Loss of ATP-dependent Transport Activity in Pseudoxanthoma Elasticum-associated Mutants of Human ABCC6 (MRP6)*

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Mutations in the ABCC6 (MRP6) gene cause pseudoxanthoma elasticum (PXE), a rare heritable disorder resulting in the calcification of elastic fibers. In the present study a cDNA encoding a full-length normal variant of ABCC6 was amplified from a human kidney cDNA library, and the protein was expressed in Sf9 insect cells. In isolated membranes ATP binding as well as ATP-dependent active transport by ABCC6 was demonstrated. We found that glutathione conjugates, including leukotriene C4 and N-ethylmaleimide S-glutathione (NEM-GS), were actively transported by human ABCC6. Organic anions (probenecid, benz bromarone, indo methacin), known to interfere with glutathione conjugate transport of human ABC1 and ABC2, inhibited the ABCC6-mediated NEM-GS transport in a specific manner, indicating that ABCC6 has a unique substrate specificity. We have also expressed three missense mutant forms of ABCC6, which have recently been shown to cause PXE. MgATP binding was normal in these proteins; ATP-dependent NEM-GS or leukotriene C4 transport, however, was abolished. Our data indicate that human ABCC6 is a primary active transporter for organic anions. In the three ABCC6 mutant forms examined, the loss of transport activity suggests that these mutations result in a PXE phenotype through a direct influence on the transport activity of this ABC transporter.

Pseudoxanthoma elasticum (PXE) is a heritable disorder affecting several different elastic tissues including the skin, the elastic Bruch’s membrane of the eye, and the arterial system.

Calciﬁcation of elastic fibers within the extracellular matrix in these tissues contributes toward an inelastic and lax dermal phenotype, angioid streaks in the retina and subsequent vision loss, and a variety of vascular abnormalities including gastrointestinal bleeding and premature atherosclerosis. It has been demonstrated recently and very unexpectedly that mutations in a gene coding for an ABC transporter protein, ABCC6 (MRP6), are responsible for the development of PXE, which is traditionally thought of as a connective tissue disease (1–4).

ABC proteins have been found in each genome studied, and the most recent annotation of the human genome sequence revealed 48 ABC-protein genes, which were grouped into seven sub-classes (see nutrigene.4t.com/humanabc.htm). The human ABCC subfamily consists of 12 members, and most of these are identiﬁed as active membrane transporters of various organic anions (for recent reviews, see Refs. 5–8). Although the majority of the proteins in the ABCC subfamily are active pumps, there are several exceptions. The cystic ﬁbrosis transmembrane conductance regulator, ABC7 (CFTR), is a chloride channel that may also regulate other channel proteins (9), and the sulfonylurea receptors, ABC8 (SUR1) and ABC9 (SUR2), regulate the permeability of speciﬁc K+ channels (10). Mutations in the CFTR gene cause cystic ﬁbrosis (11, 12), whereas mutations in the SUR1 gene are causative in a rare genetic disorder, familial persistent hyperinsulinemic hypoglycemia of infancy (10).

Inherited diseases have already been also shown to be caused by the loss of function of active ABC transporters, e.g. missense mutations in the ABCC2 gene cause conjugated hyperbilirubinemia (Dubin-Johnson syndrome) (13, 14). Although a spectrum of mutations within the ABCC6 gene is clearly responsible for PXE, the functional relationship between altered ABCC6 gene expression and the PXE phenotype is still unknown. Recent studies reveal that human and rat ABCC6 are abundantly expressed in both liver and kidney (15–17), two organs not thought to be involved in the development of PXE. It is not known, therefore, whether the pathomechanism for PXE involves aberrant ABCC6 activity directly within the affected elastic tissues, as would be expected for a heritable connective tissue disorder, or whether PXE is more likely to be a metabolic disease (18) involving abnormal hepatic and/or renal function of ABCC6.

If the major function of ABCC6 is primary active transport, a critical step to understanding the pathomechanism of PXE would be the identiﬁcation of a physiological metabolite(s) transported by ABCC6. To date, however, there have been no data reported in this regard. One study on the rat ortholog of ABCC6 reported that an anionic cyclic pentapeptide was a...
relatively poor transported substrate for the rat Mrp6/Abcc6 (17).

