Characterization of ABCB9, an ATP Binding Cassette Protein Associated with Lysosomes*

Received for publication, March 5, 2000
Published, JBC Papers in Press, March 23, 2000, DOI 10.1074/jbc.M001819200

Fang Zhang‡, Wandong Zhang§§, Lin Liu‡, Cynthia L. Fisher‡¶, David Hui‡¶, Sarah Childs§§**, Katerina Dorovini-Zis‡‡, and Victor Ling §§

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF216494 and AF216495.

‡ Present address: Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, K1A 0R6 Canada.
¶ Recipient of a Medical Research Council of Canada Studentship.
** Recipient of a National Cancer Institute of Canada Steve Fonyo Fellowship. Present address: Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, MA 02129.
†† Recipient of a Canadian Breast Cancer Foundation Studentship.

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

We have cloned full-length human and mouse cDNAs of ABCB9, which encodes a predicted multiple-spanning transmembrane domain and a nucleotide-binding domain with Walker motifs. It is therefore designated as a “half” ATP binding cassette (ABC) transporter. Northern analysis shows that the ABCB9 mRNA is expressed at a high level in testes and moderate levels in brain and spinal cord. A splice variant mRNA deleted in the last pair of predicted transmembrane segments was shown to be expressed in human tissues. Phylogenetic analysis indicates that ABCB9 is closely related to TAP1 and TAP2, two “half” ABC proteins found in endoplasmic reticulum. ABCB9 protein colocalized with the lysosomal markers, LAMP1 and LAMP2, in transfected cells. ABCB9 protein appears to be most highly expressed in the Sertoli cells of the seminiferous tubules in mouse and rat testes. These cells have high levels of phagocytosis and secretory activities. These findings pave the way for further investigation into the potential novel function of ABCB9 in lysosomes.

The ATP binding cassette (ABC)1 transporter superfamily is one of the largest gene families found in both eukaryotic and prokaryotic cells. ABC proteins contain a multispanning transmembrane domain(s) and a highly conserved nucleotide binding domain(s) (NBD) (1). In eukaryotic cells, ABC transporters participate in the translocation of a variety of molecules across cellular membranes or intracellular organelle membranes. These tasks are divided between full transporters, which consist of two transmembrane domain(s) and two NBDs, and half transporters, which contain one of each domain. In mammalian cells, full transporters are usually found in plasma membranes, which include the multiple drug transporters, P-glycoprotein (2) and MRP (3), and the chloride channel CFTR (4). Conversely, all half transporters characterized to date are found in subcellular organelles. Half transporters are likely to function either as homodimers or heterodimers. The best-studied examples are TAP1 and TAP2, which form a heteromeric complex for translocating antigenic peptides from the cytoplasm to the lumen of the endoplasmic reticulum (ER) to bind major histocompatibility complex class I molecules (5, 6). Four half transporters (PMP70, ALDP, PMP69, and ALDR) have been found in peroxisomal membranes (7–10). PMP70 and ALDP are linked to the generic diseases Zellweger syndrome (associated with a peroxisome biogenesis disorder) and adrenoleukodystrophy (associated with an oxidation of very long chain fatty acids defect) (11, 8). In mitochondria, two half transporters (M-ABC1, ABC7) have been identified (12, 13). Mutations in the human ABC7 gene are associated with X-linked sideroblastic anemia and ataxia (14).

No ABC transporter has been reported to be localized in mammalian lysosomal membranes; however, an ABC protein, HMT1, has been found in the fission yeast vacuolar membrane, the lysosomal compartment of yeast. It is involved in heavy metal tolerance (15). Mammalian lysosomes are responsible for the breakdown of cellular components and also serve as a sink for toxins, drugs, and heavy metals. Lysosomes are required to take up a variety of substances from the cytosol and release recycled precursors to the cytosol for macromolecule synthesis. Therefore, transport activities moving a wide range of molecules across lysosomal membranes must occur. Indeed, a number of lysosomal transport processes have been shown to be ATP-dependent, but transporters have not yet been identified. Among these events are translocation of peptides to lysosomes along the pathway for selective degradation of cytosolic proteins under nutritionally depleted conditions (16), heavy metal ion translocation to lysosomes for heavy metal metabolism (17), and free oligosaccharide translocation (18). ABC proteins, known to transport a wide range of molecules from ions to proteins, are potential candidates for any one of these transport activities.

