-1}$) under conditions shown previously to measure transport in the linear range of vesicular uptake (Létourneau et al., 2005a). Uptake was stopped by rapid dilution in ice-cold TSB, and the reaction mixtures filtered using a FilterMateTM Harvester and Unifilter-96 GF/B filter plate apparatus (Packard, Meriden, CT). Radioactivity on the filters was quantified by liquid scintillation counting. All data were corrected for the amount of ^3H -labeled substrate that remained bound to the filter, which was usually <10% of the total radioactivity. Transport in the presence of AMP was subtracted from transport in the presence of ATP to determine ATP-dependent uptake. Values for the negative controls (membrane vesicles of untransfected cells) were subtracted from uptake measured for wild-type and mutant MRPs. All transport assays were carried out in triplicate and results expressed as means (\pm SD).

For MRP1 activity, LTC_4 uptake was measured by incubating [^3H] LTC_4 (50 nM, 10 nCi) with 2 μg vesicle protein for 60 sec at 23 $^\circ\text{C}$. $\text{E}_217\beta\text{G}$ uptake was measured by incubating [^3H] $\text{E}_217\beta\text{G}$ (400 nM, 20 nCi) with 2 μg vesicle protein for 60 sec at 37 $^\circ\text{C}$. MTX uptake was measured by incubating [^3H]MTX (100 μM , 125 nCi) with 5 μg vesicle protein for 20 min at 37 $^\circ\text{C}$ (Létourneau et al., 2005a). For MRP2 activity, LTC_4 uptake was measured by incubating [^3H] LTC_4 (50 nM, 20 nCi) with 6 μg vesicle protein for 3 min at 23 $^\circ\text{C}$. $\text{E}_217\beta\text{G}$ uptake was measured by incubating [^3H] $\text{E}_217\beta\text{G}$ (400 nM, 100 nCi) with 6 μg vesicle protein for 5 min at 37

°C. MTX uptake was measured by incubating [³H]MTX (1 μM, 125 nCi) with 6 μg vesicle protein for 10 min at 37 °C (Ito et al., 2001a; Létourneau et al., 2005a). For MRP3 activity, LTC₄ uptake was measured by incubating [³H]LTC₄ (500 nM, 50 nCi) with 5 μg protein at 37 °C for 5 min. E₂17βG uptake was measured by incubating [³H]E₂17βG (400 nM, 70 nCi) with 5 μg vesicle protein for 5 min at 37 °C. MTX uptake was measured by incubating [³H]MTX (1 μM, 175 nCi) with 8 μg vesicle protein for 10 min at 37 °C (Oleschuk et al., 2003; Létourneau et al., 2005a).

[³H]LTC₄ Photolabeling of MRP1 and MRP2. Membrane proteins were photolabeled with [³H]LTC₄ as described previously (Loe et al., 1996b; Koike et al., 2004). Briefly, in a final volume of 50 μl, membrane vesicles (50 μg protein) were incubated with [³H]LTC₄ (for MRP1, 0.2 μM (0.08 μCi); for MRP2, 2 μM (0.8 μCi)) and 10 mM MgCl₂ for 30 min at room temperature and then frozen in liquid nitrogen. Samples were then alternately irradiated at 302 nm for 1 min using a CL-1000 UV cross-linker (DiaMed, Mississauga, ON, Canada) and snap-frozen in liquid nitrogen 10 times. Radiolabeled proteins were resolved by SDS-PAGE, and proteins were fixed in a solution of water:isopropanol:acetic acid (65:25:10) for 30 min and washed in Amplify NAMP100 (Amersham, Baie d'Urfé, QC, Canada) for 30 min. After drying, the gel was exposed to Bioflex MSI film (InterScience, Markham, ON, Canada) for 5 days at -70 °C. The films were analyzed by densitometry using Image J software (<http://rsb.info.nih.gov/ij/>) as before. [³H]LTC₄ photolabeling of MRP3 was not measured because the affinity of this transporter for this substrate is too low (Zeng et al., 2000; Zelcer et al., 2001; Oleschuk et al., 2003).

Photolabeling of MRP1, MRP2 and MRP3 by [³²P]8N₃ATP. Membrane vesicle proteins were photolabeled with [³²P]8N₃ATP as described previously (Leslie et al., 2001; Koike et al.,

2004). Briefly, membrane vesicles (10 μg) were incubated at 4 $^{\circ}\text{C}$ (non-hydrolysis conditions) with 5 mM MgCl_2 and 5 μM [$\gamma^{32}\text{P}$]8N₃ATP (1 μCi) in a final volume of 20 μl . Membrane vesicles prepared from untransfected HEK293T cells were included as a negative control. After 5 min incubation on ice, samples were cross-linked at 302 nm in a 96-well plate for 8 min, washed twice with ice-cold Tris/EGTA/ MgCl_2 buffer (50 mM Tris-HCl pH 7.4, 0.1 mM EGTA, 5 mM MgCl_2), and then solubilized in Laemmli buffer and subjected to SDS-PAGE. After drying, the gel was exposed to film for 2-12 h.

Orthovanadate-induced trapping of [$\alpha^{32}\text{P}$]8N₃ADP by MRP1, MRP2 and MRP3.

Membrane vesicles proteins (10 μg) were incubated for 15 min at 37 $^{\circ}\text{C}$ (hydrolysis conditions) in 20 μl TSB containing 5 mM MgCl_2 , 5 μM [$\alpha^{32}\text{P}$]8N₃ATP (1 μCi) and 1 mM freshly prepared sodium orthovanadate (Koike et al., 2004). For labeling MRP3, 10 μg vesicle protein were also incubated with 5 mM CoCl_2 , 1 mM sodium orthovanadate and 25 μM [$\alpha^{32}\text{P}$]8N₃ATP (5 μCi). Membrane vesicles prepared from untransfected HEK293T cells were included as negative controls. The reactions were stopped by the addition of ice-cold Tris/EGTA/ MgCl_2 buffer, washed twice and then resuspended membrane proteins (15 μl) were transferred to a 96-well plate and exposed to UV light at 302 nm on ice for 8 min. Membrane vesicles were then solubilized in Laemmli buffer and subjected to SDS-PAGE as before. After drying, the gel was exposed to film for 12–24 h.