In the present paper, we describe the first studies of the transport activity of human ABCC6. We have established that this protein actively transports at least two anionic glutathione conjugates, and this transport is abolished by three missense mutations in ABCC6 that are known to cause PXE. These new findings establish that aberrant transport is a primary determinant in the PXE phenotype and should contribute significantly to a detailed elucidation of how such functionally null alleles of ABCC6 contribute toward abnormal elastic fiber assembly in patients with PXE.

**EXPERIMENTAL PROCEDURES**

[\(^{32}\text{P}\)-Labeling was quantitated using phosphorimaging (Bio-UV-irradiated. Thereafter the membrane proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with [\(^{32}\text{P}\)-azido nucleotide (ICN Pharmaceuticals, Costa Mesa, CA). The identity of the [\(^{32}\text{P}\)-azido nucleotide-labeled bands was confirmed by autoradiography (ECL, Amersham Biosciences). The quantitative analysis was performed using a phosphorimager (Bio-Rad). The [\(^{32}\text{P}\)-azido nucleotide-labeled bands were cut out and counted using a liquid scintillation counter.](Ref. 15)

**Results**

**Expression of Wild Type and Mutant ABCC6 and Measurement of Specific ATP Binding**—To establish an experimental system to study the functional properties of both normal and mutant forms of the human ABCC6 transporter, we have amplified a novel variant of the human ABCC6 cDNA and expressed the protein in SF9 insect cells. This approach was chosen because several mammalian ABC transporters have already been successfully expressed in insect cells. In general, these expression systems have yielded high protein levels in a functionally active state (23, 24, 30, 31). Recent expression of the functional form of the rat Mrp6/Abcc6 in SF9 cells has also been demonstrated (17).

Transcription of the ABCC6 gene occurs predominantly in the liver and the kidney (15–17); therefore, we used an amplification strategy to obtain the full-length cDNA from a human kidney cDNA library. Using this cDNA with a verified sequence, we have generated recombinant baculoviruses containing the wild type human ABCC6 cDNA as well as that of three ABCC6 mutant variants (V1298F, G1302R, and G1321S). These amino acid replacements are targeted to the C-proximal ABC domain (see Fig. 1A) and have recently been identified as ABCC6 mutants that will result in PXE (20).

SF9 cells were infected with the recombinant baculovirus supernatants, and virus clones producing the highest level of ABCC6 protein expression were selected. By using these viruses, a new protein band could be detected on the Coomassie-stained PAGE of membrane preparations from recombinant virus-infected SF9 cells. This protein band corresponded to the expected molecular mass of ABCC6 of about 150 kDa (not shown). To specifically detect the expression of human ABCC6 protein and its mutant variants, a polyclonal antibody, termed HB6, was generated against a specific ABCC6 peptide segment (see Fig. 1A).

As documented in Fig. 1B, immunostaining by HB6 antibody occurred exclusively with the 150-kDa band in the ABCC6-expressing SF9 cell membranes. Moreover, as shown by immunostaining in Fig. 1B, all three mutant forms of human ABCC6 (V1298F, G1302R, and G1321S) were expressed in about the same amount as the wild type protein (94, 78, and 88% of the same amount as the wild type protein (94, 78, and 88% of the expected molecular mass of ABCC6 of about 150 kDa (not shown)). To specifically detect the expression of human ABCC6 protein and its mutant variants, a polyclonal antibody, termed HB6, was generated against a specific ABCC6 peptide segment (see Fig. 1A).