In this report, we describe a novel mammalian ABC transporter that is localized to the lysosomes in transfected cells. We have cloned this gene from both human and mouse, and designated them hABCB9 and mABCB9, respectively (Human ABC gene nomenclature committee). The similarity of ABCB9 to the
TAP proteins has recently been noted by Yamaguchi et al. (24). The ABCB9 protein expression has been examined in mouse and rat testis, because ABCB9 mRNA is most highly expressed in this tissue. Using ABCB9-specific antisera, ABCB9 is located to the cytoplasmic compartment of Sertoli cells in the seminiferous epithelium. These cells play multiple functions in nurturing and supporting germ cells, and they possess numerous lysosomes for phagocytosis.

**EXPERIMENTAL PROCEDURES**

Construction of cDNA Libraries—Poly(A)- RNA from adult brain of C57BL/6J mouse was isolated by using Trizol Reagent and oligo(dT) cellulose columns (Life Technologies, Inc.). Double stranded cDNAs were synthesized with oligo(dT) primer (SuperScript II; Life Technologies, Inc.) and ligated to Lambda Zap II (Stratagene). The Lambda ZapII/cDNA was packaged into bacteriophage particles using the Gigapack III Gold-packaging extract (Stratagene).

Cloning of ABCB9 cDNAs—The cDNA insert of EST clone c1-ch05 (Promega Genexpress, Laboratory Genethon, Evry, France) (GenBank™ accession number F06569) was used as a probe to screen a human acute lymphoblastic leukemia CCRF-CEM cDNA library (12). Two cDNA clones, c1-l and c1-s, were isolated. Manual sequencing was performed using the dideoxynucleotide chain termination method (U.S. Biochemicals). An additional 44 nucleotides from the 5’-end were obtained using 5’- RACE of poly(A)- RNA from the CEM cell line using the Marathon cDNA Amplification Kit (CLONTECH) and the ABCB9-specific primers E1-12 (5’-G5AGGGTGCCTGGCCTACCT) and E1-7b (5’- CACTCATGAAGGCCAAAG). The polymerase chain reaction (PCR) products were cloned into pCR2.1 (Invitrogen).

For cloning the mouse ABCB9 cDNA, a 1.3-kilobase (kb) fragment of hABCB9 cDNA (nucleotides (nt) 2152–3512) was used as a probe to screen the mouse brain cDNA library with low stringent hybridization condition. The longest cDNA clone, mi-1-2, was sequenced by BigDye Terminator Cycle Sequencing Ready Reaction using a Model 310 DNA Sequencer (Applied Biosystems).

DNA sequence was analyzed using GCG program software (Genetics Computer Inc.) and protein analysis using the TopPredII program (20). The nucleotide sequences for the hABCB9 and mABCB9 cDNAs have been deposited in the GenBank™ data base under accession numbers AF216494 and AF216495, respectively.

Phylogenetic analysis was performed by aligning amino acid sequences of hABCB9 and mABCB9 along with representative ABC transporters using ClustalX (21). A neighbor-joining bootstrap tree was generated from the alignment using PHYLIPv4.0 (22). The GenBank™ accession numbers for the ABC proteins employed were: TAP1, X57522; TAP2, M74447; ABC7, AB005289; UMAT, AJ003004; MDR1, 4505769; M-ABC1, AF047690; M-ABC2, AF216833; TAPL, AF216494 and AF216495, respectively.

A 3’-end-labeled 1.3-kb fragment of hABCB9 cDNA (nt 2152–3512) was hybridized to human multiple tissue Northern blots (CLONTECH). A 1.2-kb fragment of mABCB9 cDNA (nt 235–1513) was used for hybridization to multiple mouse tissue Northern blot (20 µg of total RNA from designated tissue). The same blot was hybridized to a control probe (a fragment of actin cDNA).

The first strand cDNA was randomly primed by using 1 µg of total RNA of designated tissues in 40 µl of reverse transcript (RT) reaction. Different aliquots of RT reaction (as indicated below in Fig. 3C) were amplified by PCR with primers E1-13A (5’-TGTTGGCTCAACATCATTACGGC), and E1-8B (5’-TCCCTCGGATCAGGCCGCGCCTACCGCAAC-3’, BamHI site underlined and 5’-TACGGAAGTCAATGGGACCTGGGACTC-3’, EcoRI site underlined) was used for PCR amplification of the hABCB9 gene. The PCR fragment was digested with EcoRI and BamHI and cloned into the pGEX2T vector to generate pGEX-1C. The GST/ABC fusion protein was expressed in DH5α cells transformed with pGEX-1C and purified according to the method of Storrie and Madden (26). The fusion protein was injected into New Zealand White rabbits. Immune serum was preadsorbed with fixed SKOV3 cells as described in Childs et al. (24).

