pABC11 (Also Known as MOAT-C and MRP5), a Member of the ABC Family of Proteins, Has Anion Transporter Activity but Does Not Confer Multidrug Resistance When Overexpressed in Human Embryonic Kidney 293 Cells*

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Several members of the ABC family of proteins have been implicated in multidrug resistance associated with cancer therapies. A novel member of this gene family, designated pABC11, has been identified using degenerate polymerase chain reaction. The full-length cDNA spans 5881 base pairs and encodes an open reading frame of 1437 amino acids predicted to contain two sets of transmembrane domains and two nucleotide binding characteristic of ABC proteins. The nucleotide sequence described herein extends that of three recently reported sequences, MRP5 (Kool, M., de Haas, M., Scheffer, G., Scheper, R., van Eijk, M., Juijn, J., Baas, F., and Borst, P. (1997) Cancer Res. 57, 3537–3547), SMRP (Suzuki, T., Nishio, K., Sasaki, H., Kurokawa, H., Saito-Obara, F., Ikeuchi, T., Tanabe, S., Terada, M., and Saijo, N. (1997) Biochem. Biophys. Res. Commun. 238, 790–794), and MOAT-C (Belinsky, M., Bain, L., Balsara, B., Testa, J., and Kruh, G. (1998) J. Natl. Cancer Inst. 90, 1735–1741), in the 5' direction. Northern blot analysis detected five transcripts that were differentially expressed in several tissue types, and the gene encoding pABC11 was mapped to chromosome 3. Confocal imaging of HEK293 cells expressing a green fluorescent protein-pABC11 construct confirmed plasma membrane localization of the fusion protein. Overexpression of pABC11 resulted in reduced labeling with the fluorochromes 5-chloromethylfluorescein diacetate, fluorescein diacetate, and 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxyethyl ester but not with calcine or rhodamine derivatives, consistent with pABC11 being an anion transporter. Fluorochrome export was ATP-dependent but glutathione-independent. We also show that this export pump does not confer resistance to various classes of cytotoxic drugs but does provide small but significant resistance to CdCl2 and potassium antimonyl tartrate.

The ABC gene family encodes a group of structurally related proteins typically composed of one or two transmembrane domains (containing several membrane spanning regions) and one or two nucleotide binding domains characterized by Walker motifs (A and B) and an ATP-binding cassette signature (1, 2). Although structurally homologous, diverse biological functions have been ascribed to different members of this gene family. Some ABC proteins are involved in ion channel formation and/or regulation, such as the sulfonamide receptors (SUR1, SUR2A, and SUR2B), which form KATP channels (3), and cystic fibrosis transmembrane conductance regulator, which functions as a chloride channel (4). Other members of this family are known to confer resistance to toxic substances. In Saccharomyces cerevisiae yeast cadmium resistance factor (YCF1) contributes to CdCl2, antimony and arsenic resistance and has been shown to transport the glutathione-arsenic complex (5, 6). Studies in Caenorhabditis elegans have shown that targeted inactivation of the homologue of human multidrug resistance-associated protein (MRP),1 mrp-1, results in increased sensitivity to both CdCl2 and NaAsO2 (7). P-glycoprotein (Pgp) and MRP are known to be involved in the resistance of some cancerous cell lines to certain cytotoxic drugs (8–11). Although these two proteins share only approximately 18% amino acid identity, they are both able to confer resistance to a broad spectrum of cytotoxic agents. There is some overlap in the substrate specificities of Pgp and MRP, although the latter has a preference for more anionic substrates, particularly glutathione conjugates (11). However, with observations that drug resistance is often seen in cells not expressing Pgp or MRP, it has become increasingly apparent that these two drug pumps alone cannot account for all of the drug resistance observed. Recently, other ABC proteins have been implicated, including BCRP in anthracycline resistance and MXR1 and MXR2 in mitoxantrone resistance (12–14). Additional members of the MRP family have also been described (15, 16), and investigations have shown that MRP2 (canalicular multispecific organic anion transporter (cMOAT)), when overexpressed in Madin-Darby canine kidney cells, has drug export activity (17). Therefore, it seems that the resistance profile of a given cell line may well involve the activity of several different efflux pumps, and an understanding of the substrate specificity of candidate drug resistance genes would help to unravel the complexities of multidrug resistance.

