Published in final edited form as:

Biochim Biophys Acta. 2009 July ; 1791(7): 573–583. doi:10.1016/j.bbalip.2009.02.004.

The Role of the Photoreceptor ABC Transporter ABCA4 in Lipid Transport and Stargardt Macular Degeneration

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Abstract

ABCA4 is a member of the ABCA subfamily of ATP binding cassette (ABC) transporters that is expressed in rod and cone photoreceptors of the vertebrate retina. ABCA4, also known as the Rim protein and ABCR, is a large 2273 amino acid glycoprotein organized as two tandem halves, each containing a single membrane spanning segment followed sequentially by a large exocytosomal domain, a multispinning membrane domain and a nucleotide binding domain. Over 500 mutations in the gene encoding ABCA4 are associated with a spectrum of related autosomal recessive retinal degenerative diseases including Stargardt macular degeneration, cone-rod dystrophy and a subset of retinitis pigmentosa. Biochemical studies on the purified ABCA4 together with analysis of *abca4* knockout mice and patients with Stargardt disease have implicated ABCA4 as a retinylidene-phosphatidylethanolamine transporter that facilitates the removal of potentially reactive retinal derivatives from photoreceptors following photoexcitation. Knowledge of the genetic and molecular basis for ABCA4 related retinal degenerative diseases is being used to develop rationale therapeutic treatments for this set of disorders.

1. Introduction

The transport of lipids across biological membranes is critical for the structure, function and survival of cells. This process is typically carried out by a variety of integral membrane proteins which specifically bind and actively or passively translocate lipids across the membrane lipid bilayer. Some lipid transporters import or export lipids from cells or intracellular organelles, while others simply flip lipids such as phospholipids across the lipid bilayer to generate or maintain transbilayer lipid asymmetry critical for cell membrane structure and function [1]. Lipid substrates known to be transported across membranes include vitamins, fatty acids, phospholipids, glycolipids, cholesterol, bile salts, steroids, toxins, drugs, and metabolites.

ATP binding cassette (ABC) transporters comprise a large superfamily of proteins found in essentially all living organisms [2]. The human genome encodes 48 ABC transporters which have been organized into 7 subfamilies designated ABCA through ABCG[3]. A significant number of ABC transporters function in lipid trafficking in cells and many have been linked to severe genetic diseases [4,5]. For example, inherited defects in ALD (ABCD1), a transporter of very long chain fatty acids, is associated with adrenoleukodystrophy[6], defects in Sterolin1 and 2 (ABCG5/ABCG8), transporters for sterols, have been linked to sitosterolemia [7],

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defects in ABC1 (ABCA1) which mediate the efflux of cholesterol and phospholipid from cells causes Tangier disease [8], and defects in MDR3 and BSEP (ABCB11) involved in the transport of phosphatidylcholine and bile acids, respectively, are known to cause liver disease [9] [10] .

Members of the ABCA subfamily of ABC transporters have been implicated in the transport of various lipids across cell membranes [5]. Of these, four ABCA transporters are now known to be associated with a various genetic disorders that result from defects in lipid transport. In this review, we will focus on the biochemical properties of one of these transporters, ABCA4, and its role in lipid transport and retinal degenerative diseases associated with severe vision loss.

2. ABCA Subfamily of ABC Transporters

The ABCA subfamily of ATP binding cassette transporters is a group of ABC transporters having a relatively high degree of sequence identity and a similar structural organization. Twelve *ABCA* genes designated *ABCA1* – *ABCA13* have been identified in the human genome [5,11]. *ABCA11* is now known to be a pseudogene and therefore is no longer considered as a member of this subfamily. Two subgroups have been identified. One comprises a cluster of genes (*ABCA5*, *ABCA6*, *ABCA8*, *ABCA9*, and *ABCA10*) that map to chromosome 17q24.2–3 and code for proteins of similar size (1543 – 1642 amino acids) and sequence (53 – 78% similarity). The other group consists of *ABCA1*, *ABCA2*, *ABCA3*, *ABCA4*, *ABCA7*, *ABCA12*, and *ABCA13* genes. A cluster of 3 additional genes, *Abca14*, *Abca15*, and *Abca16*, have been found in the mouse but not the human genome [12]. Homology searches have revealed the presence of ABCA homologues in a wide variety of other eukaryotic and prokaryotic organisms [13].