Results

Expression levels of MRP1, MRP2 and MRP3 Pro mutants. We showed previously that the MRP1-Pro1150Ala mutant was expressed in transfected HEK293T cells at levels comparable to wild-type MRP1 (Koike et al., 2004). To determine if this was also true for the corresponding

MRP2-Pro1158Ala and MRP3-Pro1147Ala mutants, these mutations were created in pcDNA3.1 (+/-) based MRP2 and MRP3 expression vectors, and then transfected into HEK293T cells. Membrane vesicles were prepared from the transfected cells and the expression levels of each mutant were determined by immunoblotting. As shown in Fig. 2, all three Pro to Ala mutants (MRP1-Pro1150Ala, MRP2-Pro1158Ala and MRP3-Pro1147Ala) were expressed at levels comparable to their corresponding wild-type proteins, confirming that the Pro residue at this position is not required for expression of the MRP proteins in the plasma membrane of mammalian cells.

The Pro mutations differentially affect the transport activities of MRP1, MRP2 and MRP3. The transport activities of the mutant proteins were assessed by performing vesicular uptake assays with [³H]LTC₄, [³H]E₂17βG and [³H]MTX as substrates. As noted previously, the affinities of the three transporters for these substrates differ. For example, MRP1 has the highest uptake affinity for LTC₄ ($K_m \sim 0.1 \mu\text{M}$), followed by MRP2 ($K_m \sim 1 \mu\text{M}$) and then MRP3 ($K_m \sim 5 \mu\text{M}$) (Leier et al., 1994; Loe et al., 1996b; Cui et al., 1999; Zeng et al., 2000). Consequently, the specific assay conditions for each transporter were modified accordingly (Loe et al., 1996b; Ito et al., 2001b; Oleschuk et al., 2003; Létourneau et al., 2005a). Furthermore, to take into account the background transport activity of HEK293T cells, uptake in vesicles from untransfected cells was subtracted from the uptake in vesicles from transfected cells.

As we reported previously, LTC₄ transport by MRP1-Pro1150Ala was decreased by approximately 50% (Fig. 3A) (Koike et al., 2004). For MRP2-Pro1158Ala, LTC₄ transport was also decreased by ~ 50% while it was significantly increased (1.8-fold) for MRP3-Pro1147Ala (Fig. 3A). However, analysis of the latter data was complicated by the relatively high level of [³H]LTC₄ transport observed in the vesicles made from untransfected cells when using MRP3

assay conditions where the initial LTC₄ concentration is 10-fold higher than for the MRP1 and MRP2 assays (data not shown). Thus, because of its much higher affinity for LTC₄, the low level of endogenous MRP1 present in the HEK293T cells was suspected to be responsible for this non-MRP3-mediated transport. For this reason, the LTC₄ uptake assays for MRP3-Pro1147Ala were repeated in the presence of MAb QCRL-3 which specifically inhibits MRP1 transport activity (Loe et al., 1996b). Under these conditions, a 1.6-fold increase in LTC₄ uptake by the MRP3-Pro1147Ala mutant was still observed compared to wild-type MRP3, but background LTC₄ uptake by the vesicles from untransfected cells was decreased by about 90%. These observations confirm that, as suspected, the LTC₄ transport activity in vesicles from untransfected cells was mainly due to endogenous MRP1. They also confirm that LTC₄ transport by the MRP3-Pro1147Ala mutant is increased relative to wild-type MRP3.

In contrast to LTC₄ uptake, E₂17βG transport by MRP2 and MRP3 were unaffected by the Pro1158Ala and Pro1147Ala mutations, respectively (Fig. 3B). On the other hand, E₂17βG transport by MRP1-Pro1150Ala was significantly increased (2-fold) relative to wild-type MRP1 as expected (Fig. 3B) (Koike et al., 2004).

Also as expected for MRP1-Pro1150Ala, MTX transport was significantly increased by approximately ~ 3-fold (Fig. 3C) (Koike et al., 2004). In contrast, MTX transport by the MRP2-Pro1158Ala mutant remained the same as wild-type MRP2 (Fig. 3C), while MTX transport by the MRP3-Pro1147Ala mutant was increased relative to wild-type MRP3 but to a lesser extent than observed for MRP1-Pro1150Ala (1.5-fold vs 3-fold) (Fig. 3C).

[³H]LTC₄ photolabeling of MRP1 and MRP2. To determine if the reduced LTC₄ transport by MRP2-Pro1158Ala was associated with reduced substrate binding, [³H]LTC₄ photolabeling of this mutant was compared with wild-type MRP2. As noted earlier, the amount of [³H]LTC₄ used

for photolabeling of MRP2 was increased to account for the ~10-fold difference between MRP1 and MRP2 in apparent K_m for this substrate. As shown previously, [^3H]LTC₄ photolabeling of MRP1 was not affected by the Pro1150Ala mutation (Fig. 4A) (Koike et al., 2004). Similarly, MRP2-Pro1158Ala was photolabeled by [^3H]LTC₄ to the same extent as wild-type MRP2 despite the reduced LTC₄ transport activity of the mutant (Fig. 4B). [^3H]LTC₄ photolabeling of MRP3-Pro1147Ala was not measured because the affinity of this transporter for this substrate is too low (Zeng et al., 2000; Zelcer et al., 2001; Oleschuk et al., 2003).

Mutations MRP1-Pro1150Ala, MRP2-Pro1158Ala and MRP3-Pro1147Ala do not affect [$\gamma^{32}\text{P}$]8N₃ATP binding but decrease vanadate-induced trapping of [$\alpha^{32}\text{P}$]8N₃ADP. We previously showed that photolabeling of MRP1-Pro1150Ala by [$\alpha^{32}\text{P}$]8N₃ATP was comparable to wild-type MRP1 (Koike et al., 2004). When MRP2-Pro1158Ala and MRP3-Pro1147Ala were photolabeled with [$\gamma^{32}\text{P}$]8N₃ATP, the intensity of the signals observed with the mutant and wild-type proteins were also comparable (Fig. 5), suggesting that ATP can bind to the Pro mutants as well as it does to their wild-type counterparts.

As mentioned earlier, sodium orthovanadate has been used in experimental studies of several nucleotide binding proteins to trap protein bound ADP in a relatively stable complex nucleotide (Urbatsch et al., 1995). As reported previously, when MRP1-Pro1150Ala was exposed to vanadate (1 mM) and [$\alpha^{32}\text{P}$]8N₃ATP under hydrolytic conditions (37 °C), the trapping of [$\alpha^{32}\text{P}$]8N₃ADP was very low compared to wild-type MRP1 (Fig. 6A) (Koike et al., 2004). When the MRP2-Pro1158Ala and MRP3-Pro1147Ala mutants were examined under the same conditions, a significant decrease (50%) in the level of vanadate-induced 8N₃ADP trapping by MRP2-Pro1158Ala was also observed (Fig. 6B), while no trapping of nucleotide was detected with either wild-type MRP3 or its Pro1147Ala mutant (data not shown).