Immunofluorescence confirmation of the anti-ABC9 serum was carried out using the methods from Ref. 35. The MalE fusion protein containing the C-terminal 36 amino acids of hABCB9 was linked directly to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) for the preparation of an antigen serum. ABCB9 “positive” and “negative” sera were prepared by adsorbing the anti-ABC9 serum overnight with either the GST or the GST/ABC protein immobilized on Immobilon-P transfer membranes (Millipore), respectively.

Monoclonal antibodies to GP130 (A1/18) (25) and transferrin receptor (G1/122) were generous gift from Dr. H. P. Hauri (University of Basel, Switzerland). Polyclonal antisera to alcohol dehydrogenase was kindly provided by Dr. H. Weiner (Purdue University, West Lafayette, IN). The following antibodies are purchased: monoclonal antibodies to LAMP1 (H4A3) and LAMP2 (H4B4) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City), monoclonal antibody to Calnexin (AF18; Sträussbeler) anti-rabbit IgG conjugated to lissamine rhodamine (LRSC), and goat anti-rabbit IgG conjugated to biotin (Jackson ImmunoResearch).

**Immunofluorescence**—SKOV3 cells were grown on glass slides to 50% confluence, fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) with 5 ml CaCl₂ and 10 ml MgCl₂ (PBS-MC) for 20 min, and wash with PBS-MC three times. The cells were made permeable with 0.1% saponin in PBS-MC with 3% bovine serum albumin (BSA) for 10 min. Primary antibodies were applied in PBS-MC with 3% BSA and 0.1% saponin and incubated at 37 °C for 40 min. After three washes (PBS-MC-0.1% saponin), cells were incubated at 37 °C for 40 min for secondary antibodies (LRSC-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG) in PBS-MC-3% BSA-0.1% saponin. Cells were rinsed twice with PBS-MC-0.1% saponin, rinsed twice with PBS-MC, and then mounted with fluorescent mounting medium (Dako, Carpinteria, CA). The specimens were examined with a Zeiss fluorescence microscope.

**Immunohistochemistry**—Frozen sections of mouse and rat testis were fixed in acetone and stained with the positive or negative serum by using indirect immunoperoxidase technique. The sections were incubated sequentially with 3% H₂O₂-methanol (1:1, v/v) for 20 min, 10% normal goat serum in TBS-T (7.8 g NaCl, 0.15% Tween) for 30 min, primary antibody in TBS-T with 1% BSA for 60 min, biotin-conjugated anti-rabbit IgG in TBS-T with 1% BSA for 60 min, and streptavidin-conjugated horseradish peroxidase (Jackson Immunoresearch) in TBS-T with 1% BSA for 70 min. The binding of the secondary antibody was detected using diaminobenzidine (Sigma) and H₂O₂ (0.0015%). Tissues were counterstained with hematoxylin and mounted in Crystal/Mount (Biomedia Corp.).

**Membrane Preparations**—All procedures were performed at 4 °C. Total membranes from cultured cells were prepared by resuspending cell pellets in ST buffer (250 mM sucrose, 5 mM Tris, pH 8, 2 mM phenylmethylsulfonyl fluoride, 1 µg/ml penicillin A, 1 µg/ml leupeptin, and 1% protamin). Suspended cells were Dounce-homogenized (30 strokes) with a pestle and then centrifuged at 4500 × g for 10 min to remove nuclei and unbroken cells. Total membrane fractions were collected by centrifugation at 100,000 × g (60 min).

The metrizamide (Mz) (Accurate Chemical Co.) step gradients were performed according to the method by Storrie and Madden (26) with modifications. Briefly, monolayer cells (10⁶) were harvested according to the manufacturer’s instructions (Application Sheet 3.2: “homogenization of mammalian cultured cells”; Nycomed Pharma Co., Life Technologies, Inc.). Cell suspensions were Dounce-homogenized for 15 strokes with B pestle and then centrifuged at 1300 × g for 10 min. Supernatant (PNS or Fraction I) were overlaid on top of the first gradient (6% Percoll/Biotin Phalaenius) for 15 min and centrifuged at 50,500 × g for 40 min. Bands at the PNS/6% Percoll interface (Fraction II), the 6% Percoll/17% Mz interface (Fraction III), and the 17% Mz/35% Mz interface (Fraction IV) were collected. Fraction III was then mixed with Mz to 35% and then applied to the bottom of the layer gradient (5% Mz/35% Mz). After centrifugation at 50,500 × g (40 min), bands at the 5% Mz/17% Mz interface (Fraction V)
ABC Transporter ABCB9