All members of the human ABCA subfamily are large full transporters organized in two tandem halves. Both the N-terminal and C-terminal half consists of a multi-spanning transmembrane domain followed by a nucleotide binding domain. A characteristic feature of ABCA proteins is the presence of a large extracellular domain that separates the first transmembrane segment from a cluster of five transmembrane segments in each half of the protein [14,15].

The substrates transported by most members of the ABCA subfamily remain to be identified. However, biochemical analyses of the ABCA1, ABCA3, ABCA4, and ABCA12 transporters together with their association with inherited diseases have implicated these proteins in the active transport of lipids across cell membranes. ABCA1 plays a crucial role in the efflux of cholesterol and phospholipids from macrophage and other cells [16]. Defects in ABCA1 are known to cause Tangier disease and familial high density lipoprotein deficiency, disorders associated with defective cholesterol and phospholipid efflux from cells and a deficiency in circulating high density lipoproteins [8,17–19]. ABCA3 has been implicated in phospholipid transport required for the formation of normal lamellar bodies and surfactant secretion into the alveoli of the lungs [20–22]. Mutations in ABCA3 cause neonatal surfactant deficiency associated with abnormal lamellar bodies in the lungs and a milder respiratory disease known as pediatric interstitial lung disease. Mutations in ABCA12 are responsible for harlequin and lamellar ichthyosis, diseases of the skin generally resulting from defective lipid transport [23–25]. ABCA7 and ABCA2 have been implicated in lipid transport although these transporters have yet to be linked to any inherited diseases.

3. Cellular Localization and Structural Features of ABCA4

ABCA4 was first cloned in 1997 using two different strategies. In one approach, the gene for the Rim protein, an abundant high molecular weight photoreceptor membrane protein of unknown function first described in the late 1970's, was cloned and shown to encode a novel

photoreceptor ATP binding cassette protein [14,26,27]. In another approach, Allikmets and coworkers identified the gene defective in Stargardt disease, a relatively common, early onset macular dystrophy [28,29]. This gene located on chromosome 1p22.1 was found to encode a retina specific ABC transporter which was named ABCR. Sequence comparison of ABCR and the Rim protein indicate that they are the same ABC transporter [30], now generally known as ABCA4 for the fourth identified member of the ABCA subfamily.

3.1. Localization of ABCA4 in Rod and Cone Photoreceptor Cells

ABCA4 is almost exclusively expressed in the retina, the light sensing neural tissue at the back of the eye [28,29]. The retina consists of 5 major neuronal cell types (photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells) and one glial cell type known as Mueller cells. *ABCA4* gene expression is confined to the photoreceptor cells as determined by *in situ* hybridization [28,29]. Immunofluorescence microscopic studies have localized the ABCA4 protein to the photoreceptor outer segment, a specialized compartment of the photoreceptor cell that functions in the capture of light and its conversion into an electrical signal in a process known as phototransduction [14,31].

There are two main types of photoreceptors in the vertebrate retina, rod cells that function under dim lighting conditions and cones that are responsible for vision under normal lighting conditions, color vision, and high visual acuity. Initial immunolabeling studies suggested that ABCA4 was only present in the outer segments of mammalian rod photoreceptors [14,31]. However, in subsequent studies, ABCA4 has been localized to the outer segments of foveal and peripheral cone cells as well as rod photoreceptors of the human retina [32]. This finding is in agreement with the early studies of Papermaster et al. [33] showing that the Rim protein is present in outer segment discs of frog rod and cone photoreceptors.

The outer segment of a rod cell consists of a stack of over 1000 discs surrounded by a separate plasma membrane. Each disc is composed of two adjacent, flattened membranes that are circumscribed by a hairpin loop known as the rim region. The continuous disc membrane encloses a space known as the intradiscal space or disc lumen. The circumference of the disc is interrupted by one or more incisures that penetrate toward the center of the disc. Immunoelectron microscopic studies have localized ABCA4 to the rim and incisures of the disc membrane in both mammalian and amphibian photoreceptor cells [14,33].

