Membrane Topology of the ATP Binding Cassette Transporter ABCR and Its Relationship to ABC1 and Related ABCA Transporters

IDENTIFICATION OF N-LINKED GLYCOSYLATION SITES*

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Running Title: Membrane Topology of ABCR

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ABCR is a member of the ABCA subclass of ATP binding cassette transporters that is responsible for Stargardt macular disease and implicated in retinal transport across photoreceptor disk membranes. It consists of a single polypeptide chain arranged in two tandem halves, each having a multi-spanning membrane domain followed by a nucleotide binding domain. To delineate between several proposed membrane topological models, we have identified the exocytoplasmic (extracellular/lumen) N-linked glycosylation sites on ABCR. Using trypsin digestion, site-directed mutagenesis, concanavalin A binding and endoglycosidase digestion, we show that ABCR contains eight glycosylation sites. Four sites reside in a 600 amino acid exocytoplasmic domain of the N terminal half between the first transmembrane segment H1 and the first multi-spanning membrane domain, and four sites are in a 275 amino acid domain of the C half between transmembrane segment H7 and the second multi-spanning membrane domain. This leads to a model in which each half has a transmembrane segment followed by a large extracytoplasmic domain, a multi-spanning membrane domain and a nucleotide binding domain. Other ABCA transporters including ABC1 linked to Tangier disease are proposed to have a similar membrane topology based on sequence similarity to ABCR. Studies also suggest that the N and C halves of ABCR are linked through disulfide bonds.
ABCR, formerly known as the rim protein, is an ATP-binding cassette (ABC) transporter found in vertebrate retinal photoreceptor cells (1-3). It is localized along the rim region of photoreceptor rod outer segment disk membranes (1,4,5) and more recently has been found in human foveal and peripheral cone outer segments (6). Several studies have implicated ABCR in the retinoid cycle, possibly functioning as a retinal extruder or retinal-phosphatidylethanolamine flippase to facilitate the removal of all-trans retinal from disk membranes following the photobleaching of rhodopsin (7-9).

Mutations in the ABCA4 gene encoding ABCR have been linked to Stargardt disease, a relatively common juvenile macular dystrophy characterized by a decrease in visual acuity, progressive bilateral atrophy of the central retina and the accumulation of yellow deposits within the retinal pigment epithelial cell layer (2,10-13). Mutations in ABCA4 have also been linked to related disease variants including late-onset fundus flavimaculatus (14), cone-rod dystrophy (15), retinitis pigmentosa-like dystrophy (16,17) and age-related macular degeneration (18), although the latter remains controversial (19).

ABCR is a member of the ABCA subclass of ABC transporters (20). Like most other mammalian ABC transporters, members of this subclass consist of a single long polypeptide chain organized into two tandemly arranged halves. Each half contains a membrane-spanning domain (MSD) followed by a cytoplasmic nucleotide binding domain (NBD). The ABCA subclass is distinguished from other ABC transporter subclasses by the presence of a large domain between the first NBD (NBD-1) and the
second MSD (MSD-2) that is interrupted by a hydrophobic segment, initially designated as HH1 (21). In addition, these proteins typically contain a hydrophobic stretch of amino acids close to the N-terminus that is preceded by several positively-charged amino acids. ABCR shows a high degree of sequence identity to three other members of this subclass (ABC1 or ABCA1, ABC2 or ABCA2, ABC-C or ABCA3) for which the complete primary structure has been determined (1,2,22,23). For example, ABCR is over 50% identical in sequence and has a similar hydropathy profile to ABC1, a putative cholesterol transporter that has been linked to Tangier disease (21,24-26).

Several topological models have been proposed for ABCR and ABC1. Luciani et al. (21), first proposed a model for ABC1 in which each half of the protein contains a NBD that is preceded by a MSD having a cluster of six transmembrane segments connected through relatively short hydrophilic segments (Fig 1A). A very large N-terminal cytoplasmic domain precedes MSD-1, and a large putative cytoplasmic regulatory region interrupted by a hairpin hydrophobic segment HH1 is located between NBD-1 and MSD-2. Illing et al (1) suggested a different model for ABCR (Fig 1B). A hydrophobic segment near the N-terminus (H1) and the HH1 segment, referred to as H7, were envisioned to be transmembrane segments and each MSD was suggested to consist of five membrane spanning segments. A characteristic feature of this model is the presence of two very large exocytoplasmic (extracellular/lumenal) domains in both the N and C terminal halves of the protein and the absence of the large cytoplasmic regulatory domain. Finally, Azarian and Travis (3) and more recently Sun et al. (27) proposed a hybrid model for ABCR that has some features of the first two models (Fig 1C).
specifically, in their model, the topology of the C-terminal half is similar to the model in Fig 1A with a HH1 segment that only partially inserts into the membrane, and the N-terminal half shows structural features of the model in Fig 1B with a H1 transmembrane segment at the N-terminus. A similar hybrid model has been recently proposed for ABC1(28).

