Molecular Cloning of cDNAs Which Are Highly Overexpressed in Mitoxantrone-resistant Cells: Demonstration of Homology to ABC Transport Genes

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Abstract

Reports of multiple distinct mitoxantrone-resistant sublines without overexpression of P-glycoprotein or the multidrug-resistance associated protein have raised the possibility of the existence of another major transporter conferring drug resistance. In the present study, a cDNA library from mitoxantrone-resistant S1-M1-80 human colon carcinoma cells was screened by differential hybridization. Two cDNAs of different lengths were isolated and designated MXR1 and MXR2. Sequencing revealed a high degree of homology for the cDNAs with Expressed Sequence Tag sequences previously identified as belonging to an ATP binding cassette transporter. Homology to the Drosophila white gene and its homologues was found for the predicted amino acid sequence. Using either cDNA as a probe in a Northern analysis demonstrated high levels of expression in the S1-M1-80 cells and in the human breast cancer subline, MCF-7 AdVp3000. Levels were lower in earlier steps of selection, and in partial revertants. The gene is amplified 10–12-fold in the MCF-7 AdVp3000 cells, but not in the S1-M1-80 cells. These studies are consistent with the identification of a new ATP binding cassette transporter, which is overexpressed in mitoxantrone-resistant cells.

Introduction

Beginning with the discovery of P-glycoprotein in 1976, and the subsequent molecular cloning of the encoding gene, MDR-1, the overexpression of ABC transporters has been linked with drug resistance (1, 2). Resistance ensues from reduced intracellular drug concentrations, a result of active drug efflux. The subsequent identification of multidrug resistance associated protein, encoded by the MRP gene, heralded a new era that recognized the complexity of the problem and catalyzed the search for additional transporters (3). MDR-1 and MRP are members of an expanding superfamily of ABC proteins. This superfamily is comprised of a large and diverse group of proteins that transport solutes across biological membranes. Transmembrane domains are thought to form a pathway through which substrates cross cell membranes while two ATP-binding domains, typically located on the cytosolic surface, hydrolyze ATP to accomplish substrate transport. Mutations in ABC transporters have been identified as etiological in diseases including hyperinsulinemic hypoglycemia of infancy, adrenoleukodystrophy, and cystic fibrosis (4). The transporters MDR-1/P-glycoprotein and MRP, and possibly the multispecific organic anion transporter cMOAT, are thought to be involved in both the normal excretion of xenobiotics and in drug resistance. The ABC superfamily also includes a number of transporters without known function. Kool et al. (5) have reported four additional MRP homologues, and Allikmets et al. (6) identified 21 ABC transporter genes by searching the EST database. Thus, the potential exists to identify additional transporters that mediate drug resistance.

Recent studies have described a number of cell lines with resistance to mitoxantrone that exhibit multidrug resistance without overexpression of Pgp or MRP. In addition to mitoxantrone, these cell lines are particularly resistant to anthracyclines, and have an energy-dependent reduction in the accumulation of daunomycin and mitoxantrone. Cell lines possessing this phenotype include sublines derived by selection of leukemic cells, as well as breast, colon, and gastric carcinomas (see Ref. 7). In the present study, we describe the identification of two cDNAs that are overexpressed at very high levels in several cell lines with a mitoxantrone-resistant phenotype. These cDNAs have a high degree of homology to HUEST 157481 (HSU66681), one of the ABC transport genes identified by Allikmets et al. (6).

Materials and Methods

Cell Lines. Several mitoxantrone-resistant cell lines were used. Cultures of S1-M1 cells, derived from S1 human colon carcinoma cells, were gradually advanced to 80 μM from an original selection in 3.2 μM mitoxantrone. MCF-7 AdVp3000 cells were obtained by stepwise selection to 3000 ng/ml adriamycin in the presence of 5 μg/ml verapamil. These cells have been previously characterized as having ATP-dependent efflux of daunomycin and rhodamine, and lack overexpression of Pgp or MRP (7, 8). RNA was isolated from MCF-7 AdVp sublines maintained in 20, 200, and 3000 ng/ml adriamycin, from a partial revertant cultured for over 12 months in drug-free medium, and from another MCF-7 subline maintained in 100 ng/ml mitoxantrone (9). Sensitive parental cell lines were also used, including S1 (derived from LS 174T cells) and MCF-7. In addition, RNA was isolated from several Adria-mycin-selected cell lines previously shown to overexpress Pgp.