Although ABCA4 is primarily expressed in retinal photoreceptors, mRNA and protein expression have been found in the choroid plexus of rat brain [34]. The functional significance of ABCA4 expression in the brain, however, remains to be established.

ABCA4 has been transiently expressed in several mammalian culture cell lines including HEK 293 and COS cells [35,36]. Immunofluorescence microscopic studies have localized ABCA4 to intracellular vesicular structures of varying sizes [37,38], a distribution that has also been observed for peripherin/rds and rom-1 [39,40], photoreceptor specific proteins that also localize to the rim and incisures of photoreceptor disc membranes [40,41]. The intracellular localization of heterologously expressed ABCA4 is in marked contrast to the plasma membrane localization of heterologously expressed ABCA1, a protein that is 50% identical in amino acid sequence to ABCA4. These studies suggest that ABCA4 and ABCA1 contain distinct signals that target these transporters to intracellular membranes in the case of ABCA4 and the plasma membrane for ABCA1.

3.2. General Structural Organization

The human *ABCA4* gene consisting of 50 exons encodes a 2,273 amino acid protein having a molecular mass of 256 kDa [29,30]. ABCA4 is organized into two tandem halves, with each

half containing a transmembrane segment followed sequentially by a large exocytosolic (extracellular/lumen) domain (ECD), a multi-spanning membrane domain and a nucleotide binding domain (NBD) in both the N and C halves of the protein (Figure 1a,b) [14,15]. Other members of the ABCA subfamily have a similar membrane topology and domain organization [15,42].

The short 24 amino acid N-terminal segment containing positively charged arginine and lysine residues is predicted to reside within the cytoplasm. The large ECDs in the N-half and C-half have multiple N-linked oligosaccharide chains as confirmed by site-directed mutagenesis in combination with lectin binding and endoglycosidase digestion [15]. Numerous conserved cysteine residues in the ECDs form intramolecular disulfide bonds within each domain as well as between domains [15]. The function of the ECD domains in ABCA4 is not known, although in the case of ABCA1, these domains serve as binding sites for apoA1 [42,43]. Both the N and C halves of ABCA4 are predicted to contain five hydrophobic membrane spanning segments connected by relatively short hydrophilic loops. These membrane segments, together with the single transmembrane segments preceding each of the ECDs, most likely form the substrate binding site and translocation pathway.

In addition to these domains, ABCA4 contains an extended C-terminal segment harboring a conserved six amino acid VFVNFA motif, also found in ABCA1, but not other ABCA proteins [44]. Recent deletion and substitution mutagenesis studies indicate that this conserved motif plays an important role in the proper folding of ABCA4 and ABCA1 into a functional protein [38].

3.3 Nucleotide Binding Domains

The nucleotide binding domains (NBDs) of ABCA4 consisting of approximately 200 amino acid residues display a motif organization found in other ABC transporters. This includes a conserved A-loop, Walker A, Q-loop, ABC signature, Walker B, and an H-loop [45,46]. The Walker A motif (GxxGxGKS/T; x- any amino acid) and Walker B motif (hhhhD; h- any hydrophobic amino acid) form the ATP nucleotide binding fold (Figure 2a) [45]. The A-loop consists of a tyrosine or a hydrophobic residue that may be involved in stacking interactions with the adenine ring of ATP [47].

Homology modeling suggests that the NBDs of ABCA4 have a similar structural organization as other ABC transporters of known structure (Figure 2b). Each NBD consists of two arms forming an L-shaped structure. Arm I, termed the RecA-like core domain, has β -sheets flanked by α -helices and harbors the Walker A and Walker B sites, while arm II is α -helical and contains the signature motif [48]. A conserved glutamine in the flexible Q-loop connects the RecA-like core domain to the α -helical domain. This interaction has been implicated in transmitting conformational changes between the TMD and NBDs [49].