N-linked glycosylation is known to occur on an asparagine residue (N) within a consensus sequence (-N–X–S/T-). Since N-linked glycosylation is known to take place on the lumen side of the endoplasmic reticulum, it can be used to define exocytoplasmic domains of membrane proteins. In this paper, we have identified the N-linked glycosylation sites on ABCR using trypsin digestion of ABCR in native ROS membranes, site-directed mutagenesis of heterologously expressed ABCR, concanavalin A labeling and endoglycosidase digestion. We show that ABCR has four N-linked glycosylation sites on each half of the protein. These sites are positioned within a 600 amino acid segment between H1 and MSD-1 in the N half and a 275 amino acid segment between H7 and MSD-2 in the C half of ABCR, thereby supporting the existence of two large ECDs as depicted in the model shown in Fig1B. Other members of the ABCA subclass of transporters including ABC1 and ABC2 display considerable sequence similarity to ABCR within the ECDs, and therefore are likely to exhibit a similar membrane topology and domain organization as ABCR. We also provide evidence that the C and N halves of ABCR interact through disulfide bonds. These studies provide new insight into the organization of ABCR and other ABCA transporters in the membrane and define domains that are present on the exocytoplasmic side of the membrane.
EXPERIMENTAL PROCEDURES

Preparation and Trypsin Digestion of ROS—ROS were isolated from either frozen bovine retina or human retina from donor eyes (UBC Eye Bank) using the continuous sucrose gradient centrifugation as previously described (29). ROS (1 mg/ml protein) were lysed in hypotonic buffer (10mM Tris, pH 7.4, 1mM EDTA), pelleted by centrifugation and resuspended in 0.25ml of Tris buffer (20mM Tris, pH 8.0, 0.5mM EDTA). Trypsin digestion was carried out by incubating the ROS membranes with an equal volume of Tris buffer containing either 0.8µg (for bovine ROS) or 2.0µg (for human ROS) of L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK) treated trypsin (Sigma T1426) for 30 min on ice. The reaction was stopped by the addition of 2 volumes of soybean trypsin inhibitor (2mg/ml) in 20mM Tris, pH 7.4. The membranes were pelleted by centrifugation for 15 min in a Beckman Optima TL100.4 rotor at 30,000 rpm and resuspended in Tris buffer. In some experiments, undigested and trypsin digested ROS were incubated with 20 mM N-ethylmaleimide (final concentration) in Tris buffer for 20 min to block any reactive sulfhydryl groups prior to analysis by SDS gel electrophoresis and Western blotting as previously described (30).

Monoclonal Antibodies—The Rim 3F4 monoclonal antibody against an epitope (YDLPLHPRT) near the C-terminus of bovine ABCR has been described (1). The Rim 5B4 monoclonal antibody (6) was generated from a mouse immunized with a fusion protein consisting of amino acids 113-883 of human ABCR in frame with glutathione-
S-transferase. The binding site for the Rim 5B4 antibody (LEDTGLHHK) was
determined by epitope mapping using synthetic peptides as previously described (1).

Generation of ABCR Mutants—The human ABCR cDNA (2), a generous gift of Jeremy
Nathans, was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) as previously reported
(9) and is designated as pcDNA3/ABCR. The serine (S) or threonine (T) residue within
the consensus sequence (-N-X-S/T-) for N-linked glycosylation was replaced with an
alanine (A) to prevent glycosylation on the asparagine residue (N). ABCR (Δ8) had
mutations in eight N-linked glycosylation sites within two ECDs at the following
positions: N98 (S100A), N415 (T417A), N444 (T446A), N504 (T506A), N1469
(T1471A), N1529 (S1531A), N1588 (S1590A) and N1662 (T1664A) as shown in Fig 2.
All mutations were produced with the QuikChange Site-Directed Mutagenesis Kit
(Stratagene, San Diego, CA) using the appropriate synthetic oligonucleotides. A
truncated ABCR (N-tr-ABCR) was generated by digesting pcDNA3/ABCR with NarI to
remove the C-terminal half of ABCR. Mutations at glycosylation sites N98 (S100A),
N415 (T417A), N444 (T446), N504 (T506A) were constructed as described above and
are listed in Table I. The DNA sequences of all constructs were determined to verify the
presence of the desired mutation and the absence of random mutations.