**ATP Binding, Nucleotide Trapping, and ATPase Activity Measurements**—Isolated membranes were incubated with 5 μM 8-\(^{32}\text{N}\)-labeled ATP under non-hydrdolic conditions (at 0°C) for binding or at 37°C for nucleotide trapping. In the binding studies the membranes were incubated with \(^{32}\text{P}\)-labeled ATP and then UV-irradiated. The [\(^{32}\text{P}\)-labeled ATP in the presence of the labeled nucleotide, whereas in the case of nucleotide occlusion/trapping measurements, the membranes were first washed in the presence of 10 mM ATP and then UV-irradiated. Thereafter the membrane proteins were separated by gel electrophoresis and electroblotted to polyvinylidene difluoride membranes. [\(^{32}\text{P}\)-Labeling was quantitated using phosphorimaging (Bio-Rad). The identity of the [\(^{32}\text{P}\]-labeled nucleotide-labeled bands was confirmed by immunostaining of the same blot using the anti-ABCC6 polyclonal antibody (see above). ATPase activity was measured by colorimetric detection of inorganic phosphate liberation, as previously described (23, 28).

Vesicular transport measurements were performed using a rapid filtration method as we have previously described (28, 29). ATP-dependent tracer uptake was calculated by subtracting the radioactivity on the filter obtained in the absence of MgATP (or the presence of 4 mM MgAMP) from those obtained in the presence of 4 mM MgATP. The figures represent the mean values of at least three independent experiments. Transport values were corrected for nonspecific transport observed in the control, β-galactosidase-expressing membranes. The relative amount of uptake-competent inside-out vesicles in different membrane preparations was determined from the rate of endogenous ATP-dependent \(^{32}\text{Ca}^{2+}\) uptake (see Refs. 28 and 29). The Michaelis-Menten kinetic parameters of transport have been calculated by linear regression using a software (Origin 5.0; Microcal Software Inc., Northampton, MA).
MgATP binding. To analyze such MgATP binding by the human ABCC6 protein expressed in Sf9 cells, we used a radioactively labeled photoreactive ATP analog, 8-N3-[32P]ATP. MgATP binding to ABCC6 was studied at 0 °C under non-hydrolytic conditions, and a covalent interaction between 8-N3-ATP and the proteins was induced by UV light. We found that human ABCC6 was specifically labeled by 8-N3-[32P]ATP (Fig. 1C). This labeling required the presence of Mg2+ and could be inhibited with excess (1 mM) of MgATP (Fig. 1C).

To detect the effect of mutations on ATP binding we have determined the ATP binding capacity of the wild type and the mutant ABCC6 proteins in the presence of various 8-N3-ATP concentrations (2, 5, and 10 μM). No difference between the radioactivity incorporated into the wild type or into any of the mutants could be detected at the concentrations applied (ATP binding in the presence of 5 μM 8-N3-ATP is shown on Fig. 1C).

**Functional Studies on the Wild Type Human ABCC6**—In the following experiments we examined the ATPase and transport properties of the wild type ABCC6 protein in isolated Sf9 cell membrane vesicles. Because several previously studied ABCC/MRP homologs were shown to actively transport and/or interact with anionic compounds, especially glutathione and glucuronide conjugates, we initially examined the effects of several such compounds on membrane ATPase activity in Sf9 membrane vesicle preparations. It was found earlier that these compounds stimulated the rate of ATP hydrolysis by ABCC1 or ABCC2 (28, 32). However, we found no measurable vanadate-sensitive ATPase activity of human ABCC6 either in the absence or the presence of any of the compounds examined, which included GSH and NEM-GS (up to 10 mM), indomethacin (up to 600 μM), probenecid (up to 2.5 mM), and benz bromarone (up to 100 μM).

If the relative turnover rate of a transporter is low, ATPase measurements carried out in a native membrane background may not be sensitive enough to detect the function of an active transporter. Therefore, next we have studied the ATP-dependent uptake of labeled compounds in ABCC6-expressing membrane vesicles.

Earlier, both ABCC1/MRP1 and ABCC2/MRP2 were shown to transport NEM GS, the product of a covalent interaction between N-ethylmaleimide and glutathione (28, 29). As demonstrated in Fig. 2A, the human ABCC6/MRP6 can also transport this compound in a MgATP-dependent manner. We found that the uptake of [3H]NEM-GS was linear up to 4 min at 37 °C and significantly greater in membrane vesicles expressing ABCC6 than in the control, β-galactosidase-expressing membranes. Linear time course of uptake was recorded up to 4 min at 2 different concentrations of [3H]NEM-GS, at 4 μM (Fig. 2A) and at 100 μM (not shown). The NEM-GS uptake by human ABCC6 was completely inhibited by 1 mM orthovanadate. Uptake of NEM-GS was also eliminated after shrinking the vesicles by the addition of 1 M sucrose to the medium, indicating that the measured uptake is due to vesicular transport (Fig. 2A).