Functional ABC transporters require the formation of NBD dimers. There are three models of NBD-dimer formation. A head-to-tail dimer formation aligns the Walker A domain of one subunit with the signature domain from the opposing subunit, producing a composite of two nucleotide binding sites [50]. A back-to-back arrangement shows ATP molecules being solvent exposed and facing away from each other with their Arm I domains forming hydrophobic interactions [51]. The third arrangement demonstrates a mirror image of Walker A and signature motifs facing each other. Whether dimerization of the NBDs in ABCA4 proceeds in a head-to-tail arrangement is uncertain. Furthermore, the importance of the signature motif needs to be investigated for analysis of the dimer conformation.

4. Functional Properties of ABCA4

4.1. ATP Binding and Hydrolysis

Specific binding of nucleotides to ABCA4 was first demonstrated using photoaffinity labeling techniques. The photoreactive 8-azido ATP or 8-azido ADP covalently labeled purified ABCA4 or ABCA4 in photoreceptor membranes upon irradiation with UV light [14]. Excess unlabeled ATP or GTP inhibited 8-azido ATP labeling indicating that the labeled nucleotide binding site of ABCA4 can accommodate either adenine or guanine nucleotides. When photoaffinity labeled ABCA4 in photoreceptor membranes was cleaved with trypsin into its N and C halves, or when the N and C halves were co-expressed in culture cells, ATP labeling was detected only in the C-half of ABCA4 [37].

The ATPase activity of many ABC transporters such as P-glycoprotein has been shown to be activated by compounds known to be transported by these proteins [52–54]. This strategy was used to identify possible substrates for ABCA4 [55]. For these studies ABCA4 was immunoaffinity-purified from detergent solubilized rod outer segment membranes employing a specific monoclonal antibody (Rim 3F4) directed against a well-defined nine amino acid epitope near the C-terminus of ABCA4 [14]. Purified ABCA4 in detergent or reconstitution into brain polar lipid vesicles had a relatively low basal ATPase activity [36,55]. Over 40 different compounds were tested for their capacity to activate the ATPase activity of ABCA4. Of these compounds, two physiologically relevant retinoid compounds, all-*trans* retinal and 11-*cis* retinal, stimulated the ATPase activity of detergent solubilized and reconstituted ABCA4 several fold above the basal activity. Kinetic analysis indicated that all-*trans* retinal increased both the V_m and K_m for ATP. Other retinoid and aliphatic aldehyde derivatives including retinol, retinyl esters, nonyl aldehyde, and others failed to stimulate the ATPase activity of ABCA4. These studies provided the first clue that a retinal compound, a key molecule required for vision, may serve as the substrate for ABCA4.

In addition to retinal, the ATPase activity of ABCA4 was shown to be strongly dependent on the phospholipid composition [36,55]. Basal and retinal stimulated ATPase activity was observed for ABCA4 reconstituted into membranes consisting of soybean, brain and native photoreceptor outer segment lipids. However, the highest activity was observed for ABCA4 reconstituted into vesicles made from rod outer segment lipids known to have a high content of PE (>40%) [36]. The ATPase activity of ABCA4 reconstituted in brain polar lipid could be elevated to levels comparable to that found for outer segment lipids by the addition of excess synthetic PE. The importance of PE was further confirmed by the finding that the ATPase activity of ABCA4 reconstituted into vesicles lacking PE was not stimulated by retinal. The length or degree of unsaturation of the fatty acyl chain of PE had no significant effect on the ATPase activity of ABCA4. Taken together, these studies indicated that retinal stimulated ATPase activity is not only dependent of retinal, but also PE.

The ATPase activity of the NBD1 and NBD2 of ABCA4 expressed in *E.coli* has been studied [56,57]. Both domains exhibited low ATPase activity. The kinetic parameters (K_m and V_{max}) for the NBD2 were comparable to that reported for reconstituted ABCA4. The ATPase activity of NBD1 was considerably lower than that of NBD2. Significant differences in nucleotide specificity were reported with NBD1 capable of hydrolyzing GTP, CTP and UTP as well as ATP and NBD2 specific for ATP.