Since sodium orthovanadate could not support trapping of [α - 32 P]8N₃ADP by MRP3 in the presence of Mg²⁺, other divalent cations (Mn²⁺ and Co²⁺) were tested based on previous studies of several other ABC transporters (Cai et al., 2002; Ozvegy et al., 2002; Sauna et al., 2004). The highest level of vanadate-induced [α - 32 P]8N₃ADP trapping by MRP3 was achieved with Co²⁺, although the signal was relatively weak. Nevertheless, in the presence of Co²⁺, a significant decrease in vanadate-induced 8N₃ADP trapping by the MRP3-Pro1147Ala mutant relative to wild-type MRP3 was observed (results not shown). A band of greater intensity was observed when the concentration of [α - 32 P]8N₃ATP was increased from 5 μ M to 25 μ M, and under these conditions, a 60% decrease in vanadate-induced trapping of [α - 32 P]8N₃ADP by MRP3-Pro1147Ala relative to wild-type MRP3 was detected (Fig. 6C). Thus 8N₃ADP trapping by the MRP3 mutant was decreased as observed with the MRP1-Pro1150Ala and MRP2-Pro1158Ala mutants, although under different conditions.

Discussion

Pro residues are often important determinants of membrane protein structure and may be involved in conformational changes that occur during ligand binding, transmembrane voltage variation or, in the case of an ABC transporter, during the transport cycle (Sansom and Weinstein, 2000; Deber and Therien, 2002). These changes are presumably related to the distinctive chemical characteristics of Pro in which the NH₂-group is unavailable to take part in any H-bonding networks. In addition, the Pro ring occupies some of the space that would be filled by the side chains of neighboring amino acids, and may thus induce conformation constraints on proteins (Richardson and Richardson, 1989). The complex phenotype of the MRP1-Pro1150Ala mutant and the fact that Pro¹¹⁵⁰ is highly conserved in the ABCC subfamily,

suggested to us that this residue might serve an important functional role in other members of this class of ABC transporters (Koike et al., 2004). Ala, like Pro, is an aliphatic amino acid, but unlike Pro, its NH₂ group is available for H-bonding and its small side chain is unlikely to induce any conformational restraints. For this reason, the consequences of replacing the Pro residues in MRP2 and MRP3 corresponding to MRP1-Pro¹¹⁵⁰ with Ala on the transport and nucleotide binding properties of the mutant proteins were examined.

As described earlier, the MRP1-Pro1150Ala mutant showed substrate selective changes in its transport activity (Koike et al., 2004). Thus the increased E₂17βG transport activity of this mutant was accompanied by a 4-fold reduction in $K_m(\text{E}_217\beta\text{G})$ and apparent $K_m(\text{ATP})$. Together, these changes could conceivably explain the increased E₂17βG transport observed for this mutant. In contrast, the reduced LTC₄ transport by MRP1-Pro1150Ala was associated with a decreased V_{\max} for LTC₄ transport, while no change in $K_m(\text{ATP})$ was observed (Koike et al., 2004). The different effects of LTC₄ and E₂17βG on the $K_m(\text{ATP})$ of the mutant MRP1 indicate that the interaction of MRP1 with ATP can be selectively influenced by the substrate being transported. This substrate-selective effect on nucleotide interactions may not be exclusive to MRP1 and related transporters since the ATPase activity of P-glycoprotein (ABCB1) is also reported to be stimulated by some, but not all of its transported substrates (Sharom, 1997; Loo and Clarke, 2005). However, although the ATPase activity of P-glycoprotein is increased by vinblastine and verapamil, these drugs do not affect its $K_m(\text{ATP})$ (Ambudkar et al., 1992; Sarkadi et al., 1992). Thus, the precise relationship between substrate-stimulated ATPase activity and substrate-induced changes in $K_m(\text{ATP})$ for MRP1, P-glycoprotein and other ABC transporters is not clear.

Like MRP1-Pro1150Ala, the transport activity of the MRP2 and MRP3 Pro mutants were altered in a substrate-selective manner, demonstrating the conserved functional importance of a Pro residue at the NH₂-proximal end of CL7. However, there was substantial variability with respect to the extent to which the transport of individual substrates was affected (Fig. 3). The present study is not the first to report differences in the functional consequences of mutating a conserved amino acid in MRP1, MRP2 and MRP3. Thus, we previously showed that mutations of a conserved Trp residue in TM17 of MRP1, MRP2 and MRP3 dramatically altered the transport activity of all three transporters but, like the Pro mutants, each Trp mutant exhibited a distinct pattern of changes. Thus, mutation of MRP1-Trp¹²⁴⁶ eliminated E₂17βG and MTX transport without affecting LTC₄ transport (Ito et al., 2001b), while in addition to eliminating E₂17βG and MTX transport, mutation of MRP2-Trp¹²⁵⁴ also significantly decreased LTC₄ transport (Ito et al., 2001a). MRP3-Trp¹²⁴² mutants displayed decreased MTX and LTC₄ transport, but in contrast to the MRP1-Trp¹²⁴⁶ and MRP2-Trp¹²⁵⁴ mutants, E₂17βG transport was increased at least 2.5-fold (Oleschuk et al., 2003). Primary sequence comparisons and helical wheel projections make it clear that the molecular environments of the conserved Trp residues differ significantly in each of the three transporters. Thus, it seems likely that these differences are responsible for the marked differences in the substrate specificities of the mutants. In contrast, the immediate environments of MRP1-Pro¹¹⁵⁰, MRP2-Pro¹¹⁵⁸, and MRP3-Pro¹¹⁴⁷ are predicted to be much more similar (Fig. 1). This may, at least in part, explain why the phenotypes of the three Pro mutants although distinct, are more similar to one another than the phenotypes of the three Trp mutants.

In addition to the substrate-selective changes in transport activity caused by the Pro mutations in MRP1, MRP2 and MRP3, the interaction of the three transporters with nucleotide was also

altered. Thus, although $8N_3$ ATP binding to MRP1-Pro1150Ala, MRP2-Pro1158Ala, and MRP3-Pro1147Ala remained unchanged, the level of vanadate-induced $8N_3$ ADP trapping was very low for all three mutant transporters. This suggests that the influence of these Pro residues on the catalytic activity of the three homologs is conserved, and that mutating the conserved Pro residue somehow uncouples, at least in part, the vanadate-induced ADP trapping properties of the transporters from their substrate transport. The evidence that these two activities can be uncoupled supports the idea that ATP hydrolysis is responsible for resetting the transporter in a conformation that allows a new cycle of substrate binding and transport to begin, rather than being required for the transport process itself (Higgins and Linton, 2004; Dawson and Locher, 2006).