We then studied the concentration dependence of the NEM-GS uptake in ABCC6-containing membrane vesicles (Fig. 2B). Because the ABCC6-specific NEM-GS uptake was measurable and still linear at 4 min of incubation (in the presence of 4 mM MgATP at 37 °C), we used these conditions for the transport studies. The apparent Km value of the NEM-GS transport was found to be 282 ± 54 μM, and the maximum rate of NEM-GS transport by the human ABCC6 was 106 ± 8 pmol/mg of membrane protein/min.

Because both ABCC1 and ABCC2 actively transport LTC4, we also examined the uptake of this radiolabeled compound in the membrane vesicles containing ABCC6. We found that LTC4 was transported by ABCC6; the time course of LTC4 uptake is shown on Fig. 2C. Then we investigated the concentration dependence of the transport of LTC4 by ABCC6 (Fig. 2D). The...
ABCC6-specific LTC₄ uptake was measured after the shortest technically feasible incubation time (30 s) in the presence of 4 mM MgATP at 37 °C. We estimated a $K_m$ value of 600 nM and a $V_{max}$ value of 50 pmol/mg of membrane protein/min of the LTC₄ transport, performed by human ABCC6.

The rat Mrp6/Abcc6 protein has been shown to transport an anionic cyclic pentapeptide endothelin receptor inhibitor, BQ-123 (17). We have also determined the transport activity of this compound both for the rat and the human ABCC6 proteins (20-min incubation in the presence of 4 mM MgATP and 40 nM BQ-123 at 37 °C). An ATP-dependent uptake of the labeled peptide by both transporters was detected, but the rat ABCC6 protein showed about two times higher transport rate than the human ABCC6 under the above experimental conditions. The maximum transport rate for BQ-123 by human ABCC6 was estimated to be 6.5 pmol/mg of membrane protein/min. Because of the low transport rate the actual $K_m$ value could not be determined, and the above estimation is based on the $K_m$ value of the rat protein (17 μM, see Ref. 17).

We have also examined the transport of 17-β-estradiol-17-β-D-glucuronide, a preferred substrate of ABCC3, by human ABCC6 and found a very low, although measurable ATP-dependent uptake of this compound as well. Based on these experiments, we have concluded that, at least under the experimental conditions we applied, NEM-GS and LTC₄ were the best measurable substrates for ABCC6 transport studies.

We next examined the effects of various anionic compounds on the NEM-GS transport by ABCC6. We found that indomethacin, probenecid, and benz bromarone had an inhibitory effect. Fig. 3 shows these effects in the case of ABCC6 and compares these results to the inhibitory effects of these same compounds on the transport activities of ABCC1 and ABCC2. We found that benz bromarone (30 μM) inhibited almost completely all three transporters. However, 1 mM probenecid,
which is quite effective in inhibiting both ABCC1 and ABCC2 (92 and 78% inhibition, respectively), only slightly inhibited the NEM-GS transport by ABCC6 (30% inhibition). Indomethacin had an opposite effect; 100 μM indomethacin inhibited the ABCC6-mediated transport of NEM-GS by 79% but inhibited NEM-GS transport by ABCC1 and ABCC2 only by 23 and 4%, respectively. These experiments clearly indicated an active, specific organic anion transport by the wild-type ABCC6 protein.