COS-1 Cell Transfection and Membrane Preparation—COS-1 cells were maintained as
described (9). Typically, cells in one 10-cm dish were transfected with 30 µg of the
pcDNA plasmid containing WT or mutant ABCR using the calcium phosphate procedure
(31). COS-1 membranes were isolated 48 h posttransfection from ten 10-cm dishes as follows: cells were harvested in hypotonic buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 7.4) and recovered by centrifugation. The cell pellet was washed twice in hypotonic buffer and resuspended in the same buffer containing Complete Protease Inhibitor Cocktail (Roche Diagnostics, Laval, Quebec) at 4 °C. After 1 h, the cells were disrupted using a glass homogenizer and subsequently passed through a 28G needle. The membranes were then layered onto a sucrose gradient consisting of 5% and 60% (w/v) sucrose in gradient buffer (10 mM Tris-Cl, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.1 mM EDTA, pH 7.4) and centrifuged in a Beckman SW 27.1 rotor at 21,000 rpm for 1 h at 4 °C. The membranes, which banded as a cloudy layer on top of the 60% sucrose solution, were collected with a syringe equipped with an 18G needle, diluted with 4 volumes of hypotonic buffer, pelleted by centrifugation at 135,000 x g for 20 min in a TLA100.4 rotor (Beckman) at 4 °C. The membrane pellet was resuspended in gradient buffer, 10% glycerol and protease inhibitor cocktail to give a final protein concentration of about 3.5 mg protein/ml and stored at −80 °C until used. Typically, the expression level of the ABCR(Δ7) and ABCR(Δ8) mutants was approximately 5-6 fold lower than the expression level of the ABCR(WT) protein.

*Photoaffinity Labeling of ABCR*—Photoaffinity labeling of ABCR with 8-azido-[α-^{32}P]ATP was carried out as follows: 0.5 mg of ROS membranes and 3.5 mg of COS-1 membranes were centrifuged in a Beckman Optima TL100.4 rotor at 30,000 rpm for 15
min and the pellet was resuspended in 120 µl of 20 mM Hepes, pH 7.4, 150 mM NaCl₂ and 5mM MgCl₂. 8-azido-[α⁻³²P]ATP (ICN; 20Ci/mmoll) was dried under nitrogen, resuspended in 12 µl resuspension buffer and added to each sample to give a final concentration of 4 µM azido-ATP. The samples on ice were irradiated at a distance of 11 cm for 10 min with ultraviolet light (mineral light UV Lamp; 254nm). The membranes were then washed in 20mM Hepes, 0.5mM CaCl₂. In control experiments the photoaffinity labeling reaction was carried out in the presence of 4 mM ATP. Heterologously expressed ABCR was immunoprecipitated with the Rim 3F4-Sepharose matrix (see below) prior to analysis by SDS gel electrophoresis. Azido-[α⁻³²P]ATP labeling was detected with a phosphoimager or by film-based autoradiography.

**Immunoprecipitation**—Monoclonal antibodies were coupled to CNBr-activated Sepharose 2B beads as described previously (1). ROS membranes and COS-1 cell membranes were solubilized at 1-2 mg/ml in solubilization buffer (20mM Tris, 0.15M NaCl, and 1-2% Triton X-100) and incubated for 30 min at 4°C with the Rim 3F4-Sepharose beads pre-washed with column buffer (20mM Tris, pH 7.4, 0.15M NaCl, and 0.1% Triton X-100). The affinity matrix was washed in an Ultrafree filter unit (Millipore Corp., Bedford, MA) with column buffer by low-speed centrifugation. After extensive washing, the bound protein was eluted from the beads with 100-250 µl extraction buffer consisting of 20mM Tris, 0.15M NaCl, 0.1% Triton X-100 and 4% SDS. The eluted fraction contained 20-40 ng of ABCR/µl. Truncated ABCR proteins
were isolated in the same way except that Rim 5B4-Sepharose was used to
immunoprecipitate the protein.

\textbf{N-glycosidase F (PNGase) digestion}—Human ROS membranes (12.5 µg) or COS-1
membranes (350 µg) in 10 µl of 20 mM Tris-HCl, pH 7.4 were incubated in 1 µl of
denaturing buffer (5% SDS, 10% β-mercaptoethanol) for 10 min at 32°C and then added
to 2 µl of 10% NP40, 2 µl 10X G7 buffer (0.5M sodium phosphate, pH 7.5), 3 µl 20 mM
TrisHCl, pH 7.4, and 2 µl (1,000 units) glycosidase F (PNGase F; New England Biolabs,
Mississauga, ON). Control samples were treated in the same way except in the absence
of enzyme. After 2 h at 32°C, the samples were added to an equal volume SDS cocktail
and 15 µl of each sample was resolved on a low bis-acrylamide (0.4%) SDS
polyacrylamide gel.

\textbf{SDS gel electrophoresis and Western Blotting}—The proteins were denatured in SDS
cocktail consisting of 0.01 M Tris, pH 6.8, 4% SDS, 20% sucrose in the presence or
absence of 4% 2-mercaptoethanol and separated by SDS gel electrophoresis on 6.5 or 7
% polyacrylamide gels. Proteins were transferred at 300 mA for 40 min to either
Immobilon P membranes (Millipore Corp., Bedford, MA) for antibody labeling or
nitrocellulose membranes (Schleicher & Schuell, Keene, NH) for biotinylated
concanavalin A labeling using a semidry transfer unit (1).