A

Fig. 1. A, alignment of amino acid sequences of hABCB9 (h), mABCB9 (m), and TAPL (r). The amino acids identical among the three species are printed as dash lines in sequences of mABCB9 and TAPL. Gaps in the alignment are indicated by open squares. Sequences corresponding to the conserved Walker A and B motifs and the ABC signature sequence are indicated with black bars. The TM segments predicted by TopPred II are boxed. The consensus motifs for N-linked glycosylation are indicated with striped bars. The 43 amino acids that are absent from the splice variant are marked by the line with filled circles at each end. B, alignment of amino acid sequences of hABCB9, TAP1, and TAP2. Only a portion of the alignment is shown. Note that the 43-amino-acid deletion in the hABCB9 splice variant lines up in perfect position with exon 6 of both TAP1 and TAP2 (boxed sequences). The Walker A motif is underlined.
and the 17% Mz/35% Mz interface (Fraction VI) were collected. Protein concentration of each fraction was determined by protein assay (Bio-Rad).

**Percoll Gradient**—275 μl of Fraction V was mixed with 11 ml of 19.25% Percoll in 250 mM sucrose, 10 mM Hepes, pH 7.4, and 0.2 mM EDTA (final Percoll 18.8%), layered on top of a 1-ml cushion of 2.5 M sucrose, and centrifuged in a Type 65 rotor for 60 min at 28,000 × g. 0.5-ml fractions were collected from the bottom of the gradient. Proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) were transferred to an Immobilon-P membrane (Millipore), and Western separated by SDS-polyacrylamide gel electrophoresis (PAGE) were transferred to an Immobilon-P membrane (Millipore), and Western analysis was visualized with ECL reagent (Amersham Pharmacia Biotech).

**RESULTS**

**Characterization of the ABCB9 cDNA**—We initially identified the EST clone c-1ch05 (GenBank™ accession number F06569) as a human ABC gene (ABCB9) by using the BLASTN program (27) to search the dbEST data base with the conserved NBDs of the known ABC transporters as query sequences. This gene was of interest, because it exhibited a restricted high expression pattern to testis and brain by Northern blot analysis. An EST clone corresponding to the same gene was mapped to chromosome 12q24 by Allikmets et al. (28) as part of an investigation to classify 33 human ABC transporters; however, no further characterization of this gene was undertaken. To study the function of ABCB9, we proceeded to clone a full-length cDNA of this gene. Using the cDNA insert of the EST clone c-1ch05 as a probe to screen a human T-lymphoblast CEM cell line cDNA library, we isolated two independent cDNA clones, c1-l and c1-s. Clone c1-l consists of 3468 base pairs (bp), and an additional 44 nucleotides at the 5′-end were obtained by 5′-RACE. The full-length cDNA of hABCB9 is thus 3512 bp consisting of 288 bp of a 5′-untranslated region, an open reading frame of 2298 bp coding for 766 amino acids, and 916 bp of a 3′-untranslated region. As shown in Fig. 1A, the predicted protein sequence reveals that hABCB9 contains structural motifs of a half ABC transporter with a single NBD in its C-terminal region. Hydrophathy analysis using the Eisenberg algorithm of the TopPredII program (20) suggests the presence of 10 potential transmembrane segments in the N-terminal portion of the molecule.

A 3325-bp cDNA clone of the mouse homologue of hABCB9 (mABCB9) encoding a 762-amino acid protein was also isolated from a mouse brain cDNA library. Fig. 1A shows an amino acid sequence alignment of the human, mouse, and rat (TAPL) ABCB9 genes. The similarity between mouse and rat sequences is 99% of the amino acid identity (Table I). The percentage of amino acid identity between human and rodent is 94%, with the major differences clustered in the predicted cytoplasmic domain between the transmembrane segment 4 (TM 4) and TM 5, and the C-terminal end.

**Phylogenetic analysis of ABCB9 with known ABC transporter proteins was performed using the alignment program ClustalX (21) and the tree-generating program PAUP* 4.0 (22).** A neighbor-joining analysis showed that ABCB9 falls into the same cluster as the P-glycoprotein and TAP proteins. Within this cluster, ABCB9 appeared to be closely related to TAP1 and TAP2, which are two ABC proteins found in ER (Fig. 2). Alignment of the three proteins using the GAP program (Wisconsin GCG package) indicated an identity of hABCB9 to TAP 1 and TAP 2 of 38% and 40%, respectively (Table I). The identity between the two TAP proteins was 39%.