The basal and retinal activated activities of both NBDs were also investigated by expressing and analyzing ABCA4 containing Walker A mutations (K969M for NBD1) and K1978M for NBD2) known to inhibit ATP hydrolysis [35,37]. The single and double mutants expressed at wild-type levels and bound ATP as indicated by photoaffinity labeling measurements. The ATPase activities, however, were strongly affected by these mutations [35]. The double

K969M/K1978M double mutant and the K969M single mutant showed no basal or retinal stimulated ATPase activity, whereas the K1978M mutant in NBD2 exhibited basal ATPase activity but not retinal stimulated activity. These studies indicate that NBD1 and NBD2 are both important, but play distinct mechanistic roles.

4.2 N-retinylidene-PE as the Substrate for ABCA4

Aldehydes react with primary amines to form Schiff base products. The chromophore 11-*cis* retinal is conjugated to lysine 296 of opsin through a Schiff base linkage. All-*trans* retinal released from rhodopsin following photoexcitation reacts PE in disc membranes forms the Schiff base product N-retinylidene-phosphatidylethanolamine (N-retinylidene-PE) [58,59]. Likewise, the addition of all-*trans* retinal to vesicles containing PE produces an equilibrium mixture of N-retinylidene-PE and free all-*trans* retinal [36] (Figure 3 and Figure 4). Accordingly, the observed retinal stimulated ATPase activity of ABCA4 can result from either the free all-*trans* retinal or N-retinylidene-PE [55]. Mechanistically, ABCA4, could act to extrude all-*trans* retinal from membranes similar to the efflux of hydrophobic compounds from membranes by P-glycoprotein [60]. Alternatively, ABCA4 could transport or flip N-retinylidene-PE across the disc membrane analogous to the flipping of phospholipids across the lipid bilayer as reported for other ABC transporters [61–63].

Using a solid phase assay, N-retinylidene-PE was identified as the substrate for ABCA4 [64]. In this study, various retinoid compounds in the presence of phospholipids were added to ABCA4 immobilized on an immunoaffinity matrix. After removal of unbound material, the bound retinoid was analyzed by HPLC. When all-*trans* retinal was added to ABCA4, stoichiometric amounts of N-retinylidene-PE bound to ABCA4 with an apparent dissociation constant of 4–5 μ M. N-retinyl-PE produced by sodium borohydride reduction of N-retinylidene-PE (see Figure 4) also bound tightly to the same site on ABCA4. In contrast all-*trans* retinol or all-*trans* retinal in the absence of PE failed to bind ABCA4. The Schiff base of N-retinylidene-PE can exist in either a protonated or unprotonated state. Recent spectrophotometric measurements indicate that the unprotonated form of N-retinylidene-PE binds to ABCA4 (Zhong and Molday, unpublished results).

The effect of nucleotides on the binding of N-retinylidene-PE was studied in order to further evaluate the possible role of N-retinylidene-PE as a substrate for ABCA4 [64]. Addition of ATP or GTP quantitatively released N-retinylidene-PE from ABCA4. These studies indicate that N-retinylidene-PE binds to a high affinity site on ABCA4 in the absence of nucleotide. Addition of ATP (or GTP) induces a protein conformational change that causes N-retinylidene-PE to dissociate from ABCA4. Together, these studies provide strong biochemical evidence that N-retinylidene-PE is the lipid substrate for ABCA4.

5. Transport Mechanism

On the basis of biochemical studies and structural analysis, a general scheme has been proposed for the transport of substrates across membranes by ABC transporters [46,65–68]. In the initial step, the substrate binds to a high affinity site within the transmembrane domain (TMD) of the ABC transporter. This induces a protein conformational change which alters the affinity of the NBDs for ATP. The binding of ATP causes the NBDs to come in close contact with each ATP present in the dimer interface. Dimerization of the NBDs induces a conformational change in the TMD resulting in the conversion of the high affinity to low affinity substrate binding and the release of the substrate on the opposite side of the membrane as part of the transport process. ATP hydrolysis results in the loss of NBD dimerization. Finally, the dissociation of ADP from the NBDs returns the transporter to its high affinity substrate binding state.