Western blots were blocked in 1% skimmed milk in PBS (20 mM sodium
phosphate, pH 7.4, 150 mM NaCl) for 30 min at room temperature and subsequently labeled with the Rim 3F4 or Rim 5B4 hybridoma culture fluid diluted approximately 1:20 in PBS containing 0.1% milk for 1 h. After washing in PBS containing 0.05% Tween-20, the blots were labeled with sheep-anti-mouse Ig-peroxidase for enhanced chemiluminescence (Amersham Pharmacia Biotech, Baie d’Urfé, Québec) as previously described (1).

Concanavalin A labeling was carried out as follows: The nitrocellulose membrane containing the transferred proteins was blocked in 5% bovine serum albumin (Sigma, St. Louis, MO) in PBS containing 0.05% Tween 20 (PBS-T) at 37 °C for 1 h. The membrane was then incubated with 5% bovine serum albumin in PBS containing 5 µg/ml biotinylated concanavalin A (Pierce, Rockford, IL) for 1 h at room temperature, washed extensively with PBS-T, and finally incubated with peroxidase-conjugated streptavidin (diluted 1:40,000) (DAKO, Mississauga, Ontario) for detection by enhanced chemiluminescence.
RESULTS

Both the N- and C-terminal Halves of ABCR in ROS Are Glycosylated—Previous concanavalin A binding and endoglycosidase digestion studies have shown that ABCR is a membrane glycoprotein with one or more oligosaccharide chains (1,32). To determine if the glycosylation sites are located on the N-terminal half, the C-terminal half, or both halves of the protein, ABCR in ROS membranes was cleaved in half with trypsin (1) and the complex was immunoprecipitated with the Rim 3F4 monoclonal antibody coupled to Sepharose. The fragments were separated on SDS gels and analyzed on Western blots probed with monoclonal antibodies to an epitope within the N-terminal half (Rim 5B4) and the C-terminal half (Rim 3F4) and with concanavalin A. Fig 3 shows that trypsin efficiently digested both bovine and human ABCR into a 118-120 kDa N-terminal fragment and a 114-116 kDa C-terminal fragment. The site of trypsin cleavage of bovine ABCR has been shown to be at K1309 (1). Concanavalin A labeled both fragments indicating that the N and C terminal halves of ABCR are glycosylated.

N-linked Glycosylation Sites Are Present within the Two Large Exocytoplasmic Domains (ECDs) of ABCR—Primary structural analysis indicates that there are 17 consensus sequences (-N-X-S/T-) for N-linked glycosylation in human ABCR. However, most of these are present in the NBDs and in or near the putative transmembrane segments and therefore are not predicted to be glycosylated. We reasoned that the glycosylation sites most likely reside within the large hydrophilic
domains of ABCR. Using the model of Illing et al.\cite{1}, we selected the 8 consensus sequences for N-linked glycosylation located in ECD-1 and ECD-2 for detailed studies (Fig 2). A series of ABCR mutants were constructed in which the serine (S) or threonine (T) residues in the consensus sequences were replaced with an alanine (A), thereby preventing glycosylation (Table I).

In initial studies three constructs were made: one containing mutations in the four consensus glycosylation sequences within ECD-1 (ABCR (Δ4N)); one containing mutations in the four consensus sequences within ECD-2 (ABCR (Δ4C)); and one with mutations at all eight sites (ABCR (Δ8)). The WT and mutant ABCR proteins were expressed in COS-1 cells, immunoprecipitated and analyzed on Western blots labeled with the Rim 3F4 antibody and concanavalin A. The expression levels varied for the different proteins. Typically, the ABCR (Δ4) mutants expressed at roughly 75\% that of WT, whereas the ABCR (Δ8) expression was considerably lower, and generally less than 20\% that of WT. Similar amounts of the expressed ABCR protein were applied to the lanes of a SDS polyacrylamide gel for comparative analysis. Fig 4A shows that both the expressed WT and mutant proteins migrated as a single polypeptide with apparent molecular mass (~220 kDa) similar to that of native ABCR of ROS. Concanavalin A labeled ABCR(WT) and ABCR(Δ4N) and ABCR (Δ4C) with four glycosylation sites mutated, but not ABCR(Δ8) with all eight sites mutated.

The effect of N-glycosidase F treatment on native and expressed ABCR was also analyzed on a low bis-acrylamide SDS gel that could resolve small differences in protein mobility. Western blots in Fig 4B show that N-glycosidase F treatment resulted in a
small increase in mobility of native ABCR in ROS membranes and WT ABCR in COS-1 cells. The deglycosylated ABCR co-migrated with the ABCR(Δ8) mutant which itself was insensitive to glycosidase treatment. Concanavalin A did not label ABCR after N-glycosidase F treatment (data not shown).

Azido-ATP photoaffinity labeling was carried out to determine if the expressed ABCR(Δ8) mutant was folded properly with respect to ATP binding. Fig 4C shows that both the ABCR(Δ8) mutant and ABCR(WT) were labeled with azido-ATP. As previously reported (1), excess cold ATP eliminated azido-ATP labeling confirming the specificity of the labeling reaction (data not shown).

