ABCR, also known as ABCA4, is a member of the superfamily of ATP binding cassette transporters that is believed to transport retinal or retinylidene-phosphatidylethanolamine across photoreceptor disk membranes. Mutations in the ABCR gene are responsible for Stargardt macular dystrophy and related retinal dystrophies that cause severe loss in vision. ABCR consists of two tandemly arranged halves each containing a membrane spanning segment followed by a large extracellular/lumen domain, a multi-spanning membrane domain, and a nucleotide binding domain (NBD). To define the role of each NBD, we examined the nucleotide binding and ATPase activities of the N and C halves of ABCR individually and co-expressed in COS-1 cells and derived from trypsin-cleaved ABCR in disk membranes. Whole ABCR or membranes from control infected cells were photoaffinity labeled with 8-azido-ATP and 8-azido-ADP, only the NBD2 in the C half bound and trapped the nucleotide. Co-expressed half-molecules displayed basal and retinal-stimulated ATPase activity similar to full-length ABCR. The individually expressed N half displayed weak 8-azido-ATP labeling and low basal ATPase activity that was not stimulated by retinal, whereas the C half did not bind ATP and exhibited little if any ATPase activity. Purified ABCR contained a tightly bound ADP, presumably in NBD1. Our results indicate that only NBD2 of ABCR binds and hydrolyzes ATP in the presence or absence of retinal. NBD1, containing a bound ADP, associates with NBD2 to play a crucial, non-catalytic role in ABCR function.

ABCR, formerly known as the Rim protein, is a member of the ABCA subclass of ATP binding cassette (ABC) transporters that is found along the rims and incisures of rod and cone photoreceptor outer segment disk membranes (1–4). It is thought to function in the transport of all-trans-retinal or retinylidene-phosphatidylethanolamine across the disk membrane following the photobleaching of rhodopsin (5–7). This transport process enables all-trans-retinal to be effectively reduced to all-trans-retinol by retinal dehydrogenase on the surface of disk membranes as a key step in the recycling of all-trans-retinal to 11-cis retinal for regeneration of rhodopsin and cone opsins. Importantly, it prevents the buildup of retinal and retinylidene-phosphatidylethanolamine in disk membranes that otherwise would further react to form toxic directinol side products (8, 9).

The importance of ABCR in photoreceptor biology is highlighted by the finding that over 300 mutations in the ABCR gene have been associated with a variety of clinically distinct autosomal recessive retinal degenerative diseases including Stargardt macular dystrophy, fundus flavimaculatus, cone-rod dystrophy, and retinitis pigmentosa (10–17). Stargardt macular dystrophy, the most common disease associated with mutations in ABCR, is an early onset disease characterized by progressive loss of central vision, delayed dark adaptation, accumulation of yellow deposits known as lipofuscin within the central retina, and atrophy of macular region of the retina and underlying retinal pigment epithelial cells. Abcr knockout mice exhibit many of these features including delayed dark adaptation, accumulation of lipofuscin deposits containing the dieryl compound A2E in retinal pigment epithelial cells, and slow progressive photoreceptor degeneration (7).

ABC transporters typically consist of two multi-spanning membrane domains (MSDs) that serve as a pathway for the translocation of a substrate across membranes and two ATP binding cassettes or nucleotide binding domains (NBDs) that provide the energy for substrate transport (18). In eukaryotic ABC transporters, these domains are typically found either on a single long polypeptide chain as in the case of CFTR and the multidrug resistance proteins, P-glycoprotein and MRP1, or as a complex of two identical or similar “half-molecule” subunits each having an MSD and an NBD as found in the TAP1/TAP2 ABC transporter associated with peptide antigen processing. ABCR belongs in the first category, because it consists of a single 2273-amino acid polypeptide comprised of two tandemly arranged halves (2, 10). Each half contains an MSD followed by a cytoplasmic NBD. A distinguishing feature of ABCR and other members of the ABCA subfamily is the presence of a large exocytotic (extracellular/lumen) domain that connects the first transmembrane segment to the MSD in each half of the protein (2, 19, 20).
The ATP binding properties and hydrolytic activities of individual NBDs have been examined for a number of ABC transporters by either generating half-molecules from enzymatic cleavage of full-length molecules or by co-expressing half-molecules. In the case of P-glycoprotein, the NBDs of the N and C halves both exhibit ATP binding properties and ATPase activity, but interaction of both halves is required for drug-stimulated ATPase activity and ATP-dependent drug transport (21, 22). NBD1 on the N-half of MRP1 is preferentially labeled with 8-azido-ATP when the N-half is expressed by itself or together with the C-half, whereas NBD2 on the C-half traps 8-azido-ADP, but only if this half is co-expressed with the N-half (23, 24). The functioning of MRP1 as a leukotriene C4 transporter also requires the interaction of both halves of MRP1 (25). Nucleotide binding studies have also been carried out on TAP1/TAP2, CFTR, and SUR1, a subunit of the pancreatic β-cell ATP-sensitive (KATP) channel (26–28). In these proteins, stable ATP binding occurs at NBD1, whereas ATP hydrolysis and/or ADP trapping takes place at NBD2. These studies indicate that the nucleotide binding and enzymatic properties of each NBD differ significantly for these ABC transporters. To date analysis of the nucleotide binding and ATPase activity of the individual NBDs in context of the half-molecules has not been studied for any of the ABCA subfamily members.

To define the functional role of each NBD of ABCR, we have analyzed the ATPase activity, ATP/ADP photoaffinity labeling, ADP trapping, and nucleotide content of full-length and the N and C halves generated by individual or co-expression in COS-1 cells and trypsin cleavage of ABCR in ROS disk membranes. Here, we show that the N and C halves of ABCR associate to form a complex having basal and retinal-stimulated ATPase activity and 8-azido-ATP labeling properties similar to full-length ABCR. The two NBDs have remarkably different, although interdependent functions. NBD2 in the full-length or NC complex binds and hydrolyzes ATP, whereas NBD1 appears to contain a tightly bound ADP suggesting that this domain may play a non-catalytic function. In contrast, the C-half does not bind detectable levels of ATP and shows little if any ATPase activity. Our studies restrict possible mechanisms by which ABCR utilizes energy from ATP hydrolysis to transport substrates across photoreceptor disk membranes.

MATERIALS AND METHODS
Monoclonal Antibodies and Plasmids—The Rim3F4 and Rim3B4 monoclonal antibodies directed against defined epitopes on the C-half and N-half of ABCR, respectively, and the Rho ID4 monoclonal antibody to the nine-amino acid C-terminal sequence of rhodopsin have been described previously (2, 3, 29). Human ABCR cDNA, generously provided by J. Nathans, The Johns Hopkins University, was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) to produce pcABCR. The cDNAs coding for the N-half (amino acids 1–1325) and C-half (amino acids 1326–2273) of ABCR were constructed by PCR using pcABCR as a template and the following primers: N-half, GAGCCCTGTTGGCCGCGCCACGTGGT (FseI) and GCTTCTACAGGC GGCGCCCATGTGGT (BspI); C-half, GAGCCCTGTTGGCCGCGCCACGTGGT (FseI) and GCTTCTACAGGC GGCGCCCATGTGGT (BspI). The restriction sites used to insert the PCR products are indicated in parentheses and underlined when included within the primer sequence. The base coding for the ID4 epitope is shown in italics. The PCR products were digested with the indicated restriction enzymes and ligated into pcABCR that had been digested with the same enzymes. K969M and K1978M mutations were inserted by QuikChange site-directed mutagenesis following previously published procedures (5, 30) with the following primers: CACATC GGGGCGCCCATGTGGT and GACATGAGACCCCATCATCGGATCC (K969M); GTAATGACCGGATCC (K1978M). The K969M and K1978M mutations, respectively, were cloned into the original pcABCR[K969M]. To create the C-half[K1978M] mutant, the HindIII/BspI digested PCR product from above (for constructing the C-half) was used to replace the 4-kb HindIII/BspI fragment of pcABCR[K969M]. The cDNA for the N-half-14D of ABCA1 (amino acids 1–1302) was made by replacing the 4.2-kb PstI/BamHI fragment of pcABCA1 with a 1.9-kb PCR product amplified with the following primers: CACATC GGGGCGCCCATGTGGT and GACATGAGACCCCATCATCGGATCC (K969M). The cDNA for the N-half-14D of ABCA1 (amino acids 1303–2261) containing the 3F4 epitope was synthesized by PCR (2.9 kb) with the following primers: ACTGAT TGGCGCCGGGAAACATGGAATCCAGAGAGACAGACTTG and TGCGGCCGCGGGAACATGGAATCCAGAGAGACAGACTTG and TCCGCTAGTTTAAACTCA (bases coding for the 3F4 epitope in italics) and cloned into pcPE4 (Invitrogen) at the NotI/KpnI sites. All PCR amplified sequences were confirmed by automated DNA sequencing.

Transfection of COS-1 and 293-EBNA Cells—Monkey kidney fibroblast cell line COS-1 was maintained in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal bovine serum. Human embryonic kidney 293-EBNA cells (Invitrogen) were passaged in the above Dulbecco’s modified Eagle’s medium containing 0.25 g/liter G418. Cells were plated on 10-cm dishes and transfected the following day with 30 g of plasmid per dish using the calcium phosphate method. The next day, cells were rinsed with 1 ml EDTA in phosphate-buffered saline, pH 7.4, and supplied with complete medium for 24 h.Treatment of Membranes— pcPE4 transfected cells were maintained in 60-cm dishes and the cell homogenate from one or two 10-cm dishes was diluted rapidly with 200 ml of Buffer C (10 mg/ml sonicated soybean phospholipids, 10% glycerol, 1 mM DTT, 100 mM NaCl, 3 mM MgCl2, 50 mM NaHEPES, pH 7.4) containing 10 mM CHAPS and COMPLETE protease inhibitor mixture (Roche Applied Science) and stirred on ice for 15 min at 4 °C. The supernatant after a 10-min centrifugation at 90,000 × g (TLA100.4 rotor in a Beckman Optima TL ultracentrifuge) was mixed with 50 ml of Rim3F4-Sepharose 2B beads for 1 h at 4 °C. The beads were washed six times in Buffer C containing 10 mM CHAPS and eluted with 0.2 mg/ml Rim3F4 peptide. Purified protein (24 ml) was incubated with 9 ml of 50 mM lipid (1:1 mixture of dioleoylphosphatidylethanolamine and brain polar lipid, by weight) and 3 ml of n-octylglucoside for 30 min on ice. The mixture was diluted rapidly with 200 ml of Buffer E (1 mM DTT, 1 mM EDTA, 140 mM NaCl, 10% glycerol, 25 mM NaHEPES, pH 7.4) and passed through 200 ml of Extran-4 gel (Fischer). The flow-through containing the reconstituted protein was used for determination of ATPase activity.

ATPase Activity—ATP hydrolysis was measured using 50 µM [α-32P]ATP and thin layer chromatography as described previously (30). The all-trans-retinal concentration was determined spectrophotometrically (extinction coefficient ε345 nm = 42,880 M−1 cm−1). Protein concentration was estimated from the eluate before reconstitution by laser densitometry of Coomassie Blue-stained gels using bovine serum albumin as a standard. This method gives an overestimation of the actual protein content after reconstitution (hence lower specific activity), because recovery from the Extran-4 gel column is less than 100%. Direct protein measurements after reconstitution by densitometry of Western blots were about half of that in the eluate. However, the latter method gave variable results, so we adopted a cGTPase-discontinuous gel filtration protocol consisting of 5 and 60% sucrose for 30 min at 77,000 × g (TLL555 rotor).

8-Azido-ATP Photoaffinity Labeling— Membranes (50–150 µg of protein) in 50 µl of labeling buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM MgCl2) were incubated with 1–5 µM 8-azido-[α-32P]ATP (20 Ci/mmol, PerkinElmer Life Sciences) or 8-azido-[γ-32P]ATP (13 Ci/mmol; GTP [17 Ci/mmol]) in labeling buffer (18 m M CHAPS and COMPLETE protease inhibitor mixture) for 1 h at 37 °C. The mixture was diluted rapidly with 200 µl of Buffer E (1 mM DTT, 1 mM EDTA, 140 mM NaCl, 10% glycerol, 25 mM NaHEPES, pH 7.4) and passed through 200 µl of Extran-4 gel (Fischer). The flow-through containing the reconstituted protein was used for determination of ATPase activity.
and added to 200 μl of 2% Triton X-100 in TBS. After 30 min on ice, the cleared extract was mixed with 25 μl of antibody coupled to Sepharose 2B for 1–2 h at 4 °C. The beads were washed four times in TBS containing 0.2% Triton X-100 and eluted twice, 30 μl each, in 4% SDS, 0.2% Triton X-100, TBS.

8-Azido-ATP Trapping—Membranes were incubated with 5 μm 8-azido-[α-32P]ATP in 50 μl of labeling buffer with or without 800 μm sodium orthovanadate for 10 min at 37 °C. All-trans-retinil and 50 μm DTT were added where indicated. Binding was stopped by the addition of ice-cold 20 mM Tris-HCl, pH 7.4, and the membranes were collected by centrifugation and washed twice more with TBS. One sample exposed to orthovanadate was washed in the presence of 800 μm orthovanadate. In some experiments MgATP (10 mM) was included in the wash step but had no effect. The membranes were suspended in 30 μl of 20 mM Tris-HCl, pH 7.4, irradiated with UV light for 10 min on ice, diluted to 200 μl with TBS, and solubilized as described above for azido-ATP binding.

Trypsin Cleavage of Bovine ABCR—Bovine ROS were isolated as described previously (31) and treated with 1.6–4.0 μg/ml trypsin for 30 min at 0 °C (19). The reaction was stopped by the addition of 5-fold excess of soybean trypsin inhibitor.

8-Azido-ADP Photoaffinity Labeling—Thoroughly washed ROS membranes were labeled with 5 μm 8-azido-[α-32P]ADP (16.8 Ci/mmol; Affinity Labeling Technologies) for 15 to 30 min as described for 8-azido-ATP labeling. DTT at a final concentration of 30 mM was added to quench the reaction. After 15 min, ice-cold Tris-EDTA buffer (0.5 mM EDTA, 10 mM Tris-HCl, pH 7.4) was added, and the membranes were washed five times by centrifugation at 55,000 × g for 15 min (TLA45). The membranes were suspended in 50 μl of Tris-EDTA buffer, and an equal volume of buffer containing trypsin (4 μg) was added. After 30 min on ice, the membranes were treated with trypsin inhibitor (50 μg). Cold Tris-EDTA buffer was added, and the membranes were pelleted by centrifugation.

To remove tightly bound nucleotides, membranes were treated as follows (32). The washed membrane pellet (4 μg) was resuspended in 1 ml of 100 mM NaSO4, 50% glycerol, 3 mM MgCl2, 50 mM NaHEPES, pH 7.5, and dialyzed against three changes of the same buffer at 4 °C (2 × 500 ml for 3 h each, 1000 ml overnight). The sample (100 μl) was diluted with labeling buffer and photoaffinity labeled as described above.

The washed membranes (4 μg) were resuspended in 1 ml of 50 mM NaHEPES, pH 7.5, 100 mM NaSO4, 50% glycerol, 3 mM MgCl2, and 10 mM CHAPS, stirred in a glass tube at 4 °C for 1 h, and dialyzed against three changes of resuspension buffer. The solubilized membranes, 100 μl, were centrifuged through a column of Sephadex G-50 (previously equilibrated with labeling buffer). The volume was made up to 100 μl with labeling buffer, and the sample was photoaffinity labeled as described above. This was then passed through another column centrifugation procedure to remove unbound label. Half of the labeled sample was subjected to trypsin digestion as described above.

Determination of Adenine Nucleotide Content—Typically, 48 mg of ROS membranes were used to purify ABCR by photoaffinity chromatography with or without nucleotide treatment during solubilization. Nucleotide determinations were done in triplicate. The solubilized, immunopurified ABCR was concentrated to about 400–500 μl by centrifugation (8000 × g for 30 min) using a 2-ml Amicon Centricon (8000 molecular weight cut-off). Approximately 400 μl of concentrated ABCR in an acid-washed glass tube was heated in a water bath for 10 min. A tube with buffer only was similarly treated and used as the background subtract. This method was chosen so as to avoid the possibility of any released ATP being hydrolyzed by ABCR (see analogous situation with F1-ATPase (33)). The test tubes were then placed on ice and cooled for 10 min followed by centrifugation in a table-top centrifuge at 5000 × g for 5 min. Samples containing known amounts of ATP and ADP were similarly treated so as to determine the percent hydrolysis of nucleotides under similar conditions. Usually, duplicates of 10, 100, and 1000 nM concentrations of ATP and ADP were used. An aliquot of each sample was used for ATP determinations (100 μl for each assay). The remaining 200-μl sample was used to convert ADP to ATP by addition of pyruvate kinase and phosphoenolpyruvate, and the solution mixture, prepared as described by Hamp (34). The luminometric procedure outlined by Sigma in their ATP bioluminescent assay kit (technical bulletin BAAB-1) was used to assay for ATP. Samples (100 μl for ATP and 10 μl for ATP + ADP) of each fraction were made up to 100 μl, if necessary, with a buffer of 25 mM HEPES-KOH, pH 7.5, 24 mM magnesium acetate, 5 mM EDTA, and 2 mM DTT. This was then incubated at 37 °C for 10 min and assayed (1:1 ATP assay mix at room temperature. The relative intensity was noted after 10 s. The instrument used in these assays was the LKB 1250 luminometer linked to an LKB 1260 display and recorder unit. Light intensity was calibrated by comparison with light emissions from known concentrations of ATP (usually in the 100 nM range). Bound ADP was calculated from the difference between total nucleotide (after conversion of ADP to ATP with pyruvate kinase and phosphoenolpyruvate) and the concentration of ATP (determined without conversion of ADP to ATP). Hence the moles of ATP and ADP present were calculated. Essentially, the procedures outlined by Beharry and Bragg (35, 36) were followed. Note that only acid-washed glassware was used throughout the experiments, and all the precautions mentioned in the papers above were observed.

Subcellular Localization of ABCR in Cos-1 Cells by Immunofluorescence Microscopy—The subcellular distribution of full-length ABCR and the N- and C-half-molecules expressed in COS-1 cells was determined by immunofluorescence microscopy (Fig. 1). Rather than localizing predominantly in the endoplasmic reticulum (ER) and Golgi, which is expected for transiently overexpressed membrane proteins, ABCR was typically associated with intracellular vesicles of varying sizes. Clusters of several large vesicles were observed in some cells, whereas numerous small vesicles spread throughout the cytoplasm were seen in other cells. These intensely labeled vesicles do not appear to be artifacts, because mutating a single amino acid in ABCR (D646H) changed the distribution from vesicular to perinuclear (ER/Golg), a pattern typically observed for misfolded proteins. ABCR did not co-localize with a number of organelle markers (catalase for peroxisomes, LAMP-2 for late

RESULTS

Localization of ABCR in Transfected Cells by Immunofluorescence Microscopy—The subcellular distribution of full-length ABCR and the N- and C-half-molecules expressed in COS-1 cells was determined by immunofluorescence microscopy (Fig. 1). Rather than localizing predominantly in the endoplasmic reticulum (ER) and Golgi, which is expected for transiently overexpressed membrane proteins, ABCR was typically associated with intracellular vesicles of varying sizes. Clusters of several large vesicles were observed in some cells, whereas numerous small vesicles spread throughout the cytoplasm were seen in other cells. These intensely labeled vesicles do not appear to be artifacts, because mutating a single amino acid in ABCR (D646H) changed the distribution from vesicular to perinuclear (ER/Golg), a pattern typically observed for misfolded proteins. ABCR did not co-localize with a number of organelle markers (catalase for peroxisomes, LAMP-2 for late

### Fig. 1

Immunofluorescence localization of ABCR in COS-1 cells. Cells expressing the full-length ABCR, N-half (amino acids 1–1325), C-half (amino acids 1326–2273), and both halves (NC halves) were labeled with Rim3B4 (N-half) or Rim3F4 (ABC, NC halves, C-half) and Cy3-conjugated anti-mouse immunoglobulin for analysis by immunofluorescence microscopy. Full-length ABCR and co-expressed NC halves localize to both intracellular vesicles and the ER-Golgi network. The individually expressed N and C halves localize predominantly in the ER-Golgi network.
endosomes, LysoTracker for lysosomes). The expression pattern of the N-half or C-half when expressed alone was mostly perinuclear indicative of ER localization. However, when the two halves were co-expressed, a significant fraction of the protein was found in vesicular structures like those seen in cells transfected with wild-type, full-length ABCR.

**The Two Halves of ABCR Associate When Co-expressed**—The N and C halves of ABCR, each containing a transmembrane domain followed by an NBD (Fig. 2A), were expressed individually by single transfections or together by co-transfection in COS-1 cells. Fig. 2B shows Western blots of COS-1 cell extracts, flow-through (unbound) fractions, and peptide-eluted (bound) fractions of the expressed full-length ABCR (~220 kDa) and the N (~140 kDa)- and C (110 kDa)-half-molecules isolated on a Rim3F4-Sepharose matrix (2). When the two halves were co-expressed (NC), about 50% of the N-half (detected with the Rim5B4 antibody), whereas the remainder was in the flow-through fraction. The N-half by itself did not bind to the Rim3F4-Sepharose matrix nor did it co-purify with the C-half when the N and C halves were individually expressed and mixed together prior to immunoaffinity purification. Coomassie Blue-stained gels showed that full-length ABCR and co-expressed/co-purified N and C halves were the predominant proteins observed in the peptide-eluted fraction from the Rim3F4 immunoaffinity column.

**ATPase Activity of Expressed N and C Halves**—The basal and retinal activated ATPase activity of full-length ABCR and the N and C halves individually or co-expressed in COS-1 cells was determined after immunoaffinity purification and reconstitution into lipid vesicles. Fig. 3, A and B shows that both the full-length ABCR and co-expressed N and C halves purified on a Rim3F4 column were stimulated 1.2-fold by all-trans-retinal. The specific activity of the full-length protein, however, was generally higher than the co-expressed half-molecules. In contrast, the ATPase activity of the individual N-half with a nine-amino acid 1D4 epitope tag (N*), required for immunoaffinity purification was lower and not stimulated by retinal (Fig. 3C). The 1D4 tag did not affect the functional interaction of the N and C halves, because co-expression of the N* and C halves gave similar basal and retinal-stimulated activity as co-expression of the untagged N and C halves (data not shown). The individually expressed C-half showed little if any activity (Fig. 3D).

**Effect of Walker A Lysine to Methionine Mutations on ATP Hydrolysis and Binding**—The conserved lysine residue in the NBD Walker A motif of ABC proteins is critical for the hydrolysis of ATP. Mutation of this lysine to methionine in P-glycoprotein abolishes basal and drug-stimulated ATPase activity (37, 38). With ABCR, the lysine to methionine substitution in the NBD1 (K969M) and NBD2 (K1978M) or in both (K969M/K1978M) significantly reduced the basal ATPase activity of ABCR and abolished retinal activation (Fig. 4A). Retinal-stimulated ATPase activity was also abolished when the N-half was...
co-expressed with the K1978M C-half mutant (amino acid number represents that of the full-length ABCR).

We examined the ATP binding of these mutants using the photo-reactive ATP analogue 8-azido-[α-32P]ATP. The photoaffinity labeling intensities of the single mutants (K969M and K1978M) were similar to wild-type ABCR relative to the N-terminal NBD (NBD1) of MRP1, CFTR, and SUR1 or the NBD of TAP1 is responsible for high affinity ATP binding, and the C-terminal NBD (NBD2) or TAP2 is more important for ATP hydrolysis and ADP trapping. The unexpected finding that ATP binding occurs only on the C-half of ABCR prompted us to examine the ATP binding properties of ABCA1, a member of the ABCA subfamily that is most similar to ABCR. Fig. 6 shows that the two halves of ABCA1 were labeled equally well in contrast to ABCR.

We also examined the possibility that more C-half than N-half is recovered on Rim3P4 beads and therefore displays more azido-ATP label. This is unlikely to be a problem, because we have already shown that any excess C-half that is not associated with N-half does not label with azido-ATP (Fig. 5B). Nevertheless, Fig. 6, lane 3 shows that when the two halves of ABCR were co-immunoprecipitated with Rim5B4 (which binds the N-half of ABCR and should only purify C-half that is bound to the N-half), the C-half was still labeled more strongly.

Azido-ATP/ADP Trapping by Co-expressed N and C Halves of ABCR—To gain more insight into the properties of the NBDs of ABCR, we carried out trapping experiments using 8-azido-[α-32P]ATP. As shown in Fig. 7, more azido ATP/ADP was trapped by ABCR under hydrolyzing conditions (37°C) than at 0°C. This binding was not dependent on sodium orthovanadate (Fig. 7A) or influenced by the presence of all-trans-retinal (Fig. 7B). Co-expression of the N and C halves further revealed that as with ATP binding, ATP/ADP trapping occurred in NBD2 of the C-half.

Determination of Adenine Nucleotide Content of ABCR—Our results showing that NBD1 of ABCR does not bind or trap significant amounts of 8-azido-[α-32P]ATP suggest that this site already may be occupied by a nucleotide. To determine whether ABCR contains a tightly bound adenosine nucleotide, the nucleotide content of immunoaffinity purified ABCR from ROS was determined by the ATP luciferase/luciferin luminescent assay. As shown in Table I, purified ABCR contained 0.91 moles of bound non-exchangeable adenosine nucleotide, essentially all of which was in the form of ADP. This bound nucleotide was not replaced by GDP, because the addition of GDP during the solubilization of ABCR resulted in only a marginal decrease in the level of ADP (Table 1). ADP was also not lost or replaced during hydrolysis of the ATP analogue 8-azido-[α-32P]ATP, because no binding of this analogue or its hydrolytic product was observed.

To determine whether ABCR could bind additional ADP, we added 2 mM ADP during the solubilization of ABCR. After removal of unbound ADP during immunoenzymatic purification of ABCR, the amount of ADP bound was determined. Table I shows that ABCR bound just over 2 moles of ADP under these conditions, indicating that ABCR contains an additional lower affinity ADP site, presumably NBD2.

To further identify which NBD binds ADP, we carried out 8-azido-[α-32P]ADP photoaffinity labeling experiments
ABCR in ROS membranes similar to those performed with 8-azido-[\alpha-^{32}P]ATP. As shown in Fig. 8A, 8-azido-[\alpha-^{32}P]ADP labeled the full-length and the C-half of ABCR, indicating that NBD2 is accessible to ADP, as well as ATP. In contrast, the N-half was not labeled suggesting that the NBD1 is not accessible to exogenously added nucleotide. Membranes were also photolabeled in the presence of retinal, the putative substrate for ABCR (Fig. 8A, lanes 3 and 4), and retinal plus ATP to determine whether the binding of azido-ADP would be enhanced under turnover conditions. No significant difference in photoaffinity labeling was observed under these conditions. Together, these studies indicate that the NBD1 of ABCR contains a tightly bound, non-exchangeable ADP.

Several conditions used previously to remove tightly bound nucleotides from other nucleotide-binding proteins were applied to membrane-bound and solubilized ABCR. Treatment of membrane-bound (Fig. 8B) or CHAPS-solubilized ABCR (Fig. 8C) with EDTA/Na₂SO₄/glycerol treatment used to remove tightly bound nucleotides from the proton-translocating ATP-synthase complex (32, 33, 39), did not alter the photoaffinity labeling pattern; that is, only the C-half of ABCR was labeled under non-hydrolysis conditions (0 °C) but not under hydrolysis conditions (37 °C).

FIG. 5. Azido-ATP photoaffinity labeling of the N and C halves of ABCR. A, membranes from transfected cells expressing either full-length ABCR (lanes 1 and 2) or co-expressing the N and C halves (lanes 3 and 4) were photoaffinity labeled with 1.5 \mu M 8-azido-[\alpha-^{32}P]ATP in the absence or presence of 1 mM ATP. The expressed protein was isolated on a Rim3F4-Sepharose matrix prior to analysis by SDS-PAGE and phosphorimage analysis. Left panel, Coomassie Blue-stained gel. Right panel, azido-ATP labeling. B, membranes from transfected cells expressing full-length ABCR, the N-half, or both halves (NC halves) were labeled with 8-azido-[\alpha-^{32}P]ATP and isolated as above. Similar amounts of protein were loaded in each lane of the gel as judged by staining with Coomassie Brilliant Blue (not shown). C, rod outer segment membranes were incubated with (+) or without (−) trypsin and subsequently labeled with azido-[\alpha-^{32}P]ATP. ABCR and the associated N and C complex were purified on a Rim3F4-Sepharose matrix. Left panel, azido-ATP labeling of the full-length ABCR (220 kDa) and the C-half (114 kDa); N-half was not labeled. Right panel, Western blots labeled for the full-length ABCR and C-half with the Rim3F4 antibody and the N-half with the Rim5B4 antibody. D, ROS membranes were incubated with 5 \mu M 8-azido-[\alpha-^{32}P]ATP at 0 or 37 °C for 10 min, UV irradiated for 5 min at 0 °C, and then treated with (+) or without (−) trypsin. Full-length ABCR and C-half were labeled under non-hydrolysis conditions (0 °C) but not under hydrolysis conditions (37 °C).

Fig. 6. Azido-ATP binding to the N and C halves of ABCA1. Membranes from cells expressing the N and C halves of ABCA1 engineered to contain the 3F4 epitope in the C-half and ABCR were photoaffinity labeled with azido-[\alpha-^{32}P]ATP, isolated by immunoprecipitation, and analyzed on an SDS gel. Coomassie Blue-stained gel (left panel) and azido-ATP labeling of co-expressed N and C halves of ABCA1 (lane 1) and ABCR (lane 2) isolated on a Rim3F4-Sepharose matrix, and ABCR (lane 3) isolated on a Rim5B4-Sepharose matrix. Molecular mass markers are shown on the left. The positions of the N and C halves are indicated by arrows on the right. Both the N and C halves of ABCA1 label with 8-azido-ATP, whereas only the C-half of ABCR is intensely labeled.
under these conditions. In another series of experiments, membrane-bound ABCR was covalently labeled in the presence of 30% dimethyl sulfoxide and 4 mM MgCl₂, conditions known to partially strip bound nucleotides from the F₁-ATPase (40, 41). Under these conditions 8-azido-ADP also only labeled the C-half of ABCR (data not shown). Together, these results indicate that the N-half contains a tightly bound ADP that resists removal or exchange, whereas the C-half binds and traps ATP and ADP.

**DISCUSSION**

In this study, we have examined the functional properties of the two NBDs of ABCR by expressing the two halves individually and together in COS-1 cells and by analyzing tryptic-generated half-molecules from native ABCR in ROS membranes. Co-expressed N and C halves exhibit properties similar to the full-length ABCR. The N-half co-purifies with the C-half on a Rim3F4 immunoaffinity matrix indicating that there is a strong association between these halves as found for trypsin cleaved native ABCR. The C-half, but not the N-half, is intensely photoaffinity labeled with 8-azido-ATP in both native ABCR and the NC complex. The purified NC complex also displays basal and retinal-stimulated activity characteristic of full-length ABCR. Finally, a significant fraction of the co-expressed NC complex, like full-length ABCR, exits from the ER to intracellular vesicles, indicating that this protein complex is properly folded and assembled so as to pass through the quality control system of the ER. In contrast, the individually expressed halves exhibit different properties. The N-half displays weak 8-azido-ATP labeling and low basal ATPase activity, and the C-half does not labeled with 8-azido-ATP and shows little, if any, ATPase activity. The association between the N and C halves probably occurs at an early stage of biosynthesis in the ER, because the separately expressed half-molecules mixed together during or after membrane solubilization do not associate. This is consistent with the idea that the individually expressed half-molecules are sufficiently misfolded and possibly aggregated such that they are retained in the ER and do not associate *in vitro*. The TAP1 and TAP2 subunits, as well as the P-glycoprotein and MRP1 half-molecules, have also been re-
ported to require co-expression in the same cell for functional heterodimerization (21, 23, 25, 42).

The detailed mechanism by which ABC transporters utilize ATP hydrolysis to fuel the transport of substrates across membranes is not well understood. However, in the case of P-glycoprotein, the two NBDs are structurally and functionally similar although asymmetrically arranged in the protein (22, 43, 44). Both NBDs are required for activity, because covalent inactivation at one site prevents ATP hydrolysis at the other site (37). In one proposed model, ATP hydrolysis occurs alternately at one of the sites to promote drug transport (45). In another model, ATP hydrolysis occurs at one site in a random fashion to initiate drug transport, whereas ATP hydrolysis at the second site is required to return P-glycoprotein to its original state. Analysis of other ABC transporters including MRP1, CFTR, SUR1, and TAP1/TAP2, however, indicate that the two NBDs of these transporters show distinctive, but interdependent, nucleotide binding and hydrolysis properties. In the case of CFTR, NBD1 is a site of stable ATP interaction, whereas NBD2 exhibits rapid ATP turnover (28). For MRP1, NBD1 is preferentially labeled with 8-azido ATP, whereas ADP trapping occurs at NBD2 (23). Furthermore, ATP binding to NBD1 increases the binding and hydrolysis of ATP and trapping of ADP at NBD2 indicating that these domains act in a cooperative manner (24). For the TAP1/TAP2 transporter, ATP hydrolysis by TAP2 is essential for peptide transport across membranes, whereas ATP hydrolysis at TAP1 is not required (26).

The two NBDs of ABCR, like those of TAP1/TAP2, CFTR, and MRP1, exhibit distinct properties. They are only 35% identical in sequence and bind, trap, and hydrolyze nucleotides differently. Photoaffinity labeling studies of trypsin-digested ABCR and the co-expressed half-molecules indicate that NBD2 binds and traps 8-azido-ATP and -ADP and hydrolyzes 8-azido-ATP. Unlike other ABC transporters including ABCA1, however, 8-azido-ATP and 8-azido-ADP do not label NBD1 in native ABCR and label NBD1 weakly in the expressed NC complex even in the presence of the putative retinal substrate. The weak labeling on NBD1 observed in the NC complex may be because of the presence of a small amount of poorly assembled complex.

What is the basis for the failure of NBD1 of ABCR to bind exogenously added nucleotide? It is not because of the inability of the NBD1 to bind and hydrolyze nucleotides, because it has been shown that this domain expressed in bacteria is capable of hydrolyzing ATP and other nucleotides (46). Furthermore, the individually expressed N-half also exhibits low nucleotide binding and ATPase activity. Instead, it appears to be because of the presence of a tightly bound, non-exchangeable ADP sequestered within the NBD1 of full-length ABCR. Immunoaffinity purified ABCR contains one tightly bound ADP molecule. This nucleotide was not removed or exchanged with added GDP. Furthermore, agents that are known to release tightly bound nucleotides in other proteins do not promote 8-azido-ADP labeling of NBD1 suggesting that these conditions do not remove or exchange the bound ADP in ABCR. Finally, purification of ABCR in the presence of ADP increases the number of bound nucleotide to ~two ADP molecules per ABCR molecule, consistent with the binding of an additional nucleotide, albeit more weakly, to NBD2. Taken together, these results suggest that NBD1 in ABCR contains a tightly bound ADP, the accessibility of which is blocked through interaction with NBD2. In contrast the NBD2 site is accessible to exogenous nucleotide. ATP binding and hydrolysis at this site, however, is dependent on interaction with NBD1.

When the NBDs of ABCR were expressed without the transmembrane domains, both NBD1 and NBD2 exhibited ATPase activity (46, 47). However, there were significant differences in nucleotidase activity and selectivity, consistent with the notion that the two NBDs of ABC proteins are functionally distinct. The ATPase activity of the NBD1 (V_{max} = 30 nmol/min/mg for the 43-kDa soluble protein) is comparable with the individually expressed N-half (Fig. 3C). This suggests that the N-half when expressed by itself can bind and hydrolyze ATP although relatively inefficiently. In contrast, the C-half did not bind detectable amounts of azido-ATP (Fig. 5B) and displayed ATPase activity that is significantly less than that of NBD2 (V_{max} = 140 nmol/min/mg) and close to baseline levels. It appears that the individually expressed C-half is highly misfolded and aggregated resulting in a functionally inactive protein that cannot associate with the N-half in vitro.

Previously, Sun et al. (48) have proposed a mechanism for the coupling of ATP hydrolysis to substrate transport. On the basis of analysis of naturally occurring and synthetic mutations, they proposed a model in which NBD1 is responsible for basal ATPase activity of ABCR, whereas both NBD1 and NBD2 participate in the retinal-stimulated ATPase activity and its coupling to substrate transport. An implicit assumption in this model is that both NBDs function in the binding and hydrolysis of ATP. Our studies, however, are inconsistent with this model. As discussed above, analysis of both trypsin-digested ABCR and co-expressed half-molecules indicate that NBD2 is the principal site of ATP binding and hydrolysis in the presence and absence of retinal substrate. NBD1 on the other hand appears to contain a tightly bound ADP that plays a crucial role in orchestrating the activity of NBD2 through direct interaction. Mutations in NBD1, therefore, may abolish retinal-stimulated ATPase activity of ABCR by affecting the interaction of NBD1 with NBD2. The decrease in basal ATPase activity in these mutants may result from a similar mechanism. Alternatively, a low basal ATPase activity of ABCR may arise from activity of NBD1 generated in a small fraction of the expressed protein that has been altered during solubilization and reconstitution. Mutations in this domain would thus directly decrease the observed basal activity. Our results support a model in which ATP binding and hydrolysis at NBD2 is responsible for transport, whereas NBD1 plays an essential, although non-catalytic role in this process.

Our finding that co-expression of the N and C halves of ABCR results in a functional protein may have applications in gene therapy for Stargardt macular dystrophy. Adeno-associated viral vectors provide a relatively safe, efficient, and stable means to transfer genes and associated regulatory elements into retinal and retinal pigment epithelial cells (49, 50). However, adeno-associated viral vectors with a packaging limit of about 4.8 kb prevents its application with ABCR having a cDNA of 6.8 kb even without regulatory elements. However, it may be feasible to package the cDNAs coding the N and C halves into separate adeno-associated viral vectors containing a photoreceptor cell-specific promoter. Co-expression in photoreceptor cells should result in a functional complex that could slow or prevent the onset of recessive Stargardt macular dystrophy.

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