The mechanism by which N-retinylidene-PE is transported across the disc membrane by ABCA4 remains to be determined. However, biochemical studies of ABCA4 are consistent with the ATP switch model [68](figure 5). N-retinylidene-PE binds to a high affinity site on ABCA4 in the absence of ATP [64]. This binding causes a conformational change which is relayed to the NBDs as revealed by a substrate induced change in the K_m for ATP hydrolysis [36,55]. The addition of ATP even at low temperature (conditions where ATP hydrolysis does not occur) results in the dissociation of N-retinylidene-PE from detergent solubilized ABCA4. This suggests that the binding of ATP and presumably dimerization of the NBDs is sufficient to cause a conformation change in the TMD resulting in the loss in the high affinity substrate binding site and release of the substrate [64,69]. As suggested in the ATP switch model, ATP hydrolysis and dissociation of ADP is envisioned to return ABCA4 to its initial state. The role of each of the NBDs is not understood. The model depicted in Figure 5 shows ATP binding and hydrolysis at both NBD1 and NBD2. However, photoaffinity labeling studies and ATPase measurements on mutant proteins indicate that the NBDs have distinct properties [35,37]. Although both NBDs are critical for transport function, the role of each NBD remains to be elucidated. A detailed understanding of the mechanism of lipid transport by ABCA4 will require the development of a transport assay, determination of the role of each NBD in the transport mechanism, and ultimately a high resolution protein structure.

6. Analysis of ABCA4 Knockout Mice

The role of ABCA4 as an N-retinylidene-PE transporter has also been proposed from studies of *abca4* knockout mice [70–72](figure 6). The photoreceptor cells of the *abca4* deficient mice exhibit a normal appearance with well-organized outer segments. This suggests that ABCA4 is not directly involved in outer segment morphogenesis or structure. Likewise, the photoresponse of these mice is normal except for relatively small changes in the recovery of the rod photoresponse after photobleaching [70,73]. However, significant light-dependent changes in retinoids and lipids have been observed [70,74]. Compared to wild-type mice, *abca4* knockout mice show a light-dependent elevation of all-*trans* retinal, protonated N-retinylidene-PE, and PE in the retina. Furthermore, *abca4* knockout mice raised under constant or cyclic lighting display a high degree of autofluorescence associated with the accumulation of lipofuscin deposits in the retinal pigment epithelial (RPE) cells adjacent to the photoreceptor cells. The lipofuscin deposits in the *abca4* knockout mice are associated with elevated levels of diretinyl compounds, including the diretinal pyridinium compound known as A2E and all-*trans* retinal dimers [70,71,75–78]. These diretinal compounds are formed through the initial condensation of all-*trans* retinal with N-retinylidene-PE in the disc membranes (see figure 4). In addition to these derivatives, potentially toxic photoreactive products of A2E and retinal dimers including epoxides and oxiranes have been reported to accumulate in *abca4* knockout mice [79,80](figure 6).

7. ABCA4 and Retinal Degenerative Diseases

7.1 Stargardt Macular Degeneration and Related Retinal Disorders

Stargardt disease is an early onset, autosomal recessive disease first described by the German ophthalmologist Karl Stargardt in 1909 [81]. It is the most common inherited macular dystrophy with a prevalence estimated to be 1 in 10,000 [82]. Characteristic features include significant loss in central vision in the first or second decade of life, progressive bilateral atrophy of the RPE and photoreceptors, yellow or white lipofuscin deposits within the macula at the level of the RPE cells associated with autofluorescence, and a delay in dark adaptation [83–87]. Elevated levels of N-retinylidene-PE in the retina and A2E and related diretinal fluorophores in the RPE layer have been found in tissue samples from Stargardt patients [71]. Fundus flavimaculatus, a retinal disorder with similar clinical features, is now considered to be a late onset, slow progressive form of Stargardt diseases [88,89].

Since the identification of the *ABCA4* gene over 10 years ago [29], an international effort has been undertaken to screen Stargardt patients for mutations in the *ABCA4* gene [30,90–101]. To date over 500 different mutations are now known to cause Stargardt disease. These include missense, nonsense, splice-site, frameshift, and small deletion and insertion mutations. Of these, missense mutations are most common and distributed throughout the protein. A subset of missense mutations reside in NBD1 (N965S, T971N, A1038V, S1071V, E1087K, R1108C, R1129L) and NBD2 (G1961E, L1971R, G1977S, L2027F, R2038W, R2077W, R2106C, R2107H). Several of these, including N965S, T971N, E1087K, L1971R, G1977S, reside inside or close to the Walker A and B motifs [29][[90,95,97,100] [92,102].