Together, these results indicate that heterologously expressed ABCR, like native ROS ABCR, is glycosylated on both the N-terminal and C-terminal halves of the protein. One or more of the four sites in each of ECD-1 and in ECD-2 are glycosylated and responsible for concanavalin A binding to ABCR.

*All Four Sites within ECD-2 of ABCR Are Glycosylated*—To determine which sites within ECD-2 of ABCR are glycosylated, four ABCR(Δ7) constructs were made. All four glycosylation sites of ECD-1 and three of the four sites in ECD-2 were mutated, leaving one site available for N-linked glycosylation (Table I). These mutants expressed at similar levels as the ABCR(Δ8) mutant. As shown in Fig 5 all four ABCR(Δ7) mutants labeled with concanavalin A indicating that all four sites in ECD-2 undergo N-linked glycosylation.
All Four Sites within ECD-1 of Truncated ABCR Are Glycosylated—A truncated ABCR construct (N-tr-ABCR(WT)) was made consisting of amino acid residues 1-1323. Alanine mutations were introduced at the serine or threonine residues within all four glycosylation sites of ECD-1 (N-tr-ABCR(Δ4)) or in three of the four sites (Table I). Fig 6A shows that concanavalin A labeled the truncated protein containing the four intact glycosylation sites (N-tr-ABCR(WT)) and proteins with three of the four sites mutated (N-tr-ABCR(Δ3-N98), N-tr-ABCR(Δ3-N417), N-tr-ABCR(Δ3-N446), N-tr-ABCR(Δ3-N506)). No concanavalin A labeling was observed for the N-tr-ABCR(Δ4) with all four glycosylation sites mutated. We further investigated the ability of the truncated proteins to bind ATP. Fig 6B shows that azido-ATP labeled both N-tr-ABCR(WT) and N-tr-ABCR(Δ4) indicating that these truncated proteins retained their ability to bind ATP.

Analysis of Undigested and Trypsin Digested ABCR under Disulfide Reducing and Nonreducing Conditions—The migration behavior of ABCR on SDS gels was analyzed under disulfide reducing and nonreducing conditions. Fig 7 shows that ABCR migrates more slowly under nonreducing conditions than reducing conditions indicating that ABCR contains one or more disulfide bonds that affect its structural properties. The migration behavior of trypsin treated ABCR under reducing and nonreducing conditions was also compared. Under reducing conditions, trypsin effectively cleaved ABCR into the N and C halves (see also Fig 3). Interestingly, under nonreducing conditions the trypsinized ABCR co-migrated with undigested ABCR indicating that the N and C
halves were held together by disulfide bonds.
Discussion

Membrane Topology and Structural Features of ABCR—Computer-derived hydropathy profiles and comparative protein analysis serve as a useful starting point in developing working models for the topology of novel membrane proteins. Such analyses, however, can lead to the formulation of different topological models as in the case of the ABCA subclass of transporters. Experimental data is needed to distinguish between the different models and identify important domains that are present on each side of the membrane for structure-function analysis.

In this study we have identified the exocytoplasmic (intradiskal) N-linked glycosylation sites on ABCR and used this information to discriminate between several proposed topological models for ABCR as depicted in Fig 1. Both the N and C terminal halves of native and heterologously expressed ABCR bind concanavalin A indicating that both are glycosylated. Site-directed mutagenesis studies further reveal that ABCR has eight N-linked glycosylation sites, four of which reside within the large hydrophilic ECD-1 situated between H1 and MSD-1 on the N-terminal half, and four reside within the hydrophilic ECD-2 between H7 and MSD-2 on the C-terminal half of ABCR (Fig 8A). This organization is consistent with the model proposed by Illing et al. (1). The finding that N1588 on human ABCR is glycosylated is consistent with earlier protein sequence data suggesting that the equivalent asparagine in native bovine ABCR of ROS is modified with an oligosaccharide chain (1). The H7 segment, previously thought to only partially insert into the bilayer in a hairpin loop, is envisioned to traverse the lipid
bilayer in order to connect the cytoplasmic NBD-1 to exocytoplasmic ECD-2. ABCR and other ABCA transporters (see below), therefore, do not contain an extended cytoplasmic regulatory domain as first proposed (21), although they may have a shorter regulatory segment between NBD-1 and H7. The H1 segment is also proposed to be a transmembrane segment. It contains a sufficient number of hydrophobic amino acids to span the lipid bilayer. Furthermore, this segment is preceded by several positively charged amino acids, a characteristic signature of membrane proteins having their N-terminus localized on the cytoplasmic side of the membrane (33). Studies reported here suggesting that H1 of ABCR is a membrane spanning segment is consistent with recent studies (28) showing that the analogous segment in ABC1 serves as a signal-anchor sequence important in the translocation of the ECD-1 into the exocytoplasmic space.