In this study, we isolated a novel member of the ABC gene family, designated pABC11, using a degenerate polymerase chain reaction strategy. During the course of our work, an almost identical sequence, MOAT-C, has been published (18); therefore, differences between the two sequences will be high-

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1 The abbreviations used are: MRP, multidrug resistance-associated protein; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxyethyl ester; bp, base pair(s); BSO, DL-buthionine-(S,R)-sulfoximine; CMFDA, 5-chloromethylfluorescein diacetate; EGFP, enhanced green fluorescent protein; FDA, fluorescein diacetate; kb, kilobase pair(s); MTN, multiple tissue Northern; nt, nucleotide(s); PBS, phosphate-buffered saline; Pgp, P-glycoprotein; TMR, tetramethylrhodamine chloride; AM, acetoxyethyl ester.

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lighted in this paper. Our sequence also extends that of an expressed sequence tag (277145) previously identified as an ABC protein (19), which Kool et al. (15) termed MR5P. Examination of a panel of drug-resistant cell lines revealed that MR5P was up-regulated in three cisplatin-resistant lines but unchanged in others, leading to the conclusion that the role of MR5P in this kind of drug resistance is uncertain (15). In this study, we have developed a stable cell line that overexpresses pABC11 (MOAT-C/MRP5), examined the subcellular localization of the heterologous protein, and investigated whether it is able to confer resistance to cytotoxic agents. Our results demonstrate that expression of pABC11 results in increases in fluorochrome transport and resistance to certain heavy metal compounds.

**EXPERIMENTAL PROCEDURES**

**Cloning of pABC11**—1 μg of human pancreas total RNA (CLONTech) was reverse transcribed using Superscript II reverse transcriptase (Life Technologies, Inc.) and an oligo(dT)16 primer (Perkin-Elmer). This first strand cDNA was used as a template for polymerase chain reaction (PCR) amplification with degenerate primers NDBG1 (5′-1)-and NDGA (5′-1) (20). AmpliTaq polymerase (Perkin-Elmer) was used with the following cycling parameters: 2 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s followed by 33 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s and 1 cycle of 72°C for 5 min. A diagnostic restriction digest (HindIII and BamHI) was used to eliminate cystic fibrosis transmembrane conductance regulator products, and the remaining fragments were cloned into pCR2.1 (Invitrogen) and sequenced using a dRhoda- mine terminator cycle sequencing kit and a 310 Genetic Analyser (PE Applied Biosystems). A brain cDNA library using first strand cDNA was reverse transcribed using Superscript II reverse transcriptase (Life Technologies, Inc.) and an oligo(dT)16 primer (Perkin-Elmer) and an oligo(dT)16 primer (Perkin-Elmer). A brain cDNA library using first strand cDNA was reverse transcribed using Superscript II reverse transcriptase (Life Technologies, Inc.) and an oligo(dT)16 primer (Perkin-Elmer) and an oligo(dT)16 primer (Perkin-Elmer). A brain cDNA library using first strand cDNA was reverse transcribed using Superscript II reverse transcriptase (Life Technologies, Inc.) and an oligo(dT)16 primer (Perkin-Elmer).

**Northern Blot Analysis—**Multiple tissue Northern (MTN) blots containing 2 μg of poly(A)+ RNA of various tissues per lane (CLONTech and Origen technologies Inc.) were probed with pABC11-specific fragments originating from 5′ (nt 104–1094), mid (nt 2421–2880), and 3′ (nt 3943–4395) regions of the cDNA. DNAs were radiolabeled with [α-32P]dCTP using Rediprime reagent and hybridized overnight in Rap-Hyb buffer (Amersham Pharmacia Biotech) at 60°C. Blots were washed in 0.2× SSC at 60°C (twice, 15 min each) and autoradiographed at −80°C for varying lengths of time. Blots were also incubated with a control β-actin probe.