In addition to Stargardt disease, mutations in *ABCA4* are known to cause more severe retinal degenerative diseases including autosomal recessive cone-rod dystrophy and a recessive form of retinitis pigmentosa (RP) [91,98,102,103]. Cone-rod dystrophy is a retinal disorder in which cone degeneration is more severe than rod degeneration as assessed by a more marked reduction in the cone b-wave of the photopic electroretinogram than the rod b-wave of the scotopic electroretinogram. Individuals with cone-rod dystrophy typically experience severe loss in visual acuity and color vision, and show evidence of significant degeneration of the macula. RP is a particularly severe retinal degenerative disease associated with night blindness, reduction in peripheral vision and progressive loss in central vision most often leading to complete blindness. RP patients also display narrowed retinal vessels, bone-spicule pigmentation and a waxy pallor of the optic disk. Over 40 different genes are known to be associated with various forms of RP. Genetic studies have linked selected mutations in *ABCA4* with a subset of autosomal recessive RP known as RP19 [102]. Affected individuals show an early loss in central vision and atrophy of the macula along with the characteristic features of RP.

In addition to these disorders, mutations in *ABCA4* have been reported to increase one's risk of developing age-related macular degeneration (AMD). In an initial report individuals heterozygous with selected mutations in *ABCA4* known to cause Stargardt disease were found in 16% of patients with AMD [28]. Subsequent studies, however, indicate that mutations in *ABCA4*, if indeed they are associated with AMD, are a minor cause of this disease [97].

Together, these studies suggest that mutations in *ABCA4* cause a spectrum of related retinal degenerative diseases with variable clinical severity. For the recessive disorders, Stargardt disease has the mildest phenotype, cone-rod dystrophy a moderate to severe phenotype, and RP the most severe phenotype. A model has been proposed in which the severity of the disease phenotype is inversely correlated with the residual functional activity of the mutant *ABCA4* [92,104,105]. In this model, mutations in both alleles that result in complete loss of *ABCA4* activity or produce a mutant protein with a detrimental effect on photoreceptor survival cause the RP phenotype. Compound heterozygous individuals with only residual *ABCA4* function develop cone-rod dystrophy. Finally, Stargardt disease is associated with mutations that retain partial functional activity of *ABCA4*. Although it is likely that there is a correlation between residual *ABCA4* activity and disease phenotype as suggested by this model, other factors including the effect of mutations on the trafficking of *ABCA4* to photoreceptor outer segment disc, age of diagnosis, progression of the disease, and the general genetic variability of the population also contribute to the observed phenotype [106,107].

7.2. Effect of Disease linked Mutations on the Properties of *ABCA4*

The effect of a number of disease-causing mutations on *ABCA4* protein expression and ATP binding and hydrolysis has been investigated by heterologous expression in HEK293 or COS7 cells [35,38]. Mutations resulting in internal amino acid deletions and introduction of charge residues in the putative transmembrane segments were found to significantly reduce protein expression suggesting that these mutations result in a highly misfolded protein that is rapidly

degraded by the cell [35]. A number of mutants including many in the NBDs expressed at protein levels similar to wild-type ABCA4, but exhibited reduced ATP binding and hydrolysis. Some mutations affect only the retinal activated ATPase activity such as the G1975D and K1978M mutations in NBD2, whereas a large number of mutants affect both the basal and retinal stimulated ATPase activity of ABCA4 as exemplified by the L541P mutation in ECD1, T971N in NBD1 and E2096K in NBD2 [35].