Transport Studies of Mutants ABCC6 Variants Causing PXE—Based on these results and to analyze the transport activity of the PXE-causing ABCC6 mutations, we studied the NEM-GS and the LTC4 uptake in Sf9 cell membrane vesicles containing three ABCC6 variants, V1298F, G1302R, and G1321S. As documented in Fig. 4, Sf9 membrane vesicles containing these proteins at about the same levels of expression as the wild-type ABCC6 (see Fig. 1) were essentially inactive in the transport of both NEM-GS as well LTC4. In these experiments, the transport activities for the mutant proteins were tested in the presence of both 4 and 40 μM NEM-GS (4-min incubations, Fig. 4A) or in the presence of 50 nM LTC4 (30-s incubations, Fig. 4B). The vesicular NEM-GS or LTC4 uptake was only slightly higher than that we have found in β-galactosidase-expressing membranes, reaching a maximum of 10–25% of the wild type activity (Fig. 4, A and B). No measurable uptake of radiolabeled BQ-123 or 17-estradiol-17β-D-glucuronide could be detected in these mutant variants, but the transport rates of wild type ABCC6 were already too low to draw any significant conclusions from these uptake experiments.

Nucleotide Trapping by the Wild Type and PXE Mutant ABCC6—We have studied the transition state formation (adenine nucleotide trapping) by ABCC6 and its mutant variants expressed in Sf9 cell membranes. Isolated membranes of Sf9 cells expressing wild type or mutant (V1298F, G1302R, and G1321S) ABCC6 transporters were incubated in the presence of 2 μM Mg-8-N3-[γ-32P]ATP and either orthovanadate (1 mM) or AlF4 (2 mM) was added as a trapping anion. Incubation was carried out for 5 min at 37 °C; thereafter, the membranes were washed twice with excess MgATP (10 mM) and finally irradiated with UV (see “Experimental Procedures”).

Human wild type ABCC6 was labeled by 8-N3-[γ-32P]ATP under these conditions, demonstrating that the protein formed a trapped nucleotide transitory complex in the presence of vanadate (Fig. 4C) or AlF4 (not shown). The labeling was specific, since it was not observed if Mg2+ ions were omitted from
the reaction mixture, and it required the presence of an inhibitory anion (not shown). Two of the transport-negative PXE mutants (V1298F and G1321S) could not be labeled under these nucleotide-trapping conditions (Fig. 4C) if either vanadate or AlF 4 was present as a complex-stabilizing anion. The G1302R mutant, however, could form the occluded nucleotide intermediate state, as incorporation of radioactive label was detected under catalytic conditions in this mutant (Fig. 4C).

DISCUSSION

The recent findings by several investigators (1–4) that mutations in the ABCC6 gene are responsible for the PXE phenotype are intriguing because it is quite unclear how abnormalities in the function of a membrane-embedded ABC transporter will influence elastic fiber assembly, deposition, and/or function within the extracellular matrix. The current study was initiated to specifically address the transport capabilities of both normal and mutant ABCC6 proteins, particularly as nothing is known about the physiological substrates for human ABCC6.

As we have documented in this study, the normal human ABCC6 protein and three of its mutant forms have been successfully expressed in Sf9 insect cells. These expressed proteins, with apparent molecular masses of about 150 kDa, correspond to the under-glycosylated form of ABCC6 and were found to bind MgATP specifically (see Fig. 1). These data indicate that the ABCC6 proteins can be expressed in a native, correctly folded form in the insect cell membranes.

Active transporters in the ABC subfamily (MRP-like transporters) preferentially transport anionic compounds such as glutathione and other organic anion conjugates (5–8) and play fundamental roles in detoxification processes. ABCC1 transports LTC 4, a glutathione conjugate, with very high affinity (33); ABC2 (34) and ABC3 (35) also transport this compound but with a much lower affinity.

In this study we detected an ATP-dependent, LTC 4-transport activity for human ABCC6 (Fig. 2, C and D), and the transport was completely inhibited by 1 mM orthovanadate, which is a well-known non-covalent inhibitor of the ABC ATPases. Our results indicate that human ABCC6, like ABC2 and ABC3, is a low affinity transporter of this glutathione conjugate. It is worthwhile to note that it was reported that rat ABCC6 was not able to transport LTC 4 (17).

ABCC3/MRP3 preferably transports 17-β-estradiol-17-β-glucuronide (36). We found, however, that human MRP6 expressed in Sf9 cells had only a very low activity in the vesicular uptake of this conjugate.