**Chromosomal Localization—**A monochromosomal somatic cell hybrid DNA panel (human on a mouse/hamster background) was obtained from the National Institute for Medical Research (Hinxton Hall, Cambridge, UK). A primer pair combination that yielded species-specific products was used to amplify the panel: 5′-AGGACATCC-CAAAGGAAG-3′ (nt 417–436) and 5′-GAAAGCCACGAAAAGT-CATACAG-3′ (nt 543–520). 200 ng of each DNA was used per 25-μl polymerase chain reaction with cycling parameters of 95°C for 15 min, followed by 33 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min, and a final cycle of 72°C for 10 min.

**Cytotoxicity Assay—**This was as described previously, with slight modifications (22). Cells were plated in 100 μl of medium (2.5×10^4/ml). After overnight culture, cytotoxic compounds were added in 100 μl of medium and incubated for a further 48 h. Remaining adherent cells were fixed with formaldehyde (3.5% [v/v]) in PBS) and stained with crystal violet (0.1% [w/v] in PBS) and then the dye was quantitated at 550 nm absorption in a Molecular Devices plate reader. Each agent was tested at several concentrations with three replicates per concentration, and the percentage of cytotoxicity was calculated from the formula 100 (a − b)/a, where a and b are the mean absorbances without and with the test agent, respectively.

**Fluorochrome Uptake as Measured by Flow Cytometry—**5-Chloromethylfluorescein diacetate (CMFDA), fluorescein diacetate (FDA), 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF AM), and tetramethylrhodamine chloride (TMR) were obtained from Molecular Probes; other reagents were obtained from Sigma. Cells were detached by trypsin treatment with 0.25% trypsin, 0.02% EDTA in PBS, and rinsed twice with PBS. Cells were suspended using a 1% formaldehyde in PBS. Samples were analyzed by flow cytometry using a FACScan with the FL1 setting for fluorescein derivatives and PI2 for TMR and daunomycin. In some experiments, a modified protocol was used to measure efflux in which the cells were labeled at 4°C, washed in cold PBS, and resuspended in medium. Replicate cultures were then analyzed after incubation at 37°C for various times thereafter.

**Fluorochrome Uptake as Measured by 96-Well Plate Fluorometer—**Cells were plated in 100 μl of medium (3 × 10^4/ml and cultured overnight, and 100 μl of fluorochrome (5 μM in medium) was added. After incubation for 30 min at 37°C, cells were washed with Dulbecco’s modified Eagle’s medium without phenol red, fixed in 100 μl of 1% formaldehyde in PBS, and analyzed with a Fluostar plate fluorometer (excitation at 485 nm and emission at either 520 nm for fluorescein derivatives or 640 nm for TMR). Assays were set up with four or five replicates, and the data are expressed as the mean ± S.D. after subtraction of background, which was fluorescence exhibited by cells in the absence of fluorochrome.

**RESULTS**

**Cloning of a Novel ABC Protein—**A novel ABC-related sequence, pABC11.1 (377 bp in length) containing a putative ABC signature (FSVGERQLCIARAL), together with a Walker B motif (ILILD), was amplified from pancreatic cDNA. A brain-derived expressed sequence tag clone (H17207) was found to be 97% identical to pABC11.1; therefore, we screened a brain cDNA library in order to obtain a full-length cDNA. The resulting clone clustered with a series of overlapping rapid amplification of cDNA ends products yielding a clone of staining 5881 bp length (GenBank™ accession number AF146074) and encoded an open reading frame of 1437 amino acids. During the course of this work, three sequences of varying lengths were deposited in GenBank™ that are almost identical to pABC11: MOAT-C (AF104942) (18), 5838 bp; SMRP (AB005659) (23), 4939 bp; and MR5P (U83661) (15), 2058 bp. Our sequence extends the longest of these, MOAT-C, by 71 bp.
in the 5′ direction, and in addition, the first 19 bp of MOAT-C differ from the corresponding sequence in pABC11. There are a further six nt changes between our sequence and that of MOAT-C. One of these differences lies within the predicted 5′ untranslated region (nt 186 pABC11/nt 115 MOAT-C); two other alterations are silent and do not result in amino acid substitutions (nt 919 pABC11/nt 848 MOAT-C and nt 1342 pABC11/1271 MOAT-C), and a further difference (G at 1939 pABC11 compared with A at 1866 MOAT-C) results in a conservative substitution of Val in pABC11 to Ile in MOAT-C. However, 2-bp alterations within a single codon result in a nonconservative substitution of a Ser in pABC11 for a Gly in MOAT-C (nt 1394 and 1396 of pABC11/nt 1323 and 1325 of MOAT-C). The sequence of SMRP (23) confirms the pABC11 sequence in the latter two positions. SMRP begins at nt 1049 of pABC11 and contains an insert of 114 bp at position 1601. This insert contains a stop codon in frame with the longest possible open reading frame, which led Suzuki and colleagues (23) to predict that a truncated ABC protein would result from this cDNA.