Confocal Microscopy. Cells were cultured for 48 h in 35 mm of polystyrene-coated microwell plates. A Zeiss LSM 410 confocal scanning laser microscope equipped with a 150 mW omnichrome Ar-Kr laser exciting at 568 nm was used to detect mitoxantrone fluorescence. Emitting light passed through a 590-nm long-pass filter.

RNA Isolation, Northern blot Analysis, Probe Labeling, and Quantitative PCR assay. RNA was isolated by the STAT-60 method according to manufacturer’s directions (Tel-Test, Inc., Friendswood, TX). Northern blot analysis was performed by standard methods. Labeling of cDNAs and of individual probes was accomplished using the Rediprime II random prime labeling system according to the manufacturer’s instructions (Amersham Corp., Arlington Heights, IL). A semiquantitative PCR analysis followed previously described methods (7), with primers identified from the sequence analysis. Primers used were as follows (see Fig. 2 for residue numbers): MXR1 5′ PRIMER: 1979 5′TGCCCAAGGCTCTAGGCAACAGC 2000; 3′ PRIMER: 2150 5′CACACTAGGGCACTAACC 2152; MXR2 5′ PRIMER: 1976 5′TGCCCAAGGCTCTAAGGCAACAGC 2000; 3′ PRIMER: 2150 5′CAATACCTGAGGGCTTG 2152; HUEST 5′ PRIMER: 2283 5′TCTCTCAGCTAGAACCATGG 2283; 3′ PRIMER: 2374 5′TCACCGTGGCTTTTTCAC 2375.

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The abbreviations used are: ABC, ATP binding cassette; MDR-1, multidrug-resistance-1 gene; MRP, multidrug-resistance-associated protein; TAP, transporter associated with antigen processing; EST, Expressed Sequence Tag; Pgp, P-glycoprotein; ER, endoplasmic reticulum.

\(^{1}\) Unpublished data.

\(^{2}\) The nucleotide sequence data reported in this manuscript have been deposited at the NCBI/GenBank Data Library under accession numbers AF093771 and AF093772.
Construction and Screening of cDNA Library. A cDNA library was constructed using mRNA from S1-M1-80 cells and the SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (Life Technologies, Inc., Rockville, MD). Synthesis of the cDNA library followed manufacturer’s instructions, with the exception that cDNAs <1000 bp in size were excluded. Electroporation of DH10β Electromax Cells was followed by plating on agar containing X-gal/IPTG, after which 7680 individual white colonies were picked and plated in individual wells in eighty 96-well plates. Quadruplicate filters were made and two each were probed with 32P-radiolabeled cDNA synthesized using either S1 or S1-M1-80 RNA. After obtaining adequate exposures, the filters were washed and reprobed with radiolabeled cDNA from either S1 or S1-M1-80 RNA; this second time, the filters were switched and reprobed with the alternate cDNA. Only colonies (wells) in which differences could be consistently demonstrated were transferred to a second 96-well plate and underwent repeated screening. The choice of which colonies (cDNAs) to pursue further was based on a consistent pattern of differential expression. Three cDNAs that were felt to be differentially expressed were further pursued. These three cDNAs were chosen to sequence MXR1 and MXR2. Ten cDNA-specific primers were chosen to sequence each cDNA in both the 5’ and the 3’ directions, allowing confirmation of sequence differences between the two cDNAs. The T7 Sequenase Version 2.0 kit from Amersham Corp. was used for manual sequencing. The ThermoSequenase Cy5.5 dye terminator cycle sequencing kit from Amersham Corp. was used for cycle sequencing. Cycle sequencing reactions were analyzed on the Visible Genetics OpenGene automated sequencing system. All sequences were confirmed with both methods in both directions.