Previously, it has been reported that mutant forms of P-glycoprotein can adopt multiple membrane topographies which differ in glycosylation and mobility on SDS gels (34). Multiple topologies does not appear to be the case for the ABCR proteins examined in this study. Both native and heterologously expressed wild-type and mutant ABCR proteins all migrate as a single polypeptide as analyzed on Western blots labeled with monoclonal antibodies and concanavalin A as well as for autoradiograms detecting azido-ATP labeling. Furthermore, glycosidase F treatment of native ABCR in ROS membranes and heterologously expressed WT ABCR results in a deglycosylated protein that migrates more rapidly as a single band. This protein comigrates with the ABCR(Δ8) mutant that lacks the ability to undergo N-linked glycosylation.

An inherent problem with the model, however, is the number and identity of
transmembrane segments in MSD-1 and MSD-2. Hydropathy plots typically predict seven transmembrane segments in each half of ABCR and other ABCA transporters. One segment is H1 in the N-half (H7 in the C-half) and six are in the MSD. However, since H1 and H7 are most likely transmembrane segments as discussed above, each MSD must have an odd number of transmembrane segments to satisfy the general topological organization of the protein within the membrane, i.e. ECDs located on the exocytoplasmic side and the NBDs on the cytoplasmic side of the membrane. Therefore, either one of the predicted transmembrane segments within the MSD is not a membrane spanning segment or alternatively a more hydrophilic segment, not predicted to span the lipid bilayer, is in fact a transmembrane segment. In the case of P-glycoprotein, there is strong evidence that this transporter contains six transmembrane segments in each half of the protein (35,36). Studies are currently underway to define the number and identity of transmembrane segment in the MSDs in ABCR.

Oligosaccharide chains of glycoproteins have been implicated in a number of biological processes including intracellular recognition, protein folding, protein stability and intercellular sorting. The role of the N-linked oligosaccharide chains on ABCR is not known at the present time. However, three of the four N-linked glycosylation sites in ECD-1 and all four sites in ECD-2 are conserved for human, bovine and mouse ABCR suggesting that these sites are important. Our studies indicate that expression levels are reduced when all or most of the glycosylation sites are removed, although these mutants still retain the capacity to bind ATP. These results suggest that glycosylation may play a role in stabilizing ABCR against degradation during biosynthetic processing in the
endoplasmic reticulum. A similar effect has been reported for P-glycoprotein (37). In this study deglycosylated P-glycoprotein was shown to be active as a transporter. However, the number of cells that confer drug resistance was significantly lower than for WT P-glycoprotein. This has been interpreted to indicate that the oligosaccharide chains are important in stabilizing the protein and/or for the sorting of P-glycoprotein to the plasma membrane.

To date over 180 mutations in ABCR have been linked to Stargardt macular degeneration and related retinal dystrophies (2,10-13,19) (38). Missense mutations are distributed evenly throughout the ABCA4 gene. A scan of disease causing mutations reveals that one mutation (S100P) in a N-linked glycosylation site has been found in a patient with Stargardt’s disease (38). This mutation would prevent the occurrence of N-linked glycosylation on N98, but it may also affect the folding of the protein due to the introduction of a proline residue. Analysis of the expression level and ATPase activity of the S100P mutant may provide insight into the effect of this mutation on the structure-function relationships of ABCR as has been carried out for other disease-linked mutations (27).

_Disulfide Bonding in ABCR—_ ABCR migrates more rapidly under disulfide reducing conditions than under nonreducing conditions indicating that ABCR contains one or more disulfide bonds that contribute to its structural properties. At the present time it is not known if ABCR contains only intramolecular disulfide bonds or if intermolecular disulfide bonds also exist that result in the formation of a disulfide bond.
mediated oligomeric complex in the membrane. However, the finding that trypsin-treated ABCR separates into an N and C half under disulfide reducing conditions, but stays together as a large polypeptide that co-migrates with undigested ABCR under nonreducing conditions suggests that the N and C halves of ABCR are linked together through one or more disulfide bonds. These disulfide bonds most likely occur between cysteine residues that are located in ECDs exposed to the oxidizing environment of the disk lumen. ECD-1 and ECD-2 of human ABCR contain 9 and 5 cysteine residues, respectively, all of which are conserved in bovine and mouse ABCR. On this basis, we suggest that the ECD-1 and ECD-2 regions are in close proximity to one another and stabilized by one or more disulfide bonds (Fig 8). The importance of cysteine residues in the ECDs is underscored by their involvement in disease states. Several missense mutations in cysteine residues within ECD-1 (C54Y, C75G) and ECD-2 (C1488R, C1490Y) have been linked to Stargardt disease(13,19,38,39). In addition, missense mutations leading to the introduction of a cysteine residue within the ECDs have also been found in individuals with Stargardt disease(13,40). Removal or introduction of cysteine residues into exocytoplasmic domains can alter disulfide bonding. This in turn can cause misfolding of the protein leading to a loss in function. This has been demonstrated for peripherin-2, a disk membrane protein that consists of a large exocytoplasmic domain containing an intermolecular and several intramolecular disulfide bonds.(30,41)