A series of pairwise global alignments were performed between pABC11 and the ABC proteins SUR1, MRPI, cMOAT, and YCF1 using CLUSTAL W (parameters: gap penalty, 3; cost to open gap, 5; cost to lengthen gap, 25; and PAM250 matrix). pABC11 shared a similar degree of identity with cMOAT (29%), MRPI (28%), SUR1 (27%), and YCF1 (27%).

**Northern Blot Analysis Indicates Differential Expression of Multiple Species of mRNA and the Chromosome Mapping Panel Confirms Location of Gene on Chromosome 3**—Probing of MTN blots with various pABC11 fragments revealed that several species of pABC11 are expressed at different levels in a variety of tissues (labeled A–E in Fig. 1). The largest of these transcripts, species A (>10 kb), hybridized to both 5′ and 3′ probes. Species B (approximately 6.0 kb) was considered to correspond to our cloned cDNA (5.8 kb), as it hybridized to both 5′ and 3′ probes. Two additional species, C (5.5 kb) and D (2.4 kb), hybridized to the 5′ probe but not the 3′ probe.

The relative proportions of the various transcripts differed between the tissue types tested. For instance, skeletal muscle expresses almost equal quantities of A and B, whereas B is the predominant species in brain. Similarly high levels of A, B, C, and D are seen in fetal liver, whereas only very low amounts of A and B are detected in adult liver. Species E was detected in heart, liver, skeletal muscle, and kidney after hybridization to the 3′ probe but not the 5′ probe, and its estimated size is 1.2 kb.

To test whether pABC11 homologues exist in other species, we probed a rat MTN with a fragment corresponding to the least conserved region of this cDNA (probe MID). Even after washing at high stringency, an intense signal was seen in thymus and brain (Fig. 1D), with at least two transcripts cross-hybridizing and most similar in size to the human A and B forms.

Using gene-specific primers, we mapped pABC11 to chromosome 3. This confirmed the chromosomal location reported by previous authors (15, 18, 23).

**Establishment of a Stable Cell Line Expressing an EGFP-pABC11 Fusion Protein and Subcellular Distribution of EGFP-pABC11**—A stable, clonal cell line HEKc10 was established after transfection of wild type HEK293 cells with an EGFP-tagged pABC11 construct and selection in G418. A control G418-resistant clone that did not overexpress EGFP-pABC11, HEKc5, was also obtained from the same transfection experiment. Cells were viewed by fluorescent microscopy, and digital images were captured. As expected, there was no difference in endogenous fluorescence of HEKc5 cells when compared with wild type HEK293 cells. The majority of fluorescence in HEKc10 cells could be visualized at the plasma membrane (Fig. 2). Some punctate fluorescence was also observed; it most probably corresponds to protein trafficking through the Golgi. The distribution of fusion protein in HEKc10 cells was clearly different from that of wild type EGFP, which could be seen in abundance throughout the cell, including within the nucleus (results not shown). Confirmation that HEKc10 cells were expressing the fusion protein was obtained by fluorescence-activated cell sorter (see Fig. 4) and Western blot analysis (Fig. 2, inset, lane 2). The band visualized on our Western blot was diffuse and was estimated...
to be 220 kDa, which is larger than the predicted molecular mass of 166 kDa. This increase in size and diffuse appearance is characteristic of glycosylated proteins (there are eight potential glycosylation sites in pABC11).