Results

Mitoxantrone accumulation was assayed by confocal microscopy in the S1 and S1-M1-80 cells (Fig. 1). After a 2-min incubation, low levels of accumulation are observed in the S1 cells, whereas in the S1-M1-80 cells accumulation is only observed in vesicles. The mitoxantrone fluorescence in S1-M1-80 cells at 2 min most likely represents residual mitoxantrone, because it is comparable with that observed in control S1-M1-80 cells that, despite incubation out of mitoxantrone for 1 week, have residual mitoxantrone fluorescence in vesicles (data not shown). After 10 min of incubation, the fluorescence in S1 cells is intense, although there is little fluorescence in the S1-M1-80 cells, other than that in the vesicles. ATP depletion increases the accumulation in the S1-M1-80 cells (data not shown). Similar results were observed in experiments with the MCF-7 AdVp3000 cells, although without vesicle formation. The results were confirmed with radiolabeled mitoxantrone accumulation studies, which demonstrated a 50% reduction in accumulation in S1-M1-80 and a 60% reduction in MCF-7 AdVp3000 cells compared with their respective parental cells after a 1-h incubation (data not shown). The difference between the dramatic results observed with the confocal microscopy and the radioactive drug accumulation assay may be related to the sensitivity of the assay: the concentration of radioactive mitoxantrone is 20 nM, whereas the concentration of mitoxantrone used in the confocal studies is 5 μM.

This phenotype prompted a search for a putative ABC transporter using cDNA differential hybridization. Using the strategy described in “Materials and Methods,” 7680 individual clones were screened and three potential candidates were identified as clones that appeared to be overexpressed in resistant S1-M1-80 cells. One clone was a false positive.
positive, because it was similarly expressed in both parental and resistant cells. However, Northern analysis with the other two cDNAs, designated MXR1 and MXR2 (for mitoxantrone resistance), confirmed marked overexpression of a 2.8–3.4-kb mRNA in the resistant S1-M1-80 cells (Fig. 2). The somewhat diffuse signals identified in the Northern blots used in this initial screening, as well as those shown in Fig. 3, are consistent with the existence of several similar transcripts differing in length. Sequencing of the two clones established both the similarities and differences to each other and to an EST previously identified as an ABC transporter (HUEST 157481; Ref. 6). A high degree of homology was found, but poly(A) tails in both MXR1 and MXR2 distinguished these as shorter versions of HUEST 157481. The full-length sequence presented in Fig. 2 includes sequence data from the EST database for HUEST 157481 and additional sequence obtained in one of our laboratories from several placenta cDNAs. This latter sequence was used to confirm and extend the HUEST 157481 sequence. The predicted amino acid sequence is shown, as well as features distinctive of ABC proteins, including the Walker A and B motifs and the putative transmembrane domains. Differences in MXR1 and MXR2 relative to the HUEST 157481 sequence are noted by symbols above the sequence and are described in the figure legend.

An open reading frame was identified in these cDNAs and a homology search based on the predicted amino acid sequence was performed. The alignments of highest significance with either MXR1 or MXR2, and with the full-length sequence were ABC transporters, beginning with the yeast ATP-dependent permease precursor (P25371, 27% identity). The most common alignment was with amino acid sequences for white protein genes from various species (e.g., for MXR1: mosquito, U88851, 27% identity; fruit fly, U64875, 26% identity; mouse, Q64344, 25% identity; human, U34919, 25% identity).

Results of Northern analyses using the MXR1 cDNA and RNA from several parental and multidrug-resistant sublines are shown in Fig. 3A and B. Overexpression is noted in two different S1-M1-80 RNA preparations; and in an earlier step of the selection, maintained in 3.2 mM mitoxantrone, with higher levels observed in the subline maintained in 80 μM drug. Overexpression in a series of sublines isolated during the stepwise selection of MCF-7 AdVp cells is noted, with progressively higher levels in sublines maintained at 20, 200, and 3000 ng/ml adriamycin, as well as lower levels in a partial revertant. Overexpression is also noted in Fig. 3B in MCF-7 MX100 cells, growing in 100 ng/ml mitoxantrone. No apparent expression of MXR1 is observed in seven unrelated MDR-1 expressing sublines included in Fig. 3B. The result of a Southern analysis probed with the MXR1 cDNA, shown in Fig. 3C, provides evidence of gene amplification, with the copy number in the MCF-7 AdVp3000 cells estimated to be approximately 10–12 times that in parental MCF-7 cells. Interestingly, S1-M1-80 cells do not appear to have gene amplification. Similar results were obtained using MXR2 as a probe (data not shown).