**Identification of a Splice Variant of hABCB9**—A shorter cDNA clone of hABCB9 (c1-s) was isolated that had the same sequence as c1-l, except for four internal deletions of 2 bp (from nt 1350 to 1351 and from nt 1902 to 1903), 14 bp (nt1015–1028), and 129 bp (nt1550–1688). Among those, only the 129-bp deletion is in-frame. To determine whether these “deletions” were present in native mRNA or were cloning artifacts, we performed RT-PCR amplification of RNA from CEM cells, the same cell line from which the cDNA clone was isolated. A series of primers were chosen such that each PCR product would include the 129-bp deletion and one or two of the small deletions in c1-s cDNA. Two bands corresponding to c1-s and c1-l were detected from all PCR reactions. The c1-s bands were subcloned for sequence analysis, which showed that the 129-bp, in-frame deletion was found among all PCR clones examined, whereas none of the three small frameshift deletions were found. We concluded that the three frameshift deletions seen in c1-s were cloning artifacts, whereas the 129-bp deletion exists as an isofrom of hABCB9 mRNA.

Southern blot analysis of genomic DNA indicates that there is only one copy of the hABCB9 gene in the human genome (data not shown); therefore, these two forms of mRNA are likely to be derived by alternative splicing. It is known that intron/exon boundaries are often conserved among closely related members in a gene family. Because the genomic structure of the hABCB9 locus is unknown, we checked the genomic arrangement of the two half ABC transporters, TAP1 and TAP2 (NCBI accession numbers X66401 and S57528). Fig. 1B displays a portion of the aligned amino acid sequence of these three proteins and indicates that the 129-bp deletion in hABCB9 corresponds to exon 6 of both TAP1 and TAP2. This

---

**TABLE I**

|            | hABCB9, human | mABCB9, mouse | TAP1, rat | TAP1, human | TAP2, human |
|------------|---------------|---------------|---------|-------------|-------------|
| %          | 100/100       | 95.3/94.0     | 100/100 | 100/100     | 100/100     |

**FIG. 2. Phylogenetic relationship of ABCB9 to other ABC transporters.** A neighbor-joining bootstrap tree was derived from predicted amino acid sequences of representative ABC transporters within the same subgroup. Sequences were aligned using ClustalX, and the tree was generated from the alignment using PAUP* 4.0. MDR1 protein was split into the N-terminal half (n) and the C-terminal half (c) in the analysis. The bootstrap values adjacent to each branch indicate the percentage of times each group was recovered in 500 replicates. (h, human; r, rat; Sceri, Saccharomyces cerevisiae; Spo, Schizosaccharomyces pombe.)
finding strongly suggests that the short form of hABCB9 mRNA is a splice variant that has skipped one exon. This presumptive 129-bp exon encodes 43 amino acids encompassing the last hydrophobic region (predicted TM9 and TM10) before the ATP binding domain (see Fig. 1A). This region corresponds to TMs of P-glycoprotein that are involved in drug binding (29). The splice variant protein may be speculated to have a substrate specificity different from that of the regular hABCB9.

We further examined the existence of this mRNA splice variant in normal tissues by RT-PCR amplification of RNA from human brain and testis, because hABCB9 is expressed relatively highly in these two tissues (Fig. 3A). A pair of primers, flanking the 129-bp deletion region in c1-s, is expected to generate RT-PCR products of 266 and 395 bp from mRNAs corresponding to the cDNA clones c1-s and c1-l, respectively.

RT-PCR products from testis and brain showed two bands of the expected sizes, with the c1-l band being more prominent (Fig. 3B). The c1-s band appears to be less abundant in testis than in brain. These data indicate that the c1-l and c1-s forms of hABCB9 mRNA are coexpressed in both human brain and testis. However, the ratio of these two forms are different.

Northern Blot Analysis of ABCB9 mRNA Expression—The expression patterns of ABCB9 in a panel of normal adult human tissues was investigated by Northern blot analysis. As shown in Fig. 3A, most tissues examined appeared to express low levels of hABCB9, however, relatively high expression was found in the testis, and moderate expression was found in brain, spinal cord, and thyroid. In addition to the expected band (about 3.7 kb), an additional 2.2-kb band was also detected in the tissues in which hABCB9 are highly expressed. The probe used for this Northern blot contains a 1.3-kb
hABCB9 sequence at the 3′-end; therefore, the 2.2-kb band likely presents a specific degradation product or another splice variant.

ABCB9 mRNA expression patterns in mouse tissues were similar to human: high expression was found in mouse testis, and moderate expression was found in brain and spinal cord. It should be noted that, unlike the human blot where poly(A) RNA was used, total RNA was used in the mouse blot (Fig. 3B). Therefore, it is possible that a lower level of expression of mABCB9 in other mouse tissues may not be detected due to the relative insensitivity of the assay.