Reduced levels of vanadate-induced trapping of $8N_3$ ADP have been observed in other TM15/CL7 mutants of MRP1. Thus, MRP1 mutants of Arg¹¹³⁸ and Arg¹¹⁴² exhibited reduced vanadate-induced trapping of $8N_3$ ADP and, as shown for the Pro mutants in the present study, this was not associated with an overall reduction in transport activity, since transport of some substrates was comparable to wild-type activity (Conseil et al., 2006). However, the Pro¹¹⁵⁰, Arg¹¹³⁸ and Arg¹¹⁴² MRP1 mutants differ from a double CL7 mutant described recently by Ren et al. (2006) in which Glu¹¹⁵⁷ and Gly¹¹⁶¹ were mutated to Leu and Pro, respectively. Thus, the Glu1157Leu/Gly1161Pro mutant displayed both decreased vanadate-induced $8N_3$ ADP trapping as well as decreased $8N_3$ ATP photolabeling at both NBDs (Ren et al., 2006). On the other hand, LTC₄ transport by this double mutant, like the Pro mutants in this study and the other CL7 mutants mentioned above, was markedly reduced. Unfortunately, the Glu1157Leu/Gly1161Pro mutant was not tested with other substrates, so it is not known whether the decreased transport activity of this mutant was substrate selective. Nevertheless, despite some differences, these studies together with the present study, all indicate that residues in CL7 can influence the activity

of the NBDs of MRP1. CL7 mutations have also been shown to affect the function of other ABCC proteins, including MRP2 (ABCC2), CFTR (ABCC7), MRP6 (ABCC6) and SUR1 (ABCC8), which are responsible for the genetic disorders known as Dubin-Johnson syndrome, cystic fibrosis, Pseudoxanthoma elasticum and persistent hyperinsulinemia hypoglycemia of infancy, respectively (Conseil et al., 2005). Thus the integrity of CL7 is important not only for the activity of the drug-transporting MRP proteins but also for the physiological functions of other ABCC proteins. These observations support the idea that this region may have a common role as a signaling conduit between the MSDs where substrate recognition and transport occurs and the NBDs where the energy for the transport process is generated.

Based on their crystal structure of BtuCD, a bacterial ABC importer of vitamin B12, Locher et al. (2002) have suggested that a cytoplasmic loop ('L loop') in the BtuC subunit is critical for transmitting signals from the MSDs to the NBDs (BtuD subunit) upon substrate binding. Amino acid sequence alignments of the 'L loop' in BtuC suggest that it might correspond to CL4 and CL6 of human MRP1. However, sequence similarities also exist between the L loop of BtuC and the 'EAA loop' in CL4 of CFTR, which is also present in CL5 and CL7 in MRP1 (Locher et al., 2002; Ren et al., 2006). Thus mutagenesis studies to date suggest that the proposed signaling role of the BtuC 'L loop' could be fulfilled by CL7 in MRP1 and the analogous region in related ABCC proteins. The precise role of CL7 is not yet clear, but it is reasonable to suggest that it might interact with NBD2 since many of the MRP1 CL7 mutations affect vanadate-induced [$\alpha^{32}\text{P}$]8N₃ADP trapping which occurs primarily at NBD2 in MRP1 (Gao et al., 2000).

Finally, it could be presumed, based on the high degree of sequence similarity between MRP1/MRP2 and MRP3, that their interactions with nucleotide would be the same. Our observation that Mg²⁺ does not support trapping of 8N₃ADP by MRP3 was therefore unexpected,

particularly in view of the fact that vesicular transport and $8N_3ATP$ photolabeling could be readily measured in the presence of this divalent cation. Thus it seems that the differences between MRP1/MRP2 and MRP3 may lie in their interactions with the vanadate anion since Mg^{2+} -dependent transport and $8N_3ATP$ photolabeling were observed for all three proteins and only the extent to which the proteins can trap $8N_3ADP$ in the presence of vanadate was different. Several earlier reports have shown that other ABCC proteins (e.g. MRP4 (ABCC4), and rat Mrp6 (Abcc6)), as well as the more distantly related half transporter BCRP (ABCG2), require divalent cations other than Mg^{2+} to detect $8N_3ADP$ trapping (Cai et al., 2002; Ozvegy et al., 2002; Sauna et al., 2004). As observed for ABCG2 and MRP4, we found that Co^{2+} was more effective than Mg^{2+} and Mn^{2+} at supporting trapping of $8N_3ADP$ by MRP3. These differences in cation dependence notwithstanding, these and other data² favor the conclusion that the conserved Pro residue in CL7 of all three transporters has a significant influence on the activity of NBD2.

In conclusion, our findings indicate that the role of MRP1-Pro1150, MRP2-Pro1158 and MRP3-Pro1147 is only partially conserved among the three MRP-related transporters. Thus, MRP1-Pro¹¹⁵⁰, MRP2-Pro¹¹⁵⁸ and MRP3-Pro¹¹⁴⁷ differ in their influence on the substrate specificity of these homologous transporters but share a common influence on nucleotide interactions at NBD2 that appears uncoupled from substrate transport.

Acknowledgment. We thank Drs. Gwenaëlle Conseil and Alice Rothnie for helpful discussions and advice, and Kathy Sparks and Sara Ikeda for technical assistance.

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Footnotes

Supported by a grant from the Canadian Institutes of Health Research (CIHR) (MOP-10519).

I.J.L. is the recipient of a CIHR Doctoral Award and A.J.S. is the recipient of a Queen's University Robert John Wilson Fellowship. S.P.C.C. is the recipient of a Canada Research Chair in Cancer Biology and is the Queen's University Bracken Chair in Genetics and Molecular Medicine.

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² Létourneau IJ, Nakajima A, Deeley RG and Cole SPC, manuscript in preparation

Figure Legends

FIG. 1. Predicted topology and partial sequence alignments of MRP-related proteins. The Walker A, Walker B and C-signature motifs are labeled A, B and C, respectively, in the two NBDs. Shown below the topology scheme is a sequence alignment of CL7 of human MRP1, MRP2 and MRP3 and several other members of the human ABCC subfamily. Swiss-Prot accession numbers: MRP1 (ABCC1), P33527; MRP2 (ABCC2), Q92887; MRP3 (ABCC3), O15438; MRP4 (ABCC4), O15439; MRP5 (ABCC5), O15440; MRP6 (ABCC6), O95255; CFTR (ABCC7), P13569; SUR1 (ABCC8), Q09428; SUR2 (ABCC9), O60706. **MRP1-Pro¹¹⁵⁰**, **MRP2-Pro¹¹⁵⁸** and **MRP3-Pro¹¹⁴⁷** are in boldface and underlined.

FIG. 2. Protein expression levels of mutants MRP1-Pro1150Ala, MRP2-Pro1158Ala and MRP3-Pro1147Ala and their corresponding wild-type proteins. Immunoblots shown are of membrane vesicles (0.5 and 1 µg protein) prepared from HEK293T cells transfected with wild-type or mutant expression vectors. MAbs QCRL-1, M₂I-4 and M₃II-9 were used to detect MRP1, MRP2 and MRP3, respectively. Relative levels of the MRP proteins were determined by densitometry and are indicated in italicized numbers below the blots. HEK refers to control membrane vesicles prepared from untransfected HEK293T cells. Similar results were obtained in 2-6 additional independent experiments.

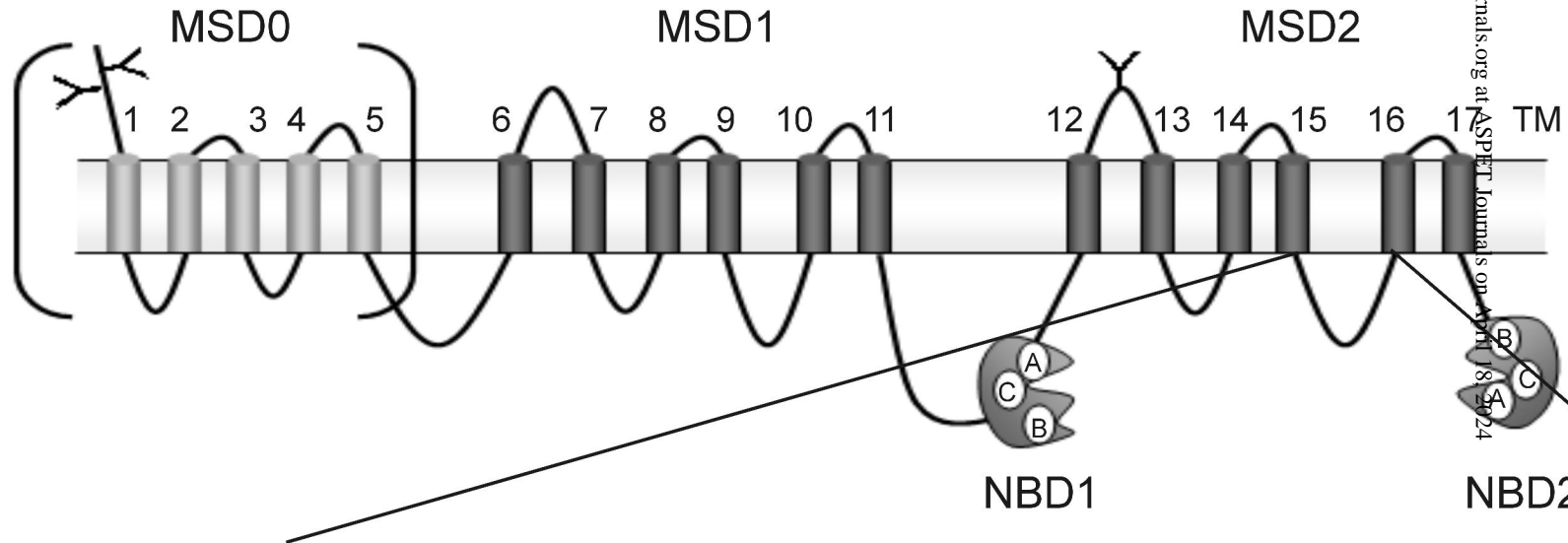
FIG. 3. Vesicular transport of ³H-labeled organic anions by mutants MRP1-Pro1150Ala, MRP2-Pro1158Ala and MRP3-Pro1147Ala. The transport activities of the Pro mutants are expressed as percent of the activity of the corresponding wild-type protein. (A) [³H]LTC₄ uptake, (B) [³H]E₂17βG uptake and (C) [³H]MTX uptake. Organic anion uptake by membrane vesicles

prepared from untransfected cells was subtracted from the uptake obtained in both the wild-type and mutant membrane vesicles. Transport conditions were as described in the “Material and Methods”. Bars represent the means (\pm SD) of triplicate determinations in a single experiment. Transport activities were corrected to take into account according to relative protein expression levels as shown in the immunoblots in Fig. 2. Similar results were obtained in at least one additional independent experiment.

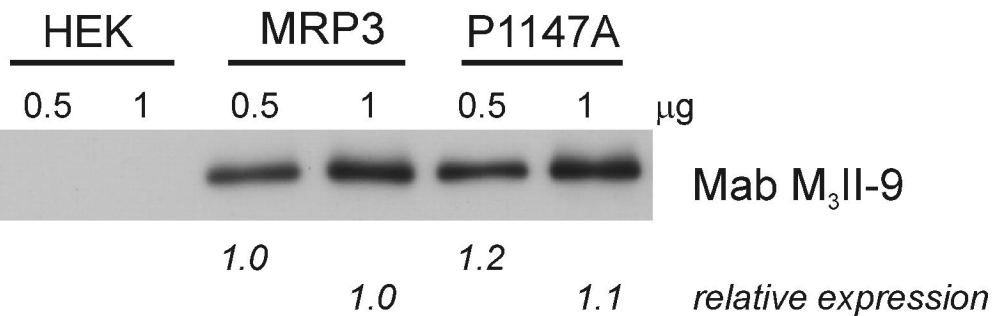
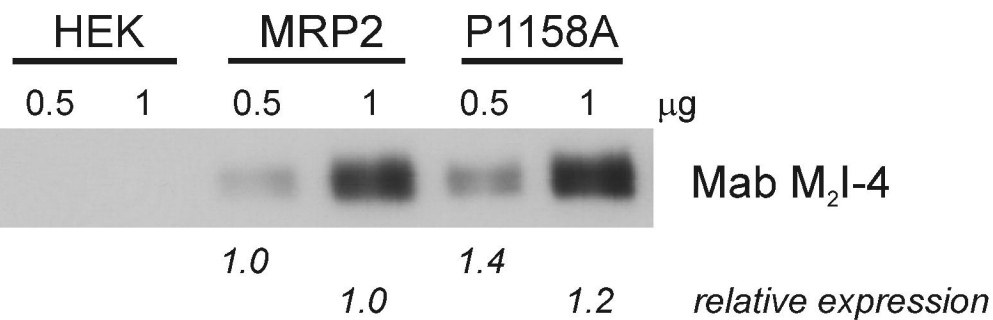
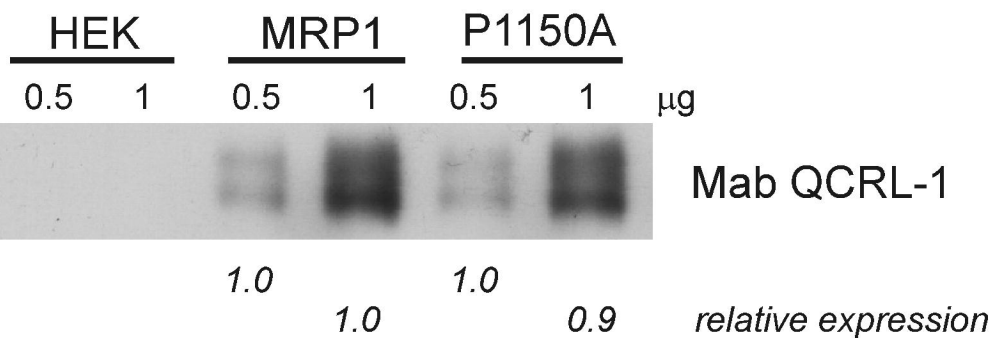
FIG. 4. [^3H]LTC₄ photolabeling of wild-type and Pro mutants of MRP1 and MRP2. Membrane vesicles (50 μg protein) were incubated with [^3H]LTC₄ (A) MRP1 (200 nM/0.08 μCi), (B) MRP2 (2 μM /0.8 μCi), irradiated at 302 nm and after resolving proteins by SDS-PAGE, gels were processed for fluorography and densitometry. Relative levels of [^3H]LTC₄ photolabeling are indicated in italics and have been corrected for any differences in protein expression levels relative to the corresponding wild-type protein. HEK refers to control membrane vesicles prepared from untransfected HEK293T cells.

FIG. 5: [$\gamma^{32}\text{P}$]8N₃ATP photolabeling of wild-type and Pro mutants of MRP1, MRP2 and MRP3. Membrane vesicle proteins (10 μg) were incubated with 5 mM MgCl₂ and 5 μM [$\gamma^{32}\text{P}$]8N₃ATP (1 μCi) and after 5 min on ice were irradiated at 302 nm at 4 °C and then resolved by SDS-PAGE. Gels were dried and exposed to film, and analyzed by densitometry. Relative levels of [$\gamma^{32}\text{P}$]8N₃ATP photolabeling are indicated in italics and have been corrected for any differences in protein expression levels relative to the corresponding wild-type protein. HEK refers to control membrane vesicles prepared from untransfected HEK293T cells.

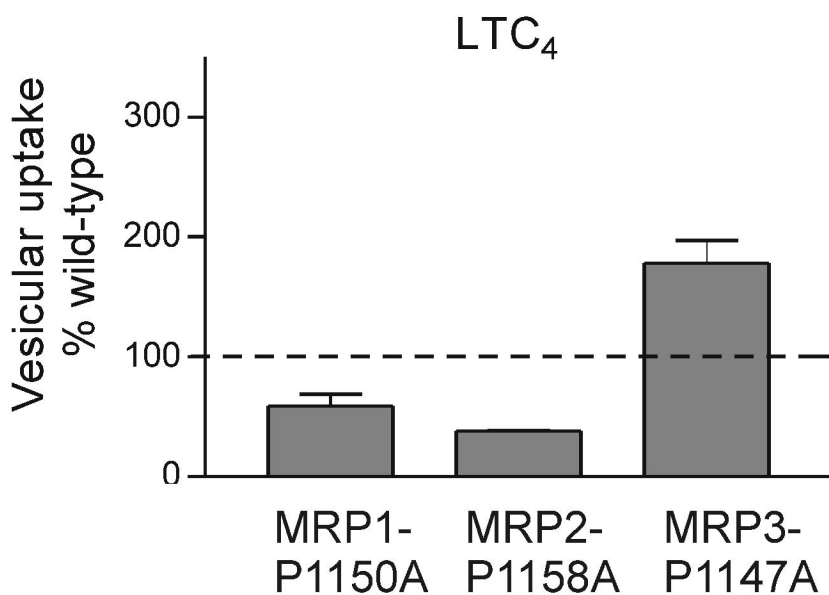
FIG. 6. Vanadate-induced trapping of [$\alpha^{32}\text{P}$]8N₃ADP by wild-type and Pro mutants of MRP1, MRP2 and MRP3. For MRP1 (A) and MRP2 (B), membrane vesicles (10 μg protein) were added to 5 mM MgCl₂, 5 μM [$\alpha^{32}\text{P}$]8N₃ATP (1 μCi) and 1 mM sodium orthovanadate. For MRP3 (C), membrane vesicles (10 μg protein) were added to 5 mM Co²⁺, 1 mM sodium orthovanadate and 25 μM [$\alpha^{32}\text{P}$]8N₃ATP (5 μCi). All reaction mixes were incubated at 37 °C for 15 min, and after washing, membrane proteins were irradiated on ice and then resolved by SDS-PAGE. After drying, the gel was exposed to film for 12 h and the film was analyzed by densitometry. The relative levels of vanadate-induced [$\alpha^{32}\text{P}$]8N₃ADP trapping are indicated in italics and have been corrected for any differences in protein expression levels relative to the corresponding wild-type protein. HEK refers to membrane vesicles prepared from untransfected HEK293T cells that were included as a negative control.



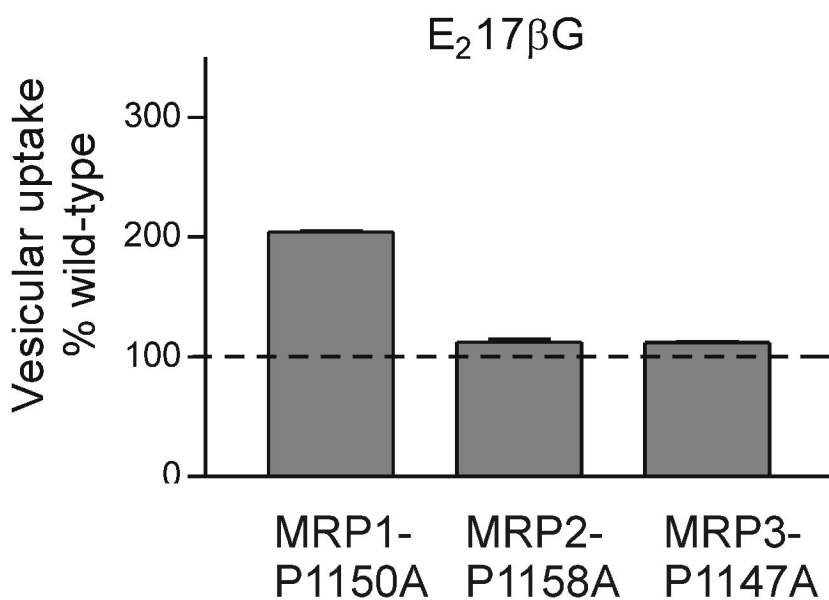
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MRP2	1149-	RRLDSVTRSPPIYSHFSETVSGLPVIRAFEHQQRFLKHNEVRIDTNQKCVFSWITS
MRP3	1138-	KRLESVSRSPPIYSHFSETVTGASVIRAYNRSRDFEIIISDTKVDANQRSCYPYIIS
MRP4	890-	KRLESTTRS P VFSLSSSLQGLWTIRAYKAEERCQELFDAHQDLHSEAWFLFLT
MRP5	1040-	KRLDNITQS P FLSHITSSIQGLATI HAYNKGQEF LHRYQELLDDNQAPFFLFTCA
MRP6	1113-	RRLESASYSSVCSHMAETFQGSTVVRAFRTQAPFVAQNNARVDESQRISFPRLVA
CFTR	1041-	KQLESEGRSPPIFTHLVTSKGLWTLRAFGRQPYFETLFHKALNLHTANWFLYLST
SUR1	1188-	QQLDDTTQL P LLSHFAETVEGLTTIRAFRYEARFQOKLLEYTDSNNIASLFLTAA
SUR2	1161-	QELDDSTQL P LLCHFSETAEGLT TIRAFRHETRFKQRMLELTD TNNIAYLFLSAA