Finally, a semiquantitative PCR assay was performed (Fig. 4) using the primers described in “Materials and Methods.” The results in the top panel were obtained using a primer pair that recognizes only the longer transcript encoded by HUEST 157481, because the 3′ primer is complementary to sequence beyond the end of MXR1 and MXR2. The middle panel, labeled MXR1, shows the results of PCR reactions using a primer pair that detects both the long HUEST 157481 transcripts and transcripts of MXR1 length; this was achieved by using a 3′ primer complimentary to sequence beyond the termination of MXR2, but before the end of MXR1 and HUEST 157481. In the bottom panel, labeled MXR2, the primer pair used detects all three transcript lengths: HUEST 157481, MXR1, and MXR2. This latter pair of primers was less efficient than the other two pairs, and only a very faint signal could be discerned with parental S1 RNA, precluding precise quantitation. The accompanying table summarizes the relative levels of expression, derived by dividing the level in the resistant cells by that in the parental cells. The data indicate that transcripts of all three lengths are overexpressed. However, the shorter versions are more highly expressed, as evidenced by the higher relative level of expression. The inference that the longer transcript (HUEST 157481) comprises a smaller fraction of transcripts in the S1-M1-80 cells is consistent with the fact that the shorter transcripts (MXR1 and MXR2) were isolated in the cloning process. It should be emphasized that the values in the tables represent relative differences, and not absolute levels. These relative differences were derived by dividing the absolute value obtained in the resistant cells by the absolute value obtained in the parental cells. In contrast to the relative levels, the absolute levels in both resistant sublines are similar as evidenced by the comparable products obtained with comparable amounts of input RNA, an observation that is consistent with the similar levels detected by Northern analysis.

Discussion

The present study describes the isolation of two cDNA clones (termed MXR1 and MXR2) from S1-M1-80 cells, which display a phenotype characterized by high levels of mitoxantrone resistance. The observation that these cDNAs recognize sequences that are overexpressed in additional cell lines with a similar phenotype suggests a causal role for this ABC transporter in mediating the multidrug-resistant phenotype of these cells. Support is also provided by the observation that progressively higher levels were found in cells maintained in incrementally higher concentrations of drug, and that lower levels were found in a partial revertant. Specificity is demonstrated by the absence of overexpression in multiple other adriamycin-selected cell lines in which overexpression of Pgp has been previously identified. Furthermore, Southern analysis confirmed amplification with an estimated 10–12-fold increase in the copy number of these sequences in the MCF-7 AdVp3000 cells, but provided no evidence for gene amplification in S1-M1-80 cells. The sequence of these cDNAs revealed >98% homology with an EST previously identified as an ABC transporter (HUEST 157481; Ref. 6). However, the length of the two cDNAs were different, suggesting the existence of either a family of genes, or most likely transcript variants resulting from differential polyadenylation. Size analysis of the mRNA by Northern blot analysis demonstrated a range of transcripts from 2.8–3.4 kb.

The number of ABC transporters expressed in drug-resistant cells has steadily expanded in recent years. Although the evidence for a role in drug resistance is most convincing for MDR-1 and MRP, expression of other ABC transporters not initially cloned from drug-resistant cells has also been reported, including cMOAT and the MRP homologues MRP 3–6 (5). Indeed, a portion of MRP6 was initially identified as the anthracycline-resistance-associated protein ARA in human leukemia cells (10). Recently, an abstract describing an ABC transporter isolated from MCF-7 AdVp cells was reported; this may represent a transcript similar to one of those described herein, or may represent a separate gene (11).