ABCB9 Protein Expression and Drug Resistance in Transfected Cells—To facilitate the study of potential ABCB9 functions, hABCB9 cDNA was subcloned into an expression vector and stably transfected into a human ovarian carcinoma cell line SKOV3. The expression of hABCB9 in transfected cells (SKOV/ABCB9) was examined by Western blot using polyclonal antiserum against a GST fusion protein containing the C-terminal 36 amino acids of hABCB9. A prominent band of approximately 72 kDa was detected using the ABCB9-specific antibody (Fig. 4A, lanes 2 and 3), which is significantly smaller than the predicted molecular mass of hABCB9 (84.5 kDa). A similar discrepancy has also been reported previously in the E. coli half ABC transporter hemolysin B, a protein with a 79.9-kDa predicted molecular mass but 66 kDa as measured by SDS-PAGE (30). These differences may be due to either the aberrant behavior of highly hydrophobic proteins on SDS gels or post-translational modification.

Using antisera preadsorbed with either GST-ABCB9 fusion protein (GST/ABC) or GST alone, an identical experiment was performed to confirm the specificity of the antibody. Treatment with GST/ABC but not with GST alone abolished the detection of the 72-kDa band (Fig. 4A). A faint band of approximately 72 kDa was also observed in the control cells, but that band was not abolished with GST/ABC-treated antiserum and therefore was not hABCB9.

Five N-glycosylation sequence motifs were found in ABCB9 protein (Fig. 1A). To determine whether hABCB9 contained N-linked oligosaccharides, a membrane preparation was digested with N-glycosidase F prior to Western blot analysis. This treatment did not change the mobility of the detected band, indicating that hABCB9 was not N-glycosylated (Fig. 4B). If the predicted topological structure of ABCB9 is such that the NBD is in the cytoplasmic compartment, then all the N-glycosylation site would be in the predicted cytoplasmic loops and unlikely to be used. Our results appear to confirm this prediction.

It is not known whether ABCB9 is able to function alone as a homodimer or needs to form a complex with another half ABC transporter. It has been demonstrated recently that transfection of a half ABC transporter, BCRP, alone was able to render cells resistant to a number of drugs (31). To determine whether ABCB9 overexpression may give rise to drug resistance, an hABCB9 high expression clone, an intermediate expression clone, and the control cells (vector-transfected cells) were exposed to the compounds colchicine, vinblastine, taxol, actinomycin D, etoposide, methotrexate, daunorubicin, and cisplatin. No difference in drug sensitivity was observed between transfected cells and control cells (data not shown).

Localization of hABCB9 Protein to Lysosomes in Transfected Cells—The subcellular localization of hABCB9 protein was determined by staining of intact permeabilized monolayers of transfected cells (SKOV/ABCB9) and control cells (SKOV3) using anti-ABCB9 antibody and FITC-conjugated secondary anti-rabbit IgG. SKOV/ABCB9 cells exhibited strong granular perinuclear staining, which was not evident in the control.
SKOV3 cells (Fig. 5A). The staining pattern suggested a subcellular organelle location of ABCB9 in SKOV/ABCB9 cells. Further analysis was performed by double immunofluorescent staining using anti-ABCB9 antibody and a panel of organelle markers (Fig. 5, A and B). Anti-ABCB9-specific staining showed a pattern different from those stained with markers of ER, Golgi, and mitochondria (MitoTracker CMXRos, data not shown), but overlapped with the lysosomal markers LAMP1 and LAMP2. Anti-ABCB9-specific staining also overlapped partially with the staining pattern of a marker of endosome (lysosomes and endosomes share some common pathways in membrane trafficking). The colocalization of hABCB9 with lysosomal marker LAMP1 was also observed in another transfected clone in which hABCB9 is moderately expressed and in hABCB9 transiently transfected cells (data not shown).

To confirm the subcellular localization of hABCB9 protein, we performed subcellular fractionation of SKOV/ABCB9 cells. Membrane proteins from the postnuclear supernatant were separated by two step gradients into the ER-mitochondria-rich fraction (Fraction IV) and Golgi-lysosome-rich fraction (Fraction V) (Fig. 6A). Western blot analysis indicated that hABCB9 protein was enriched at the Golgi-lysosome fraction. This Golgi-lysosome-rich fraction was further fractionated by Percoll gradient to resolve lysosomal and Golgi membranes (Fig. 6B). The profile of hABCB9 protein on this gradient overlapped almost completely with that of the lysosomal marker. Thus, the subcellular fraction analysis, in agreement with the immunofluorescence study, indicates that hABCB9 protein is located in the lysosome of SKOV/ABCB9 cells.