Resistance to Cytotoxic Agents by EGFP-pABC11-overexpressing HEKc10 Cells—Because of the sequence homology of pABC11 to YCF1, which confers resistance to Cd<sup>2+</sup> in yeast, we initially assessed the cytotoxic effects of CdCl<sub>2</sub> on HEKc10 cells compared with our control cells. As shown in the single experiment in Fig. 3A, HEKc10 cells demonstrated a slight increase in resistance to CdCl<sub>2</sub> toxicity at 1 μM compared with three control clones. Because all three control clones behaved similarly, only HEKc5 was used subsequently. In an additional seven experiments, HEKc10 demonstrated increased resistance to 1 μM CdCl<sub>2</sub> relative to HEKc5 (Fig. 3B). HEKc10 cells did not show enhanced resistance to the cytotoxic agents daunomycin, vincristine, mitoxantron, etoposide, cisplatin, colchicine, chloroquine, CDNB, calcein, sodium arsenite, and sodium arsenate (Table I). However, some resistance to potassium antimonyl tartrate was seen (Table I and Fig. 3C). The data in Fig. 3C are from a representative experiment but were confirmed in two additional experiments. This suggests that EGFP-pABC11 may have transporter activity, but it is clearly distinguishable in specificity from Pgp, MRP, and the less well characterized mitoxantron and cisplatin transporters (13, 24–28).

Fluorochrome Accumulation/Efflux by HEKc10 Cells—Labeling with fluorochromes enables a more direct measurement of drug efflux. Initially, HEKc5 and HEKc10 cells were incubated with fluorochromes at 37 °C and washed, and uptake was measured by fluorometry using either a flow cytometer or 96-well plate reader. In this variant of the assay (uptake assay), it is assumed that fluorochrome uptake is passive and that the amount of labeling reflects active transport out of the cell.

As analyzed by flow cytometry, HEKc5 and HEKc10 cells labeled comparably with calcein-AM, TMR, or rhodamine 123 labeling, but labeling of HEKc10 cells with BCECF-AM was greatly reduced, whereas labeling with BCECF-AM was reduced by about 50% (Fig. 5). Several conclusions can be drawn from this. First, EGFP-pABC11 is an organic anion transporter. Second, increasing the negative charge (or size) of the substrate as in BCECF and calcein reduces transport efficiency, as does introduction of a positive charge (TMR). Finally, FDA is not a structural requirement. This is significant in that FDA and CMFDA shows that the chloromethyl group on CMFDA is not a structural requirement. This is significant in that it is this group that is required for conjugation to reduced glutathione (GSH) for transport as a GSH conjugate by MRP. Therefore GSH conjugation may not be necessary for transport by EGFP-pABC11, and this is discussed further under “Discussion.”

Modulation of EGFP-pABC11-mediated Fluorochrome Efflux—Fluorochrome efflux by Pgp can be blocked by verapamil, and MRP-mediated efflux can be blocked by probenecid and sulfinpyrazone (28, 32, 33). With FDA labeling of HEKc10 cells, verapamil and probenecid had minimal effects, whereas sulfinpyrazone had a small effect (Fig. 6A). In contrast, in HEKc5 cells, which do not overexpress EGFP-pABC11, the MRP blockers probenecid and sulfinpyrazone markedly enhanced FDA...
labeling, implying that HEK293 cells constitutively express a fluorochrome exporter (perhaps of the MRP type).

The requirement for GSH conjugation in drug transport can be tested by depleting cells of GSH by treatment with the GSH synthesis inhibitor \( \text{DL-buthionine-(S,R)-sulfoximine} \) (BSO) (28, 34). BSO treatment enhanced labeling of HEKc5 cells with CMFDA but not HEKc10 cells (Fig. 6B). For HEKc5 cells, this can be interpreted as follows: GSH depletion inhibits conjugation of the fluorochrome with GSH and its subsequent export by the constitutive GSH conjugate transporter. In the EGFP-pABC11-overexpressing HEKc10 cells, export of CMFDA appears to be much less GSH-dependent, in keeping with the observations above. Similar results were obtained on labeling with FDA rather than CMFDA (data not shown).