Both ABCC1 and ABCC2 transport NEM-GS, a stable N-ethylmaleimide conjugate of glutathione, and this experimental model compound proved to be useful in studying the transport characteristics and inhibitor profile for these proteins (28, 29). In the current study, we have found that NEM-GS is also actively transported by the human ABCC6 (see Fig. 2, A and B) and that this transport was inhibited by orthovanadate.

The apparent \( K_m \) value of the NEM-GS transport was found to be 282 ± 54 μM. This value is similar to that found earlier for the ABCC1 transporter (155 ± 40 μM, Ref. 29), whereas the \( K_m \) value of NEM-GS transport by ABCC2 was an order of magnitude higher (2.5 mM, Ref. 28). As calculated from these experiments, the maximum rate of NEM-GS transport by the human ABCC6 (106 ± 8 pmol/mg of membrane protein/min) was significantly lower than transport rates determined in the same Sf9 membrane system for the human ABCC1 or ABCC2 (∼1,500 and 950 pmol/mg of membrane protein/min, respectively; see also Refs. 28 and 29). The relatively low transport activity of ABCC6 is consistent with our inability to measure the drug-stimulated ATPase activity of this protein in the Sf9 cell membranes.

In our current experiments the most efficiently transported substrates by ABCC6 were NEM-GS and LTC 4; therefore, we used these conjugates as model compounds in our further experiments. By comparing the inhibitory profiles for three organic anions (probenecid, benzbromarone, and indomethacin) on NEM-GS uptake by ABCC6, we found that this profile was significantly different for ABCC6 than for ABCC1 or ABCC2 (Fig. 3). The most effective inhibitors for ABCC6 were benzbromarone and indomethacin. These observations indicate that ABCC6 has a unique substrate and/or inhibitor specificity.

In the ABC subfamily of human proteins, in addition to several active transporters (e.g. ABCC1, -2, -3, -4, and -5) there are ion channel-forming proteins or ion channel regulators like CFTR/ABCC7, SUR1/ABCC8, and SUR2/ABCC9. Our present data suggest that ABCC6 is a genuine primary active transporter that can transport various anionic conjugates. The affinity of the transporter to LTC 4 is rather low, whereas the affinity of ABCC6 to a model glutathione conjugate (NEM-GS) is relatively high. Although the maximal rate of transport by human ABCC6 is much lower than that of the two other glutathione conjugate-transporting proteins, ABC2 and ABC3, our findings suggest that ABCC6 is more likely to be an active transporter than an ion channel regulator.

Data in the literature indicate that several ABC transporters, when expressed in Sf9 cell membranes, showed the same ATP binding and substrate transport activities as those present in mammalian cells (e.g. Refs. 23 and 31). Thus, although the search for a genuine, physiological substrate of ABCC6 still continues, the establishment of this expression system should greatly help these further studies. Also, the most efficient inhibitors can be selected on the basis of these experiments.

In ABC transporters MgATP is hydrolyzed in a catalytic reaction that involves the formation of a transition state complex, which contains an occluded nucleotide in the catalytic site (“trapped adenine nucleotide”). This complex can be stabilized by phosphate-mimicking anions like vanadate or AlF 4 (37, 38). The analysis of this partial reaction of the ATPase cycle can reveal important basic information related to the molecular mechanism of a given ABC transporter.

In the present study we have found that the human ABCC6 protein could be labeled by 8-β-[γ-32P]ATP under hydrolytic conditions; that is, at 37 °C and in the presence of Mg 2+ (see Fig. 4C). The reaction conditions were diagnostic for the catalytic intermediate formation, because the trapped nucleotide could not be chased by excess MgATP. The adenine nucleotide trapping reaction by ABCC6 required the presence of ATPase inhibitory anions, vanadate, or AlF 4. It is important to note that channel-forming or -stimulating ABC transporters like CFTR or SUR can also occlude adenine nucleotides. However, the detection of this reaction, in contrast to that in the primary active transporters, does not require the presence of stabilizing anions (39, 40), since the decomposition of this transitory complex is slow in these channel-forming or -stimulating ABC transporters. All these data reinforce our conclusion that ABCC6 is an ATP-dependent active transporter.