**ABCB9 Protein in the Sertoli Cells of Mouse and Rat Testes**—The high level of ABCB9 mRNA expression in human and mouse testis prompted our further characterization of the expression of ABCB9 protein in this tissue. For this analysis, ABCB9-specific polyclonal serum was preadsorbed with either GST or GST-ABCB9 fusion protein to deplete GST-specific antibodies or both GST- and ABCB9-specific antibodies, respectively. The depletion of ABCB9-specific antibodies was evaluated by immunostaining of SKOV/ABCB9 cells. As shown in Fig. 7A, anti-GST-depleted serum displayed perinuclear punctate staining in SKOV/ABCB9 cells; this specific staining was absent from the anti-GST/ABCB9-depleted serum. Control SKOV3 cells were not stained by either antisera (data not shown). These adsorbed sera were designated ABCB9-positive serum and -negative serum, respectively. These sera were further examined by Western blot analysis of membrane proteins from SKOV/ABCB9 cells and mouse and rat testis. Using the positive serum, immunoreactive proteins were observed in mouse and rat testes, which migrated similarly to that seen in the SKOV/ABCB9 cells. These immunoreactive bands were not seen with the negative serum (data not shown).

Immunohistochemistry was performed on frozen sections of...
adult mouse and rat testes to determine the distribution of ABCB9 protein. Both mouse and rat testis showed similar staining patterns when stained with the positive serum, which were absent with negative serum (Fig. 7, B and C). This ABCB9-specific stain was confirmed by using affinity-purified antisera, which exhibited similar staining patterns as shown in Fig. 7 (B and C). Furthermore, this stain can be competed with the GST-ABCB9 protein but not with GST (data not shown). The specific staining patterns seen with the positive serum indicates that ABCB9 protein is expressed in the Sertoli cells of the seminiferous tubules.

The Sertoli cell is a tall (75–100 μm) columnar cell that spans the seminiferous epithelium from the basement membrane to the luminal region. Its lateral surfaces consist of slender processes, which embrace differentiated sperm cells, the spermatocytes and spermatids. Elongated spermatids are embedded in apical invaginations (crypts) of Sertoli cells. The Sertoli cell can be seen to have a very elaborate shape under the light microscope (Fig. 7D). The positive staining demonstrated in Fig. 7 (B and C) represents typical Sertoli cell staining.

DISCUSSION

We have isolated full-length cDNAs of ABCB9 and examined its mRNA expression in selected tissues of human and mouse. The greatest expression was observed in testis with moderate levels in brain and spinal cord. We have localized hABCB9 to lysosomes in transfected cells by two different approaches, immunofluorescent double staining and subcellular fractionation. The cell line, SKOV3, used for transfection has previously been shown to express other ABC transporters localized to plasma membranes (24) or to mitochondrial membranes (12). Therefore, lysosomes are not the default route for transfected ABC proteins in SKOV3 cells. We conclude that lysosomes are likely the natural location of ABCB9, even though it remains to be elucidated what role it plays in that compartment.

Two classes of lysosomal and endosomal targeting signals, YXXΦ (Φ is a bulky, uncharged, amino acid) and di-leucine (or isoleucine) motifs (32), are typically found in the short C-terminal cytoplasmic tails of a group of glycoproteins that traverse the membrane once or twice. Both motif classes can be found in the ABCB9 C terminus; however, it appears that they are also found in many other ABC transporters not known to be localized to lysosomes or endosomes. Therefore, whether these motifs serve to localize ABCB9 signals for ABCB9 needs to be further clarified.

The expression of ABCB9 in Sertoli cells has allowed us to place its lysosomal localization into a functional context. One of the Sertoli cell’s specialized functions is to phagocytose residual cytoplasmic bodies, which detach from late spermatids during spermatiation. In fact, at the stage when fusion of lysosomes with the residual bodies occurs, the lysosomal turnover rate increases sharply (33). The tight junctions between Sertoli cells forms part of the blood-testis barrier separating spermatagonia from spermatocytes and spermatids. Lysosomes may also be critical for the detoxification of blood-borne compounds as part of the barrier function of Sertoli cells (for a review, see Ref. 34). Further investigations will be required to determine the subcellular localization of ABCB9 in Sertoli cells and to delineate its functional significance.

We thank Dr. Jonathan A. Sheps for phylogenetic analysis; Dr. Wayne Vogl for photographing the tissue sections and giving helpful comments; Jefferson Loa for his assistance in immunohistochemical staining; and our colleagues, Douglas Hogue, Ping Lam, Renxue Wang, and Jonathan Sheps for helpful discussions and critical reading of the manuscript.