Depletion of intracellular ATP resulted in enhanced labeling of HEKc10 cells with FDA (Fig. 6C), consistent with EGFP-pABC11 being an ATP-dependent transporter.

**Decreased Fluorochrome Labeling of HEKc10 Cells Is Due to Increased Efflux Rather Than Reduced Uptake**—In the above labeling experiments, the assumption has been made that the differences observed in fluorochrome labeling between HEKc5 and HEKc10 clones are due to enhanced efflux by HEKc10 cells. To test this directly, cells were labeled with FDA at reduced temperature (to prevent efflux), washed, and then incubated for various periods at 37 °C to measure efflux. When labeling was performed at 10 °C, HEKc10 cells showed reduced initial labeling relative to HEKc5 cells. This could be due either to differences in dye uptake or to residual efflux activity in HEKc10 at this temperature. The latter seems more likely because reactions carried out at 4 °C resulted in comparable labeling. When efflux was subsequently measured in the presence of probenecid (to block the constitutively expressed transporter), HEKc10 cells lost their label much faster than HEKc5 (Fig. 7), confirming that EGFP-pABC11 is involved in drug efflux. In this experiment, the increase in fluorescence with HEKc5 cells at 7.5–15 min is due to conversion of unhydrolyzed (nonfluorescent) FDA to fluorescent fluorescin. In HEKc10 cells, this increase was not seen, presumably due to the efflux rate of fluorescin being higher than the rate of hydrolysis of FDA to fluorescin.

**Decreased FDA Labeling of HEKc10 Cells Is Due to Preferential Export of the Hydrolyzed Product Rather Than FDA Itself**—Fluorochrome labeling was done in the standard 96-well assay with two modifications. First, phenol red-free medium was used so that changes in fluorescence of the whole well contents (cells plus supernatant) could be monitored, and second, probenecid was included in the medium to block the constitutive transporter. If EGFP-pABC11 exports FDA (nonfluorescent) in preference to its hydrolysis product fluorescin (fluorescent), then the whole well contents for HEKc10 should exhibit much lower fluorescence than for HEKc5. If anything, HEKc10 cultures had increased fluorescence (data not shown), indicating that EGFP-pABC11 preferentially exports fluorescin relative to FDA.

![Fig. 4. FACScan profiles of HEKc5 and HEKc10 cells either unlabeled (−) or labeled (+) with calcein-AM (A), TMR (B), daunomycin (C), or CM-FDA (D). Note that the scale of fluorescent intensity is logarithmic.](image-url)
DISCUSSION

A novel member of the ABC family of proteins has been cloned. We predict that the initiating codon begins at nt 198 resulting in a protein of 1437 amino acids. Although the sequence surrounding this putative initiating Met, 5'-AA-GAUGA-3', is not identical to a consensus Kozak sequence, 5'-ACCAUGG-3', the dominant purine at position -3 is present, rendering the nucleotides at positions -2, -1, and +1 less influential in determining the translational start site. Pairwise alignments indicate that this protein shares similar degrees of homology with several ABC proteins of diverse function, including SUR1, YCF1, and MRP, and that the majority of conserved residues lie within the nucleotide binding domains and the second transmembrane domain, with the NH2-terminal transmembrane domain being the most divergent portion of the protein.

Although pABC11 may be closest in amino acid composition to a subgroup of ABC proteins, its mRNA expression profile is very different. For example, cMOAT expression is restricted mainly to the liver (17), whereas multiple transcripts of pABC11 are observed in several tissue types. Although it has not been conclusively proven, we suspect that these multiple species arise through alternative splicing of a single gene, a phenomenon already reported for other ABC proteins, including SUR2 (35–37), the major histocompatibility complex-encoded peptide transporter Tap2 (38), MRP3 (39), and MRP (40). Valuable but limited information can be gained from the protein sequence, mRNA tissue distribution, and homology searching with respect to the function of this novel ABC protein.