Membrane Topology of ABC1 and Other Members of the ABCA
Sequence alignment of three members of the ABCA subclass (ABC1, ABC2, and ABC3) indicates that these proteins most likely have a similar membrane topology and domain organization as ABCR. In the N-terminal half, they all contain a H1 segment of 18-22 amino acids preceded by three or more positively charged arginine or lysine residues, a large hydrophilic domain with multiple consensus sequences for N-linked glycosylation, a MSD with multiple putative transmembrane segments and a NBD. This arrangement is repeated in the C-terminal half of the protein. Of particular note is the relatively high degree of sequence similarity between ABCR and ABC1 within the ECDs. Three N-linked glycosylation sites in the ECD-1 and two sites in ECD-2 are conserved between ABCR and ABC1 (Fig 8B). Sequences flanking these sites also show a high degree of sequence identity and conservation. The similarity between ABCR and ABC1 extends to the cysteine residues within the ECDs. All nine cysteine residues in the 600 amino acid ECD-1 and all five cysteines in the 275 amino acid ECD-2 are conserved in the corresponding positions in ABC1. Interestingly, a missense mutation in one of the conserved cysteine residues (C1478R) in ECD-2 of ABC1 is responsible for Tangier disease, an autosomal recessive disease linked to the accumulation of cholesterol esters (25). This suggests that this cysteine is important in the structure-function relationship of ABC1, possibly playing a role in the formation of critical disulfide bonds.

ABC2 and ABC3 also show considerable sequence similarity to ABC1 and ABCR and exhibit similar structural features and domain organization. However, ABC2 has more consensus sequences for N-linked glycosylation in the ECDs than ABCR or ABC1 suggesting that it may be more heavily glycosylated, and ABC3 has much smaller
ECDs that result in a shorter length of the polypeptide. Nonetheless, these members of
the ABCA subclass most likely have a similar membrane topology as ABCR.

In summary our results indicate that ABCR has one large glycosylated ECD
situated between H1 and MSD-1 in the N half and another between H7 and MSD-2 in
the C half. These domains contain conserved cysteine residues that appear to participate
in intramolecular disulfide bonds that are likely to be crucial for the proper folding of the
protein. ABC1 and related ABCA transporters are likely to have a similar membrane
topology as ABCR based on the high degree of amino acid conservation and similar
structural features. They also are likely to contain intramolecular disulfide bonds within
the ECDs that play a crucial role in protein folding and stability.

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REFERENCES


Footnotes

1 Abbreviations used are: MSD, membrane spanning domain; NBD, nucleotide binding domain; ECD, exocytoplasmic domain; ROS, rod outer segment; WT, wild-type; PBS, phosphate buffered saline; N-trABCR, truncated ABCR containing the N terminal half of the protein
**Fig.1: Proposed topological models for ABCR and ABC1.**  

**A.** Topological model first proposed by Luciani et al. (21) for ABC-1. Both the N-terminal and C-terminal halves contain a multi-spanning membrane domain (MSD-1 and MSD-2) preceded by a nucleotide binding domain (NDB-1 and NDB-2). The MSDs consist of six transmembrane segments connected by short hydrophilic loops. Two very large cytoplasmic domains are present: one comprises the N-terminus and the other, suggested to be a regulatory domain (R), is interrupted by a highly hydrophobic segment (HH1) that is envisioned to partially insert into the lipid bilayer in a hairpin loop.  

**B.** Topological model proposed by Illing et al. (1,10) for ABCR. In this model, the N-terminal half contains a transmembrane segment designated H1 near the N-terminus. A large extracellular domain (ECD-1) precedes the MSD-1 consisting of five transmembrane segments. The C-terminal half has a similar arrangement with the HH1 segment (now referred to as H7) being viewed as a transmembrane segment. A second large ECD-2 precedes the MSD-2. Numbering represents the approximate sequence locations of the various domains in ABCR.  

**C.** Hybrid topological model proposed by several groups (3,27,28) showing structural features of models in **A** and **B**. The N-terminal half is equivalent to the model in **B** and the C-terminal half is related to the model in **A**. Only one large ECD (ECD-1) is present and the HH1 segment that partially inserts into the bilayer is retained.
Fig 2. A linear diagram showing the location of the consensus sequences for N-linked glycosylation within the extracytoplasmic domains (ECDs) of ABCR. The diagram is based on the model presented in Fig 1B and shows the location of the four putative N-linked glycosylation sites in the N-terminal half and the four sites in the C-terminal half. The positions of the asparagine (N) residues are indicated. The location of the epitopes for monoclonal antibodies Rim 3F4 and Rim 5B4 are shown along with a trypsin cleavage site (1).