REFERENCES

1. Higgins, C. F. (1992) Annu. Rev. Cell Biol. 8, 67–113
2. Childs, S., and Ling, V. (1994) in Important Advances in Oncology (DeVita, V. T., Hellman, S., and Rosenberg, S. A., eds) Vol. 2, pp. 21–36, J. B. Lippincott Company, Philadelphia, PA
3. Cole, S. R., and Deacy, R. G. (1998) Biosciences 20, 931–940
4. Rijard, J. R., Rommens, J. M., Keren, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., and Tsui, L.-C. (1989) Science 245, 1066–1073
5. Towsend, A., and Towedale, J. (1993) Semin. Cell Biol. 4, 53–61
6. Meyer, T. H., van Endert, P. M., Uebel, S., Ehring, B., and Tappe, R. (1994) FEBS Lett. 351, 443–447
7. Kamijo, K., Taketani, S., Yokota, S., Osumi, T., and Hashimoto, T. (1990) J. Biol. Chem. 265, 4534–4540
8. Mosser, J., Douar, A. M., Sarde, C. O., Koschis, F., Fril, R., Moser, H., Pouatka, A. M., Mandel, J. L., and Aubourg, P. (1993) Nature 361, 726–730
9. Holzinger, A., Kammerer, S., and Roscher, A. A. (1997) Biochem. Biophys. Res. Commun. 237, 152–157
10. Holzinger, A., Kammerer, S., Berger, J., and Roscher, A. A. (1997) Biochem. Biophys. Res. Commun. 233, 261–264
11. Gartner, J., Moser, H., and Valle, D. (1992) Nat. Genet. 1, 16–23
12. Hogue, D. L., Liu, L., and Ling, V. (1999) J. Mol. Biol. 285, 379–389
13. Cisek, P., Lill, R., and Kispal, G. (1998) FEBS Lett. 441, 266–270
14. Allikmets, R., Raskind, W. H., Hutchinson, A., Schueck, N. D., Dean, M., and Koehler, D. M. (1999) Hum. Mol. Genet. 8, 743–749
15. Ortiz, D. F., Kreppel, L., Speiser, D. M., Scheel, G., McDonald, G., and Ow, D. W. (1992) EMBO J. 11, 2491–2498
16. Cuervo, A. M., and Dice, J. F. (1998) J. Mol. Med. 76, 6–12
17. Havelaar, A. C., de Gast, I. L., Snijders, S., Beerens, C. E., Mancini, G. M., and Verhejen, F. W. (1998) FEBS Lett. 436, 223–227
18. Saint-Pal, A., Codogno, P., and Moore, S. E. H. (1999) J. Biol. Chem. 274, 13547–13555
19. Yamaguchi, Y., Kasono, M., Terada, T., Sato, R., and Maeda, M. (1999) FEBS Lett. 457, 233–236
20. Gartner, J., Moser, H., and Valle, D. (1992) Nat. Genet. 1, 16–23
21. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) Nucleic Acids Res. 25, 4876–4882
22. Swofford, D. L. (1999) PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0b2a, Sinauer Associates, Sunderland, MA
23. Frangioni, J. V., and Neel, B. G. (1993) Anal. Biochem. 210, 179–187
24. Chinla, S., Yeh, R. L., Hui, D., and Ling, V. (1998) Cancer Res. 58, 4160–4167
25. Linstedt, A. D., Mehta, A., Suhani, J., Reggio, H., and Hauri, H. P. (1997) Mol. Biol. Cell 8, 1073–1087
26. Storrie, B., and Madden, E. A. (1990) Methods Enzymol. 182, 203–235
27. Altschul, S. F., Boguski, M. S., Gish, W., and Wootton, J. C. (1994) J. Mol. Biol. 265, 703–718
28. Allikmets, R., Begg, M., and Dean, M. (1996) Hum. Mol. Genet. 5, 1649–1655
29. Greenberger, L. M. (1993) J. Biol. Chem. 268, 11417–11425
30. Juranka, P., Zhang, F., Kulpa, J., Endicott, J., Blight, M., Holland, I. B., and Ling, V. (1992) J. Biol. Chem. 267, 3764–3770
31. Doyle, L. A., Yang, W., Abruzzo, L. V., Kroghmann, T., Gao, Y., Rishi, A. K., and Ross, D. D. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 15665–15670
32. Hunziker, W., and Geuze, H. J. (1996) The Sertoli Cell (Russell, L. D., and Gisvold, M. D., eds) pp. xxii–xxv, Cache River Press, Clearwater, FL
33. Harlow, E., and Lane, D. (eds) (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY