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Mechanistic Studies of ABCR, The ABC Transporter in Photoreceptor Outer Segments Responsible for Autosomal Recessive Stargardt Disease

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ABCR is an ABC transporter that is found exclusively in vertebrate photoreceptor outer segments. Mutations in the human ABCR gene are responsible for autosomal recessive Stargardt disease, the most common cause of early onset macular degeneration. In this paper we review our recent work with purified and reconstituted ABCR derived from bovine retina and from cultured cells expressing wild type or site-directed mutants of human ABCR. These experiments implicate *all-trans*-retinal (or Schiff base adducts between *all-trans*-retinal and phosphatidylethanolamine) as the transport substrate, and they reveal asymmetric roles for the two nucleotide binding domains in the transport reaction. A model for the retinal transport reaction is presented which accounts for these experimental observations.

KEY WORDS: Retinal degeneration; retinoid transporter; macular degeneration; ABC transporter.

INTRODUCTION: ABCR AND RETINAL DISEASE

This paper summarizes recent in vitro work on the photoreceptor-specific ABC transporter, ABCR. ABCR is an abundant membrane protein that is localized exclusively within the photoreceptor outer segment (Illing *et al.*, 1997; Sun and Nathans, 1997; Molday *et al.*, 2000), a specialized appendage that houses the visual pigments and other phototransduction proteins (Fig. 1). As described more fully below, data from in vitro assays with purified ABCR (Ahn *et al.*, 2000; Sun *et al.*, 1999) and from mice in which the ABCR gene has been disrupted (Mata *et al.*, 2000; Weng *et al.*, 1999) imply that ABCR transports *all-trans*-retinal, the chromophore derivative that is released from the visual pigment following light absorption.

Mutations within the gene encoding ABCR are responsible for autosomal recessive Stargardt disease (STGD; Allikmets *et al.*, 1997a; Lewis *et al.*, 1999; Nasonkin *et al.*, 1998; Rozet *et al.*, 1998; Stone *et al.*, 1998), the most common early onset form of inherited macular degeneration, as well as a subset of cases of autosomal recessive cone-rod dystrophy (Cremers *et al.*, 1998) and retinitis pigmentosa (Cremers *et al.*, 1998; Martinez-Mir *et al.*, 1998; Rozet *et al.*, 1999). Moreover, carriers of ABCR mutations may be at increased risk for age-related macular degeneration (AMD; Allikmets *et al.*, 1997b, 2000), the most common cause of loss of vision in elderly individuals in industrialized countries. Current evidence suggests that ABCR defects responsible for retinitis pigmentosa, cone-rod dystrophy, STGD, and AMD represent a continuum of severities from a complete absence of ABCR function in retinitis pigmentosa to less than 50% loss of function in AMD (Maugeri *et al.*, 1999).

Vision begins when light is absorbed by a visual pigment, a specialized G-protein coupled receptor that carries an 11-*cis*-retinal chromophore. Photoactivation isomerizes retinal from 11-*cis* to *all-trans*, after which

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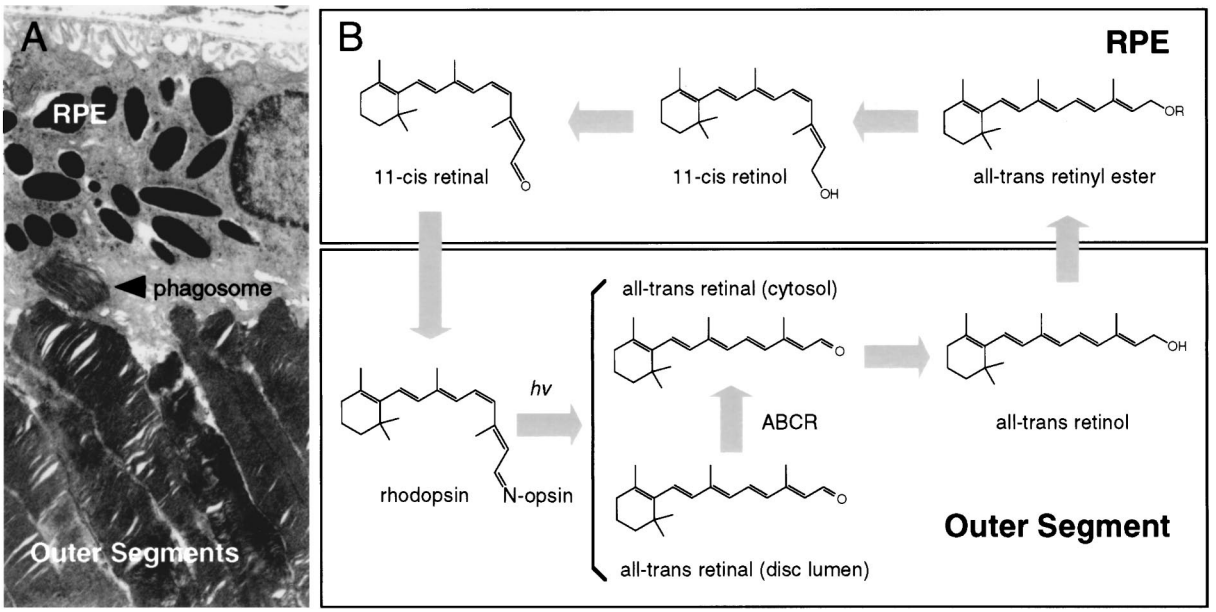

Fig. 1. Photoreceptor outer segments, the RPE, and the visual cycle. (A) Electron micrograph of mouse photoreceptor outer segments (lower) and the RPE (upper). The striations within the outer segments are derived from the internal disc membranes. In vertebrates the photoreceptor outer segment is continually renewed throughout the life of the organism. Newly synthesized outer segment proteins and lipids are incorporated at the base of the outer segment, moving outward until they reach the distal tip of the outer segment where they are engulfed by the RPE. A phagosome with a newly ingested fragment of outer segment is seen in the center of the micrograph. (B) The visual cycle. Light absorption by the visual pigment rhodopsin leads to the release of *all-trans*-retinal, which partitions into the outer segment disc membrane where it exists as a mixture of free *all-trans* retinal and as a Schiff base adduct with PE. ABCR is hypothesized to extract *all-trans*-retinal or flip the *all-trans*-retiny1–PE adduct from the lumenal face of the disc membrane to the cytosolic face, facilitating its reduction to *all-trans*-retinol by retinol dehydrogenase. Within the RPE, retinol is esterified, isomerized to the 11-*cis* configuration, oxidized to form 11-*cis*-retinal, and returned to the photoreceptor where it recombines with opsin to form rhodopsin.

all-trans-retinal dissociates from the visual pigment to be replaced by a new molecule of 11-*cis*-retinal. In rod photoreceptors, the released *all-trans*-retinal (the aldehyde derivative of vitamin A) is reduced to *all-trans*-retinol (the corresponding alcohol derivative) which is then transported to the adjacent cell layer, the retinal pigment epithelium (RPE). Within the RPE, *all-trans*-retinol is chemically isomerized to the 11-*cis* configuration, oxidized to the aldehyde, and returned to the photoreceptor. This cycle of events is referred to as the visual cycle (Fig. 1).

In vertebrate photoreceptors, a variety of experiments suggest that reduction of released *all-trans*-retinal to *all-trans*-retinol is a critical and rate-limiting step in setting the threshold of rod photosensitivity. In vitro, binding of *all-trans*-retinal to the visual pigment apoprotein, opsin, leads to a low level of activation (Buczylko *et al.*, 1996; Cohen *et al.*, 1992; Jager *et al.*, 1996; Surya *et al.*, 1995) and in vivo, the initial rise and subsequent decline in *all-trans*-retinal following intense illumination parallels the loss and recovery of visual sensitivity (Alpern, 1971; Alpern *et al.*, 1970; Fain *et al.*, 1996; Lamb, 1980; Saari *et al.*, 1998; Zimmerman *et al.*, 1974). As seen in Fig. 1(B), ABCR is hypothesized to accelerate the conversion of *all-trans*-retinal to *all-trans*-retinol by transporting either *all-trans*-retinal or a Schiff base adduct of *all-trans*-retinal and phosphatidylethanolamine (PE) from the lumenal to the cytosolic face of the internal (disc) membrane system of the outer segment, thereby making it accessible to the cytosolic enzyme responsible for its reduction.

Figure 2 summarizes our current thinking regarding the role of ABCR in retinal disease. STGD, the best studied disease associated with ABCR mutations, is characterized by a defect in dark adaptation and by a massive accumulation of lipofuscin—a heterogeneous mixture of molecular debris—within the RPE (Blacharski, 1988; Eagle *et al.*, 1980; Klein and Krill, 1967; Steinmetz *et al.*, 1991). The lipofuscin is very likely derived from photoreceptor outer segments which are normally engulfed and degraded by the RPE. Ultimately, these defects are followed by RPE and photoreceptor cell loss. The current model for ABCR's role in STGD (Mata *et al.*, 2000; Sun *et al.*, 1999; Weng *et al.*, 1999) envisions a partial block at the

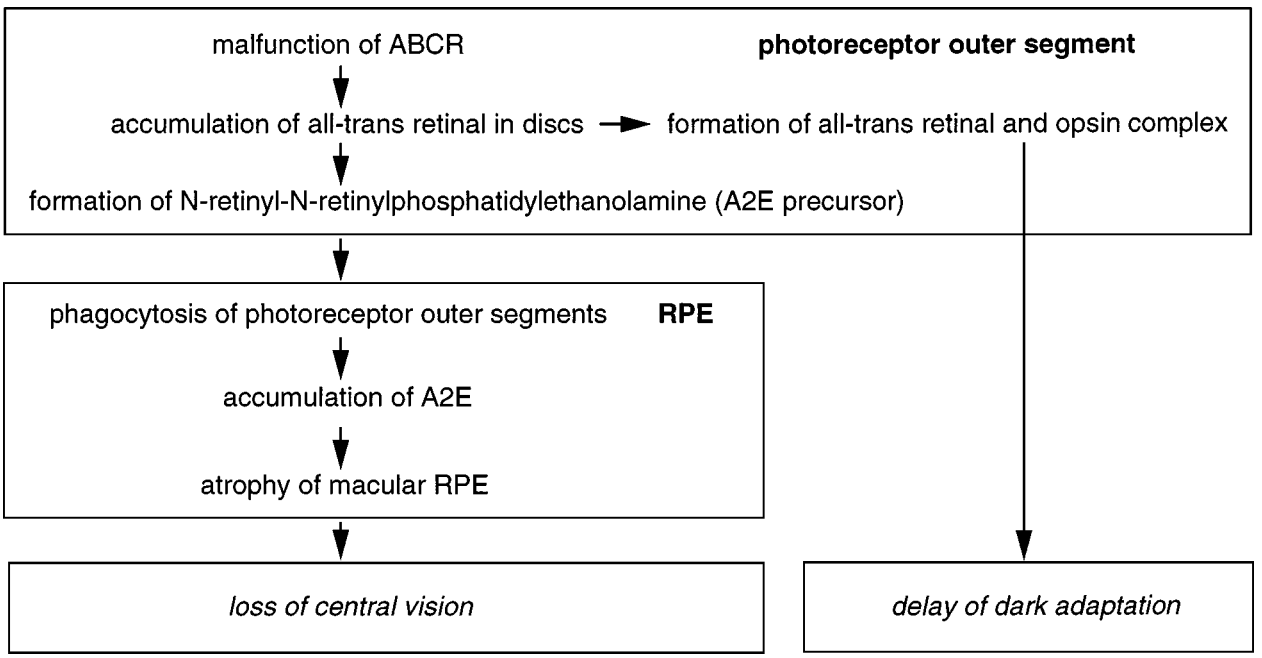

Fig. 2. Model for the pathophysiology of Stargardt disease. Top, events within the photoreceptor outer segment; center, events within the RPE; bottom, clinical consequences.

all-trans-retinal reduction step based on ABCR's proposed role in transporting *all-trans* retinal to the cytosolic face of the membrane.

The left side of Fig. 2 shows the cellular consequences of a block at the *all-trans*-retinal reduction step; these consequences are proposed to follow, at least in part, from an accelerated buildup of a diretinal adduct referred to as A2E (Eldred and Lasky, 1993; Sakai *et al.*, 1996). A2E is one component of the RPE lipofuscin that accumulates over time in STGD eyes, and to a lesser extent in normal eyes (Delori *et al.*, 1995a,b; Eldred and Lasky, 1993; Mata *et al.*, 2000). A2E most likely forms spontaneously within the outer segment when *all-trans*-retinal condenses to form a Schiff base with PE (Eldred and Lasky, 1993; Mata *et al.*, 2000; Parish *et al.*, 1998). Its accumulation in RPE cells reflects phagocytosis of photoreceptor outer segments and appears to sensitize the RPE to phototoxicity by visible light (Sparrow *et al.*, 2000). The right side of Fig. 2 shows the proposed mechanism for delayed dark adaptation, a characteristic feature of STGD (Fishman *et al.*, 1991).

We now turn to our in vitro studies with ABCR which have focused on (1) identifying the transport substrate, (2) defining the biochemical defects associated with various disease-associated ABCR sequence variants, and (3) exploring the mechanism by which ATP hydrolysis is coupled to transport.

EVIDENCE THAT ABCR TRANSPORTS RETINAL

The first clue to the identity of ABCR's transport substrate came from localization of ABCR to photoreceptor outer segments (Illing *et al.*, 1997; Sun and Nathans, 1997), which immediately suggested that it might transport a molecule uniquely important to vision. The second clue came from circumstantial evidence for the accumulation of A2E in STGD (Delori *et al.*, 1995a,b), which suggested that ABCR may play a role in the visual cycle. Taken together, these clues focused our attention on retinoids as the most attractive of the candidate transport substrates (Allikmets *et al.*, 1997a; Sun and Nathans, 1997).

Our point of departure in testing various compounds as potential transport substrates was the observation that purified and reconstituted P-glycoprotein, another member of the ABC transporter family, exhibits an increase in ATPase activity when incubated in the presence of known transport substrates, an effect that presumably reflects the coupling of ATP hydrolysis to the transport reaction (Ambudkar *et al.*, 1992; Sarkadi *et al.*, 1992; Shapiro and Ling, 1994; Urbatsch *et al.*, 1994). As a strategy for screening large numbers of candidate substrates, monitoring ATPase activity has a number of experimental advantages, including speed and sensitivity. More

importantly, it allows one to circumvent the need to develop transport assays for a large number of chemically diverse compounds which would require radiolabeled or fluorescent derivatives of each compound.

To search for transport substrates, we purified ABCR to apparent homogeneity from bovine rod outer segments, reconstituted it into lipid vesicles, and asked which of a group of 37 chemically diverse compounds could stimulate ABCR-mediated ATP hydrolysis (Sun *et al.*, 1999). The results of this survey show that several structurally unrelated compounds—including amiodarone, digitonin, and *all-trans*-retinal—are effective activators of ABCR-mediated ATPase. Among these several compounds, only *all-trans*-retinal (hereafter referred to simply as "retinal") and related retinoids show simple Michaelis–Menton activation of ABCR's ATPase, indicative of a single binding site or class of binding sites. A second distinction between retinal and the several nonretinoid activators was observed when combinations of activators were tested. Retinal added in combination with any of the nonretinoid activators produces a greater than additive effect, but the several nonretinoid activators produce only additive effects when tested in combination among themselves.

A third distinctive characteristic of ATPase activation by retinal is its effect on the initial reaction velocity measured at different ATP concentrations. In this analysis, retinal is observed to activate ABCR by an "uncompetitive" mechanism (Fig. 3(A) and (B)), a terminology borrowed from the familiar division of reversible enzyme inhibitors into competitive, noncompetitive, and uncompetitive types (Lehninger, 1975; Segel, 1976). An uncompetitive mode of activation is characterized by a downward and parallel shift in the locus of points that characterize 1/initial velocity vs. 1/[ATP] in a Lineweaver–Burk plot. In mechanistic terms, these data imply that retinal specifically interacts with and accelerates a rate-limiting step in the ATPase reaction pathway (Fig. 3(C)). This mode of action can be equivalently conceptualized as a double displacement or "ping-pong" reaction (Fig. 3(D)) in which the progression of one substrate—in this case ATP—through the reaction pathway depends upon an enzyme intermediate formed via an interaction with the other substrate, retinal. By contrast, in a "noncompetitive" mode of activation the ligand interacts with the enzyme at all points in the reaction cycle, producing an effect that is equivalent to adding more enzyme to the reaction. Noncompetitive activation is seen with amiodarone, one of the nonretinoid ABCR activators. In a Lineweaver–Burk plot, noncompetitive activation is characterized by a downward rotation in the locus of points that characterize 1/initial velocity vs. 1/[ATP] such that the $1/K_m$ intercept remains unchanged.

An uncompetitive mode of ATPase activation is precisely the behavior predicted for a transport substrate, whereas a noncompetitive mode of activation is the behavior predicted for a nonsubstrate that allosterically activates the ATPase. Taken together, the characteristics of ABCR ATPase activation by retinal strongly imply that retinal (or its PE adduct) is the transported substrate(s). Weng and colleagues independently arrived at the same conclusion based on the excessive light-dependent accumulation of retinyl–PE in the retinas of ABCR $(-/-)$ mice (Weng *et al.*, 1999). We note that the analysis of uncompetitive vs. noncompetitive modes of ATPase activation might prove to be generally useful in the identification of transport substrates for the many "orphan" transporters identified by large-scale DNA sequencing.

Whether ABCR transports both *all-trans*- and 11-*cis*-retinal in vivo, either of which can stimulate ATPase activity in vitro, remains an open question. We note that the action of retinol dehydrogenase, which exhibits a high degree of specificity for *all-trans*-over 11-*cis*-retinal (Lion *et al.*, 1975; Palczewski *et al.*, 1994; Rattner *et al.*, 2000), would reduce any *all-trans* retinal that is transported to the cytosolic face of the disc membrane, but would not be expected to alter the fate of 11-*cis*-retinal.

IN VITRO STUDIES OF DISEASE-ASSOCIATED ABCR VARIANTS

A major challenge in the genetic analysis of ABCR-based retinopathies arises from the observation that the vast majority of ABCR sequence variants identified to date are missense mutations that are rare in both patient and control populations. With the current sample size of most sequence variants, one cannot determine statistically whether a particular sequence variant is pathogenic or neutral (Allikmets *et al.*, 1997a; Lewis *et al.*, 1999; Nasonkin *et al.*, 1998; Rozet *et al.*, 1998; Stone *et al.*, 1998). A related challenge is to determine the degree to which each pathogenic variant impairs ABCR function, as genotype–phenotype analyses indicate that age of onset and disease severity correlate with different ABCR alleles. To address these questions, we have produced and functionally analyzed wild-type and mutant human ABCR from transfected 293 cells (Sun *et al.*, in press). Among 37 disease-associated ABCR variants studied, the majority were found to differ from the wild type in protein yield, ATP-binding, basal ATPase activity, and/or the modulation of ATPase activity by added retinal.

Approximately one-third of the human ABCR variants are produced with greatly reduced yield. Mutations that produce small deletions or that introduce charged

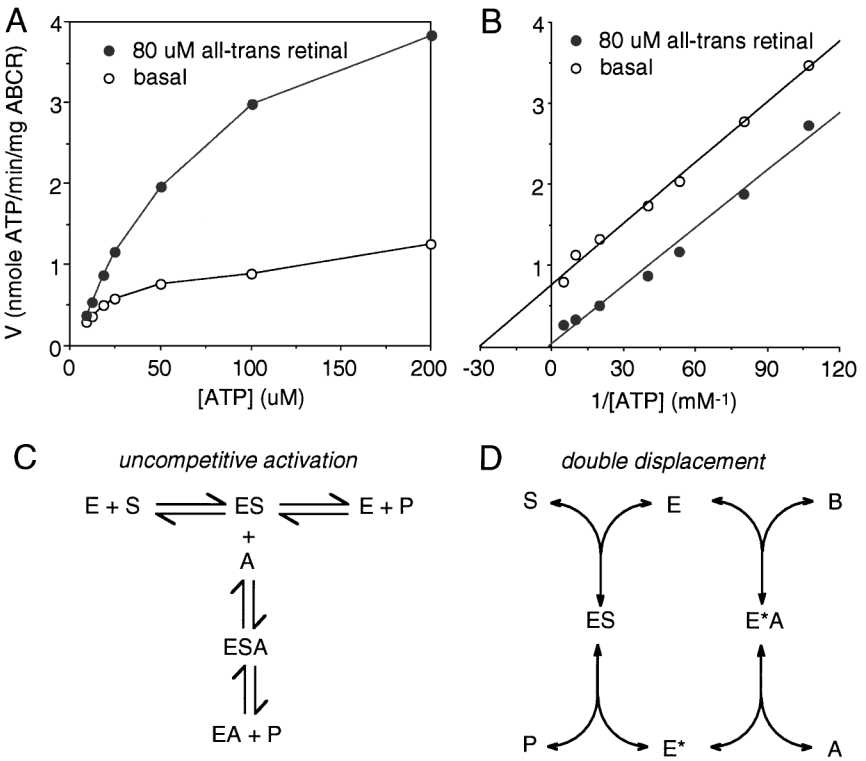

Fig. 3. K_m for ATP and V_{max} for ATP hydrolysis determined for purified and reconstituted bovine outer segment ABCR in brain polar lipid in the presence or absence of 80 μM *all-trans*-retinal. Panel (B) shows the Lineweaver–Burk plot derived from the data shown in panel (A). Based only on the intercepts of the fitted lines, the K_m for ATP and the V_{max} of ATP hydrolysis are as follows: 33 μM and 1.3 nmol/min/mg (no addition), or 725 μM and 29 nmol/min/mg (80 μM *all-trans*-retinal). *All-trans*-retinal addition therefore increases both the K_m for ATP and the V_{max} of ATP hydrolysis more than 20-fold. The parallel shift of the datapoints in the Lineweaver–Burk plot as a function of added retinal is consistent with an uncompetitive mode of enzyme activation by *all-trans*-retinal. (C) Schematic of uncompetitive activation. A, activator (*all-trans*-retinal); E, enzyme (ABCR); P, product (ADP + Pi); S, substrate (ATP). Binding of the activator specifically to the E–S complex creates a second and more efficient path to the reaction product. (D) Schematic of a double displacement or "ping-pong" reaction. The conversion of one substrate, S (ATP), to product, P (ADP + Pi), creates an altered enzyme species, E*, which is restored to the original enzyme species, E, by processing of a second substrate, A, to product, B. A and B in this model would represent retinal prior to and after transport, respectively.

amino acids into predicted transmembrane domains produce the most dramatic reductions in yield. Among the ABCR variants that are expressed with normal or nearly normal yield, labeling with α-32 P-azido-ATP reveals a subset of ABCR variants that are defective in ATP binding. Interestingly, a variety of mutations that lie outside of the nucleotide binding domains (NBDs) can impair azido-ATP labeling. Among these are L541P, predicted to reside adjacent to a transmembrane domain, and W1408R, which resides between the homologous halves of ABCR. These data suggest that ATP binding to the two NBDs is allosterically coupled to conformational changes in or near the transmembrane regions.

To examine the effect of mutation on the coupling between retinal binding/transport and ATP hydrolysis, a subset of ABCR variants associated with STGD and/or AMD were purified, reconstituted into membranes, and tested for basal and retinal-stimulated ATPase activities. Figure 4(A) and (B) illustrates the behavior of two of these variants, T971N and G1961E, which reside within NBD-1 and NBD-2, respectively. T971N shows little basal ATPase and little or no retinal-stimulated ATPase; it is also defective in azido-ATP labeling. G1961E exhibits reduced basal ATPase activity, and the ATPase activity shows *inhibition* by retinal rather than stimulation. Among the variants tested in NBD-1, T971N and A1038V have a lower

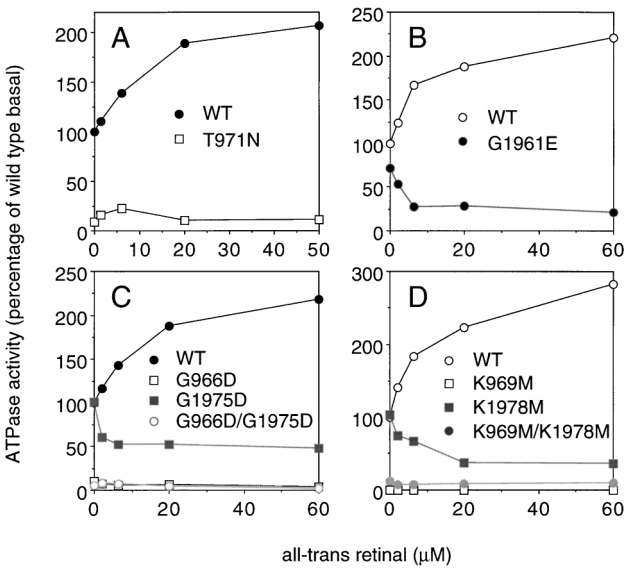

Fig. 4. Effect of *all-trans*-retinal on ATP hydrolysis by naturally occurring and synthetic mutants in the NBDs. The ATPase activity of purified and reconstituted human ABCR is measured in the presence of different concentrations of retinal. By convention the ATPase activity of the wild type in the absence of retinal, i.e., the basal ATPase, is taken as 100% activity. Within each panel, the wild-type and mutant proteins were produced, harvested, purified, and assayed in parallel. (A) Naturally occurring variant T971N in NBD-1. (B) Naturally occurring variant G1961E in NBD-2. (C) Synthetic substitutions of a conserved glycine in the Walker A motif of NBD-1 (G966D), NBD-2 (G1975D), or both (G966D/G1975D). (D) Synthetic substitutions of a conserved lysine in the Walker A motif of NBD-1 (K969M), NBD-2 (K1978M), or both (K969M/K1978M).

basal ATPase activity than N965S, and all three show little or no retinal-stimulated ATP hydrolysis. Among the variants tested in NBD-2, L1971R eliminates both basal and retinal-stimulated ATP hydrolysis, whereas G1977S and E2096K resemble G1961E in showing *inhibition* rather than stimulation of ATPase by retinal. The complete or nearly complete elimination of all ATPase activity produced by single NBD mutations—T971N, A1038V, or L1971R—implies that the two NBDs are allosterically coupled.

To more precisely define the role of each NBD, we studied two sets of synthetic single and double mutations in either the highly conserved first glycine or the lysine of the Walker A motif, GXXGXGK, within the first and second NBDs. Based on the crystal structure of the homologous NBD from the *Salmonella typhimurium* histidine permease (Hung *et al.*, 1998), the synthetic mutations are predicted to eliminate ATP binding and/or hydrolysis without affecting protein stability. All six synthetic variants—four single and two double mutants—show approximately normal yields of ABCR as determined by immunoblotting. When purified, reconstituted, and tested for ATPase activity, the synthetic mutations show (1) that mutations in NBD-1 (G966D or K969M), either alone or in combination with mutations in NBD-2 (G966D/G1975D or K969M/K1978M), abolish both basal and retinal-stimulated ATP hydrolysis and (2) that mutations in NBD-2 (G1975D or K1978M) do not alter the basal ATPase activity but lead to *inhibition* rather than stimulation of ATP hydrolysis by retinal (Fig. 4(C) and (D)), a pattern noted above for the naturally occurring NBD-2 mutations G1961E, G1977S, and E2096K.

These data indicate that, in the absence of retinal, ABCR undergoes cycles of ATP hydrolysis exclusively at NBD-1. The retinal-dependent inhibition of ATPase observed in NBD-2 mutants indicates that, without ATP binding and/or hydrolysis at NBD-2, retinal can bind to ABCR and trap it in an intermediate conformation that allosterically blocks the NBD-1 ATPase cycle.

A MODEL FOR TRANSPORT AND ATP HYDROLYSIS

Figure 5 shows a simple model that accounts for the observations described above. The crux of the model is that the NBD-1 ATPase cycle is coupled to retinal binding to an entry site on the lumenal face of the bilayer and that the NBD-2 ATPase cycle is coupled to retinal release from an exit site on the cytoplasmic face of the bilayer. The model predicts that NBD-2 mutations block retinal release; the resulting failure to empty the entry site and return it to its starting conformation presumably inhibits the initiation of a new ATPase cycle at NBD-1.

In the particular embodiment of these ideas that we depict in Fig. 5, the transport cycle is driven by the sequential opening and closing of retinal entry and exit sites. The opening and closing of the entry site is coupled to the ATPase cycle of NBD-1, while the opening and closing of the exit site is coupled to the ATPase cycle of NBD-2. The model makes no predictions regarding the exact manner in which an ATPase cycle is coupled to a cycle of binding site opening and closing. In Fig. 5 we have arbitrarily shown one part of the NBD-1 ATPase cycle coupled to the opening of the entry site and a second part of the NBD-1 ATPase cycle coupled to the closing of the entry site. It is equally plausible that NBD-1 coupling involves only opening or closing of the entry site and that the site relaxes spontaneously to the alternate state. A similar line of reasoning applies to the coupling between the exit site and NBD-2.

Transport is envisioned to involve the following seven steps (Fig. 5(B)): (1) opening of the entry site on the lumenal side of the membrane; (2) binding of retinal (or a Schiff base of retinal and PE) to the open entry site; (3) opening of the exit site on the cytosolic side of the membrane; (4) translocation of retinal from the entry to the exit site, a movement which might not be coupled to either ATPase cycle; (5) closure of the entry site; (6) release of retinal from the exit site; and (7) closure of the exit site. The sequential pattern of the four opening and closing events—entry site opens, exit site opens, entry site closes, and exit site closes—determines the directionality of transport.

In the absence of retinal (Fig. 5(C)), left), the model predicts that wild-type ABCR or mutants defective in NBD-2 would exhibit a cycle of opening and closing of the entry site coupled to ATP hydrolysis by NBD-1. Because the ATPase activity in the absence of retinal is unaffected by mutations that are predicted to eliminate NBD-2 ATPase (Fig. 4(B)–(D)), we presume that the exit site/NBD-2 ATPase cycle is activated only after retinal binds to the entry site. In the presence of retinal, wild-type ABCR proceeds through the entire transport cycle, whereas the model predicts that mutants defective in NBD-2 will be blocked at step 3 (Fig. 5(C), right). In the latter case, a fraction of the ABCR population would be drawn into a state in which retinal is bound to the open entry site, reducing turnover through the entry site/NBD-1 ATPase cycle, thereby leading to the observed *inhibition* of ATPase by added retinal.

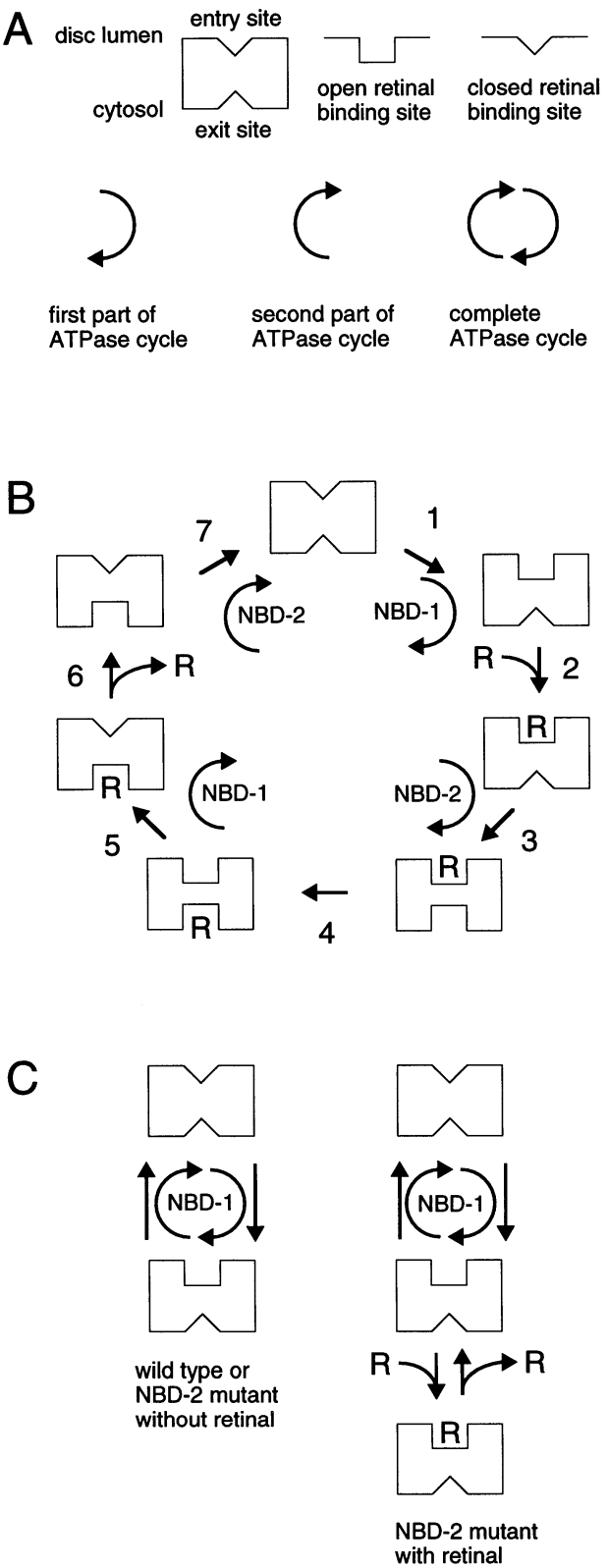

Fig. 5. Model for the ABCR reaction cycle and the effect of NBD-2 mutations and added retinal. (A) In the upper half are schematic representations of the transmembrane domain of ABCR with entry and exit sites for retinal. Each site is presumed to exist in either of two states: open (high affinity) or closed (low affinity). The lumenal and cytosolic faces of the membrane are represented, respectively, by the upper and lower sides of ABCR's transmembrane domain. In the lower half are schematic representations of a cycle of ATP hydrolysis. A single full circle represents a complete cycle of ATP binding, hydrolysis, and ADP + Pi release. (B) A transport cycle in which one complete ATP hydrolytic cycle occurs at each of the two NBDs and a single retinal or its Schiff base adduct with PE (R) is vectorially transported from the lumenal to the cytosolic face of the membrane. (C) Partial transport cycles of wild-type ABCR or of NBD-2 mutants in the absence of retinal (left), or of NBD-2 mutants in the presence of retinal (right). Because the basal ATPase activity is unaffected by NBD-2 mutations it is proposed to arise from progression through the ATPase cycle at NBD-1 coupled to the opening and closing of the entry site. In the absence of NBD-2 function, binding of retinal or retinal–PE slows the ATPase cycle at NBD-1 by reversably drawing ABCR into a semistable configuration in which the open entry site is occupied (right, bottom).

SUMMARY AND PERSPECTIVE

The experiments outlined above represent initial steps in dissecting the function of ABCR and in uncovering its role in retinal physiology and retinal disease. One major challenge for the future will be to develop an in vitro transport assay, a challenge made more difficult by the extreme hydrophobicity of retinal. If such an assay can be developed, then it will be possible to directly test the transport activity of wild-type and mutant ABCR, and to critically evaluate mechanistic models of ABCR-mediated transport.

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