A key finding in our present experiments was that three PXE-causing mutant forms of ABCC6 expressed in Sf9 cell membranes had markedly reduced transport activities, indicating that loss-of-function mutations are responsible for PXE. To date, 43 mutations in the human ABCC6 gene, associated with pseudoxanthoma elasticum, have been identified (1–4, 20, 41, 42). This current distribution pattern of PXE mutations revealed a cluster in the region of the gene encoding the C-
terminal region of the protein (20): 11 of the 23 missense mutations identified were found within the C-proximal ABC domain, whereas only two missense mutations were found in the N-proximal ABC domain. In the present study we expressed and investigated three PXE mutants, V1298F, G1320R, and G1321S, that were all within the C-proximal ABC domain (see Fig. 1A).

These mutant variants of ABCC6 were all expressed in SF9 cells at similar levels to the wild type protein (Fig. 1B) and demonstrated normal MgATP binding (Fig. 1C). These data suggest that neither mutations caused a major structural alteration in the ABCC6 protein. The expression system we utilized is not suitable to detect if the mutation resulted in a traffic-incompetent conformation of the protein, i.e. if it retained in the endoplasmic reticulum and could not be targeted to its physiological membrane compartment (for a review, see Ref. 43). However, all three mutations resulted in a complete or nearly complete loss of NEM-NS and LTC4 transport (Fig. 4).

The missense mutations analyzed in this study affect residues within the C-proximal ABC domain. Glycine 1302 is part of the catalytically important Walker A motif or P-loop (GXXGXXGKS/ST; 1299GRTGAGKS1306 in the C-proximal ABC domain of ABCC6) and is a strictly conserved residue. The P-loop wraps over the γ-phosphate of ATP, allowing the formation of extensive hydrogen bonding between the main-chain nitrogens and the phosphate, as evidenced by crystal structures of the HisP (the ATPase subunit of the histidine permease system of Salmonella typhimurium) ABC domain (44), the Rdp50cd (catalytic domain (a dimer of the ATPase subunits) of Rdp50) ABC domain (45), and the MalK (the ATPase subunit of the maltose transport system of Thermococcus litoralis) ABC domain (46).

Valine 1298 is the residue immediately preceding this motif. Sequence alignment of the ABC domains of human ABC transporters revealed that valine is the most frequently found amino acid in this position, although amino acids with different side chains (Ile, Leu, Thr, or Cys) are also tolerated. It is noteworthy that no bulky, aromatic amino acid is present in this position in any of the human ABC transporters. Sequence alignment also revealed that the glycine residue is conserved in each human ABC transporter, in positions corresponding to 1321 in the ABCC6 sequence. Thus, all the missense mutations examined here significantly affect conserved amino acids in the ABC6 protein.

In the present study we found that neither V1298F nor G1321S ABC6 could be labeled with 8-azo-[γ-32P]ATP in the presence of vanadate or AlF4 under nucleotide-trapping conditions (see Fig. 4C). These results may suggest that the above inactivating mutations resulted in an altered conformation of the catalytic site, which is not compatible with transition state formation. An alternative explanation may be that a transition state, which is not compatible with transition state inactivating mutations resulted in an altered conformation of the catalytic important Walker A motif or P-loop (47).

Interestingly, nucleotide trapping was performed by the G1320R mutant (Fig. 4C), which suggests that the transport/ATPase cycle is arrested at a later step in this transport-negative mutant. It is worthwhile to note that nucleotide trapping was detected in two transport-incompetent mutant forms of human ABCB1/MDR1 in which the mutations also affected the Walker A motives (47).

As a summary, the data presented in this paper strongly suggest that human ABC6 is a primary active transporter for several organic anions. In addition, the loss of transport activity in the three ABC6 mutant forms examined, all causing PXE, indicates that the transport function of ABC6 may be directly responsible for the development of this disease.

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Addendum—While this paper was under editorial review a report was published with the finding that an ABCC6 polymorphism is associated with variation in plasma lipoproteins (48).

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