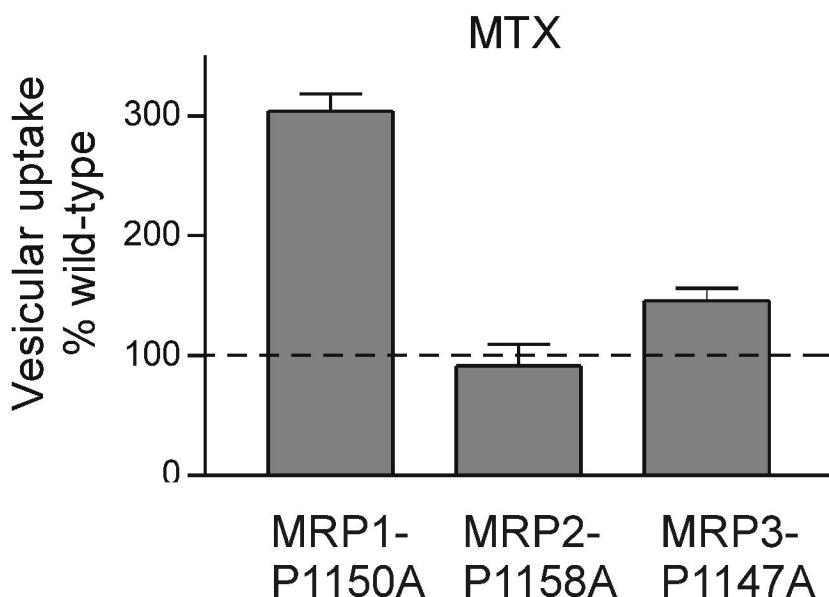
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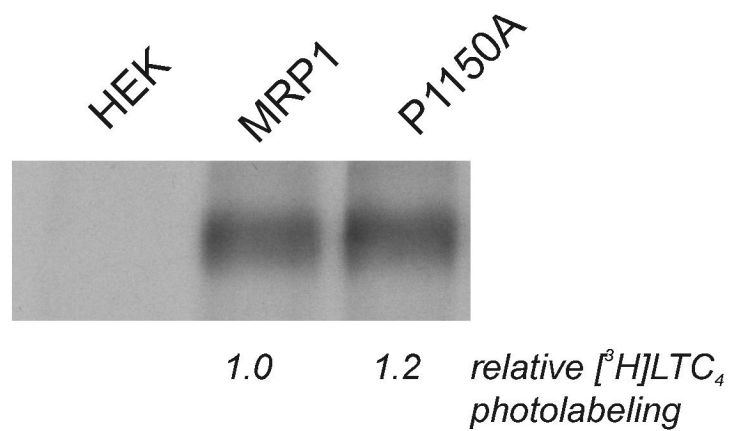
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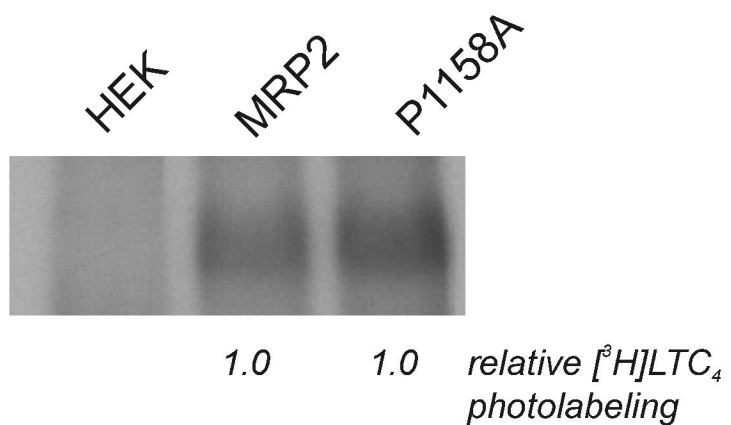
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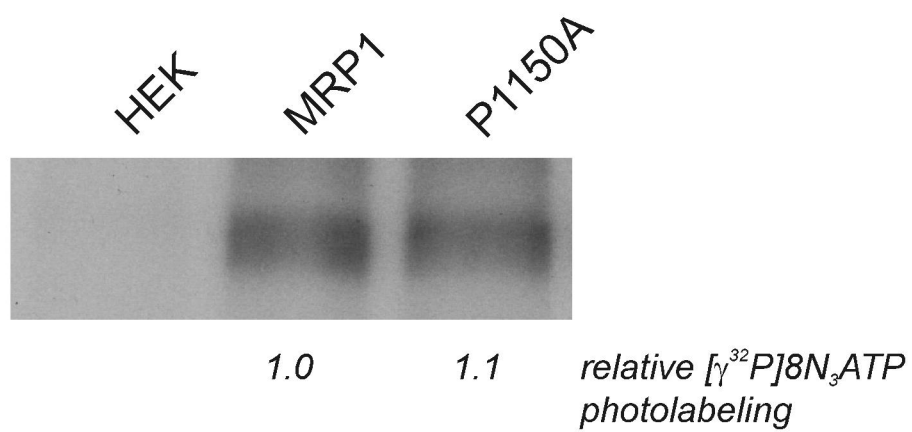
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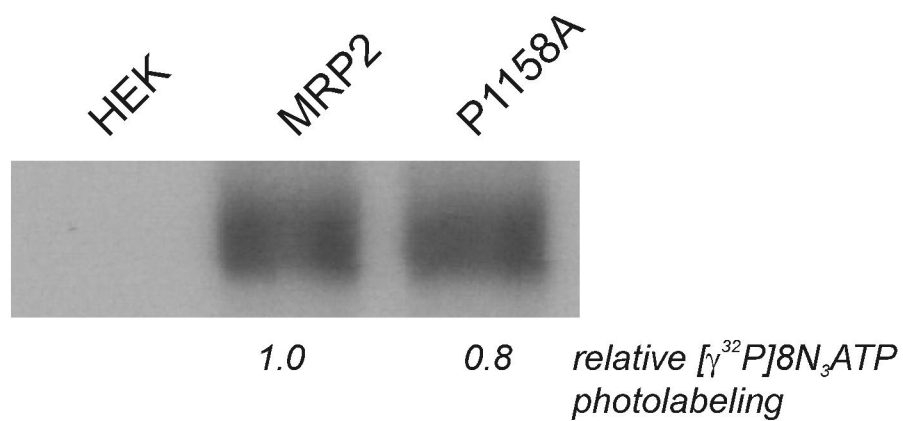
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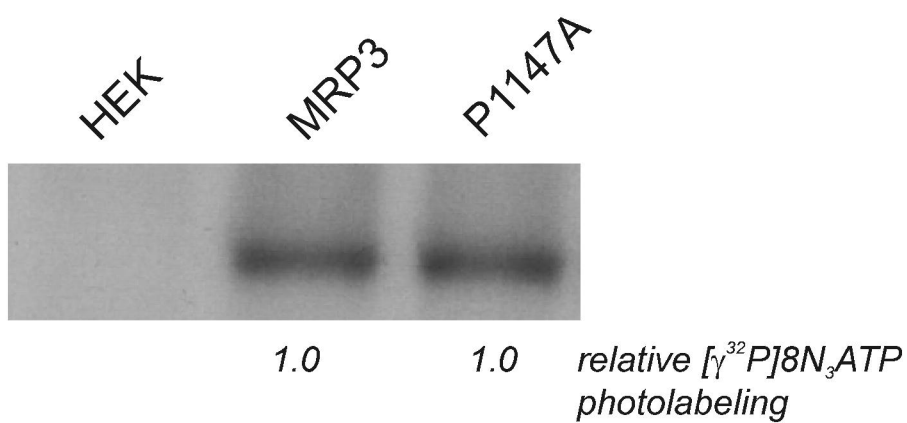
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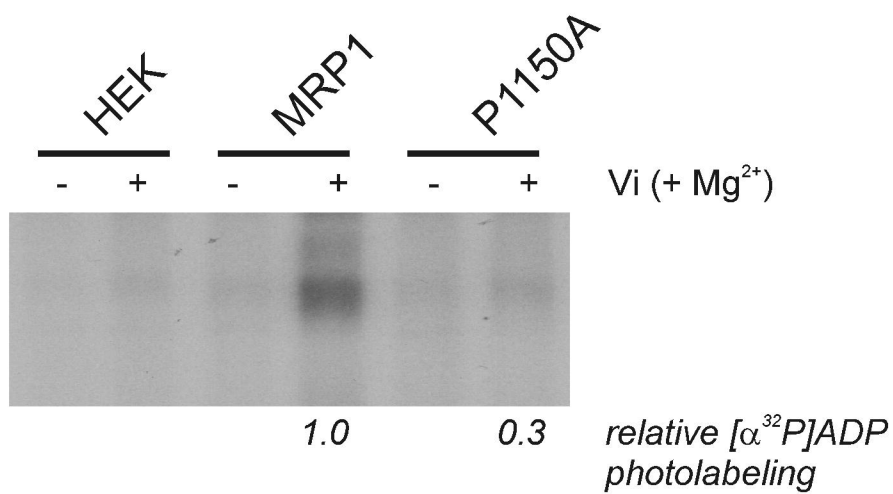
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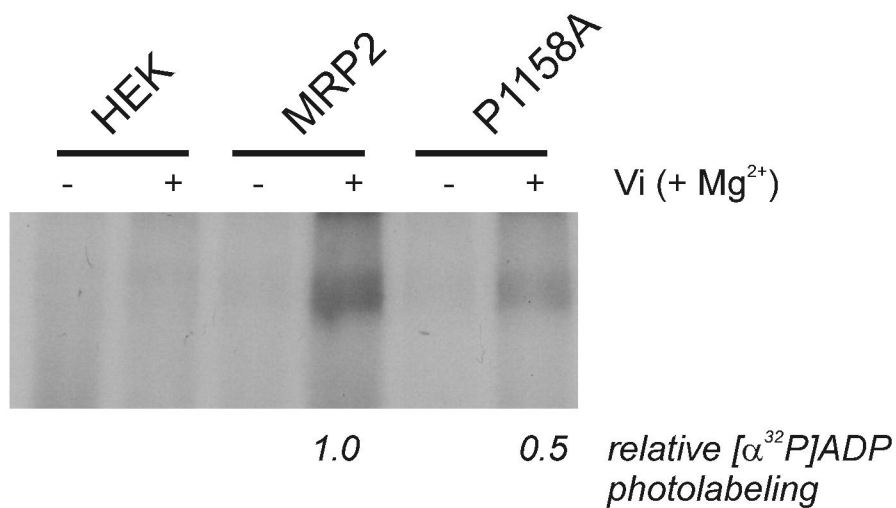
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A



B



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