Fig 3. Western blots of the trypsin generated N-terminal and C-terminal halves of ABCR labeled with monoclonal antibodies and concanavalin A. Bovine (bROS) and human (hROS) rod outer segment membranes were digested with trypsin and the ABCR complex was immunoprecipitated with the Rim 3F4-Sepharose matrix. The undigested ROS membranes and immunoprecipitated, trypsin-cleaved ABCR were separated on SDS polyacrylamide gels and Western blots were labeled with Rim 5B4 and Rim 3F4 antibodies that detected the N-terminal and C-terminal halves of ABCR, respectively, and concanavalin A (ConA). Both the N-terminal and C-terminal halves of bovine and human ABCR are labeled with concanavalin A.

Fig 4. Expression, concanavalin A labeling, glycosidase F digestion and azido-ATP labeling of ABCR(WT) and ABCR(Δ8) and ABCR(Δ4) glycosylation mutants. Wild-type (ABCR(WT)) and mutant ABCR with four deleted glycosylation sites in the N half (ABCR (Δ4N)), four deleted sites in the C half (ABCR (Δ4C)) or all eight sites deleted
(ABCR (Δ8)) were expressed in COS-1 cells.  

A. Western blots of ROS membranes and ABCR mutants immunoprecipitated from COS-1 membranes and labeled with a monoclonal antibody to ABCR (Rim3F4) and concanavalin A (ConA);  

B. Western blots of untreated (-) or glycosidase F treated (+) human ROS membranes or COS-1 cell membranes labeled with the Rim 3F4 antibody. A low bis acrylamide SDS gel was used to resolve small shifts resulting from glycosidase F treatment;  

C. Autoradiographs of 8-azido-[α-32P]-ATP photoaffinity labeled ABCR from ROS and COS-1 cell membranes. ABCR from labeled COS-1 membranes was immunoprecipitated on a Rim 3F4-Sepharose matrix prior to SDS gel electrophoresis.

**Fig 5. Expression and concanavalin A labeling of ABCR(Δ7) mutants.** Four ABCR(Δ7) mutants lacking the four glycosylation sites in the N half and three of the four sites in the C half were expressed in COS-1 cells and immunoprecipitated with Rim 3F4-Sepharose. An equal amount of protein was applied to an SDS gel. Western blots were labeled with either the Rim 3F4 antibody or concanavalin A (ConA). ROS membranes were run as a positive control.

**Fig 6. Expression and concanavalin A labeling of N-truncated ABCR (WT) and glycosylation mutants.** The N-terminal half of wild-type ABCR (N-tr-ABCR (WT)) and mutants lacking three of the four glycosylation sites (N-tr-ABCR (Δ4)) were
expressed in COS-1 cells. A, Western blots of immunoprecipitated proteins labeled with the Rim 5B4 antibody and concanavalin A (ConA). B, Phosphoimaging of COS-1 membranes photoaffinity labeled with 8-azido-[α-32P]-ATP and immunoprecipitated with Rim 5B4-Sepharose.

Fig 7. Analysis of trypsin digested ABCR under reducing and nonreducing conditions. Bovine ROS membranes were digested with trypsin and subjected to SDS gel electrophoresis under disulfide reducing (+β-mercaptoethanol) and nonreducing (-β-mercaptoethanol) conditions. Western blots were labeled with the Rim 3F4 and Rim 5B4 antibodies.

Fig 8. Structural properties of ABCR and ABC1. A, Topological model for ABCR. The four N-linked glycosylation sites in each exocytoplasmic domain (ECD-1 and ECD-2) are shown in hexagons. These domains lie between the first transmembrane segment (H1 or H7) and the membrane spanning domains (MSD-1 and MSD). The Walker A and B and active transporter signature (ATS) in the nucleotide binding domains (NBD-1 and NBD-2) are also shown. We also show a disulfide linkage between ECD-1 and ECD-2.

B, Amino acid sequence alignment of human ABCR and human ABC1 in regions flanking the conserved N-linked glycosylation sites (black boxes) in ECD-1 and ECD-2. Identical amino acids are shown with (:) and conserved amino acids are shown (.). Conserved cysteine residues are shown in gray. The high degree of identity in these and other regions of the ECD and similar hydropathy profiles and other structural features
support the view that ABC1 has a similar membrane topology as ABCR.
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Fig 1
Fig 2

Diagram showing structural elements with labels and annotations.
Fig 5

Rim 3F4

ConA

ABCR

ROS

ABCR (Δ7-N1662)

ABCR (Δ7-N1590)

ABCR (Δ7-N1529)

ABCR (Δ7-N1469)
**A**

**ECD-1**

- hABC1: MPSAGMLPWLQGIFCNVNNPCEFQSTPGEESPGRGIVSNYYNSIIARVYRDQELLNMAPESQ
- hABC1: MPSAGTLPWYQCIICNANNPCEFQRYTPGEAPGYPGVGNFNSNIVARLFSDARRLLLYSGKDT

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**ECD-2**

- hABC1: HLETEDNIVKVNFKGWALSVFLNVHAINLRAISLDRDPREEYGTQTSQPIMNITKEQ

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**Fig 8**