The precise cellular localization and function of the protein(s) encoded by these cDNAs are not yet known. A BLAST search using the amino acid sequence predicted from an open reading frame found in MXR1 revealed 25–27% identity with the white gene from various species, including human (12, 13). The product of this gene, and others in the TAP gene subfamily, represents a half-transporter, and is thought to heterodimerize, thus allowing the assembly of functional transporters. The product of the Drosophila white gene forms heterodimers with the product of either the brown gene to transport...
Fig. 2. Northern blot showing overexpression of sequences homologous to the probes used. The cDNAs used in this experiment were isolated from an S1-M1-80 cDNA library and recognize similar size messages. The lower part of the figure depicts the nucleotide and predicted amino acid sequence. The numbers on the left are those of the complete cDNA sequence and the predicted amino acid sequence. The complete sequence was obtained by combining data from HUEST 157481 with sequence results from several normal placenta cDNAs. Translation is predicted to start with the residues 205–207 (ATG), and these are highlighted. The amino acid numbers begin with this residue and are shown beneath the nucleotide residue numbers on the left. For clarity, only the sequence of HUEST 157481 and MXR1 are shown. MXR1 extends from 1121–2591, whereas MXR2 extends from 1084–2229; both MXR1 and MXR2 had poly(A) tails. Walker A (amino acids 80–89) and Walker B (amino acids 206–210) motifs are highlighted in gray. Six putative transmembrane domains are underlined. A tentative chaperonin-binding epitope is double underlined. Differences among the sequences noted in the figure are as follows: p, unlike HUEST 157481 and MXR1, which have five consecutive As (1360–1364), MXR2 has one less A in this area; #, both MXR1 and MXR2 have a Ga at 1648, a substitution that changes amino acid 482 from arginine to glycine; †, unlike HUEST 157481 and MXR1, which have two Ts at 1654–55, MXR2 has three Ts in this region; ¶, both MXR1 and MXR2 have a deletion at 1660; §, HUEST 157481 has an additional G at position 1950, which is not present in either MXR1 or MXR2 (this additional G would result in a frame shift and premature termination and is ignored in the translation); ‡, in contrast to HUEST 157481, which has five consecutive Ts (1961–1965), both MXR1 and MXR2 have 6 Ts in this area; φ, indicates the end of the MXR2 transcript; ¢, MXR1 has a deletion at position 2254 (this residue is in the 3' untranslated region).
guanine, or the scarlet gene to transport tryptophan, thus determining eye color in *Drosophila*. Similarly, TAP1 and TAP2 dimerize to transport peptides into the ER, where assembly of the MHC complex occurs (4). Although a high degree of homology to TAP1/TAP2 was not found, the role of the TAP1/TAP2 dimer for antigen transport into the ER is intriguing. A similar location could be conceived for the *MXR* gene products if they function to transport substrates into the ER.

The mitoxantrone-resistant phenotype has been previously reported in the cell lines included in this study, and in other cell line models (see Ref. 7). Typically, the phenotype includes high levels of mitoxantrone tolerance and anthracycline-resistance (although at lower levels), without cross-resistance to paclitaxel or to cisplatin. ATP-dependent efflux of rhodamine and daunomycin has been observed (7). In some of the resistant cells, compartmentalization into vesicles has been implicated as a partial explanation for resistance. Although identification of a potential ABC transporter has heretofore proven elusive, several lines of investigation have suggested that mitoxantrone is modified before transport out of the cell. Inside-out membrane vesicles from the S1-M1-80 cells do not transport mitoxantrone directly, but do transport the anionic glutathione conjugate, LTC4 (data not shown). If mitoxantrone must be metabolized before transport, it is possible that the *MXR* gene products could transport either glutathione-conjugated or glucuronidated mitoxantrone. Alternatively, if localized on the ER, the *MXR* gene products could transport UDP-glucuronide, which seems to be the rate-limiting step in glucuronidation reactions (14, 15). Raising antibodies against *MXR* will aid in determining localization and function.

It is not clear whether the three distinct 3’ regions identified, including the HUEST 157481 and our clones *MXR1* and *MXR2* represent different genes, or most likely differential polyadenylation. The presence of splice variants has not been reported for MDR-1/Pgp, but has been noted in a number of other ABC transporters. These include MRP, MDR3 (the Pgp-related phosphatidylcholine transporter), and the photoreceptor-specific transporter gene *ABCR* (16 –18).

Similarly, transcript variants resulting from alternative polyadenylation events have been described in human PMP69, the putative peroxisomal ABC-transporter (19). If variants are present, differences in mRNA stability could result. For example, expression of variants could account for differences in phenotype observed among cell lines with overexpression of the mitoxantrone transporter.

In summary, we have identified overexpression of three cDNAs with homology to ABC transporters in mitoxantrone and anthracycline-resistant cells. On the basis of homology, these cDNAs seem to be related to half-transporters that require dimerization to transport substrates. The demonstration of overexpression of an ABC transporter in cells with high levels of resistance to mitoxantrone and the anthracyclines opens anew the question of clinical significance and offers new hope for the reversal of clinical drug resistance.

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References


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