Therefore, to get further insight into its function, the effect of overexpression of the protein in HEK293 cells was investigated.

Overexpression of EGFP-pABC11 fusion protein did not increase resistance to a range of anticancer drugs or arsenic.

**FIG. 5.** Labeling of clones HEKc5 and HEKc10 by FDA, CMFDA, BCECF, calcein-AM, rhodamine 123, and TMR. The structures shown are those of the expected intracellular hydrolysis products. BCECF is a mixture, and the structure shown is one of the three possible hydrolysis products.

**FIG. 6.** Modulation of fluorochrome labeling of clones HEKc5 and HEKc10. A, FDA labeling of cells in the presence of 1 mM probenecid, 1 mM sulfipyrazone, or 25 μM verapamil. B, CMFDA labeling of cells pretreated for 24 h with 50 μM BSO to reduce intracellular GSH concentrations. C, FDA labeling of cells after ATP depletion. For ATP depletion, cells were incubated for 20 min in glucose-free medium with 50 mM 2-deoxyglucose and 15 mM sodium azide before addition of FDA in the same medium.
However, small but statistically significant increases in resistance to CdCl₂ (2.4×) and potassium antimonyl tartrate (2.9×) were seen, although it is debatable whether these increases are biologically relevant. This pattern of resistance differs from that found for overexpression of other ABC proteins. For example, with MRP (24, 41), similar levels of resistance to potassium antimonyl tartrate were found, but high levels of resistance to anticancer drugs (in the range 5–25×) were also found. This suggests a difference in specificity between pABC11 and other transporters, but other possibilities must be considered. For example, does EGFP tagging affect function? We cannot answer this question for pABC11 because attempts to express untagged or His-tagged protein were unsuccessful. However, for other ABC proteins, tagging was consistent with function (42–44). Cell background is another possible variable, but the studies on MRP cited above were also done in HEK293 cells (24). These reservations on the function of overexpressed, tagged protein also apply to its subcellular localization, which may be altered relative to the native protein, as has been seen for MRP (discussed by Tommasini et al. [45]).

Our studies on fluorochrome transport also suggest that pABC11 differs in specificity from MRP and Pgp. Like MRP, EGFP-pABC11 overexpression results in reduced labeling with BCECF-AM and CMFDA (31, 46). BCECF is also a substrate for Pgp (47). A striking observation in our study is the failure of EGFP-pABC11 to reduce labeling by calcein AM and rhodamine derivatives, both classes of compounds being substrates for MRP and Pgp, albeit with differing efficiencies (29). For EGFP-pABC11, there was a clear structure activity relationship for fluorochrome export (Fig. 5) with an increase in size and/or positive charge resulting in decreased efflux. Like MRP (28), EGFP-pABC11 appears to have a preference for the anionic hydroslys products of fluorochromes rather than the uncharged ester, which is preferred by Pgp (46). In this context, it is significant that antimonyl tartrate is also an organic anion.

Glutathione is necessary for transport of many substrates by MRP and its homologues (reviewed by Ishizawa et al. (48)) but not for calcine or BCECF (28, 46). In our studies, BSO treatment to deplete glutathione did not affect fluorochrome transport by EGFP-pABC11 (but did inhibit activity of the constitutively expressed transporter). This shows that glutathione is not necessary for EGFP-pABC11 efflux of the fluorochromes FDA and CMFDA, but we cannot exclude the possibility that it may be required for other substrates.

Although EGFP-pABC11 is functionally different from MRP, it has more in common with MRP than Pgp, as expected from the amino acid homologies. However, one final piece of evidence highlights the distinction between MRP and EGFP-pABC11. Probencid and sulfipyrazone are broadly reactive anion transport inhibitors that effectively block MRP when used in the mM range, but at this concentration, these compounds had minimal effect on EGFP-pABC11.

In conclusion, EGFP-pABC11 is an organic anion transporter that appears to be functionally different from previously described ABC transporters. However, it must be stressed that the studies described here were performed using an overexpression system, and the next step is to see whether they can be confirmed with the native protein in a more physiological setting.

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