More recently, the effect of two disease-associated C-terminal deletion mutants on N-retinylidene-PE binding and ATP binding and hydrolysis was studied [38]. An ABCA4 mutant lacking the C-terminal 30 amino acids including a conserved VFVNFA motif and associated with cone rod dystrophy showed decreased protein expression, protein misfolding associated with retention in the endoplasmic reticulum, and complete loss in retinal and ATP binding and hydrolysis activity. In contrast, a mutant lacking the C-terminal 24 amino acids but retaining the VFVNFA motif and associated with a mild form of Stargardt disease showed only a limited decrease in protein expression and relatively normal intravesicular localization, and a relatively small reduction in retinal stimulated ATPase activity. The expression and activity of these mutants are generally consistent with the observed phenotypes of the patients and highlight the importance of the conserved VFVNFA in proper protein folding and functional activity of ABCA4 [99,108].

The effect of several disease-associated mutations on the trafficking of ABCA4 to outer segments has been studied in transgenic *Xenopus laevis* [107]. A number of mutants were retained in the inner segment of the photoreceptors. This suggests that protein mislocalization as well as misfolding and diminished function can contribute to ABCA4 associated diseases.

8. Model for the Role of ABCA4 in the Visual Cycle and Retinal Degenerative Diseases

Analyses of Stargardt patients together with biochemical characterization of ABCA4 and *abca4* knockout mice have implicated ABCA4 in removal of retinoids from outer segments as part of the visual or retinoid cycle [55,69,70]. Following photoexcitation, all-*trans* retinal is released from rhodopsin and partitions into the lipid bilayer of disc membranes. Most of the all-*trans* retinal is reduced to all-*trans* retinol on the cytoplasmic side of the membrane by the retinol dehydrogenase RDH8 and possibly other NADPH-dependent dehydrogenases [109, 110]. All-*trans* retinol is then transported from photoreceptors to the RPE cell where it is converted to all-*trans* retinal ester by lecithin:retinol acyl transferase (LRAT), isomerized to 11-*cis* retinol by RPE65-isomerase, and oxidized to 11-*cis* retinal by 11-*cis* retinol dehydrogenases (Figure 3). 11-*cis* retinal is then transported back to the photoreceptor outer segment where it can recombine with opsin to regenerate rhodopsin or cone opsin [109,110]. However, a fraction of all-*trans* retinal generated from the photobleaching of rhodopsin or cone opsin reacts with PE to form N-retinylidene-PE. The acidic pH of the disc lumen can trap the protonated form of N-retinylidene-PE in the lumen leaflet of the disc membranes [111]. The function of ABCA4 is envisioned to actively transport or flip trapped N-retinylidene-PE from the lumen to the cytoplasmic side of the disc membrane. On the cytoplasmic surface, N-retinylidene-PE can dissociate into all-*trans* retinal and PE, and all-*trans* retinal can then be reduced to all-*trans* retinol by RDH8 for the resynthesis of 11-*cis* retinal and the regeneration of rhodopsin via the visual cycle. In this model the function of ABCA4 is to insure that all-*trans* retinal and N-retinylidene-PE is completely removed from disc membranes following photobleaching of rhodopsin in rod cells and cone opsin in cone cells.

Loss or decrease in ABCA4 transport activity will result in an accumulation of N-retinylidene-PE and all-*trans* retinal in disc membranes as found in *abca4* knockout mice and Stargardt patients [70,71] (Figure 5, Figure 6). N-retinylidene-PE can react with all-*trans* retinal to form

the direct derivative A2PE (figure 4) and related direct compounds in outer segments [71,75,76]. Rod and cone outer segments undergo a continual renewal process in which new membrane is added at the base of the outer segments while packets of aged outer segments are removed by shedding and phagocytosis by adjacent RPE cells. Through this process, the outer segment is renewed once every 10 days. Ingested phagosomes containing A2PE, all-trans retinal dimers and other retinoid compounds fuse with lysosomes to form phagolysosomes which contain enzymes that degrade the outer segments (Figure 6). A2PE is hydrolyzed to A2E, but cannot be further metabolized. As a result, A2E and related retinal compounds progressively accumulate as lipofuscin deposits in RPE cells [71,76,77]. Upon exposure to blue light, A2E can be converted into epoxides [79]. A2E and related epoxides can lead to loss in RPE function and cell death [75,77,78,112,113]. Since RPE cells are required for photoreceptor cell survival, the loss in RPE cells will cause the photoreceptor cell death and a loss in vision.

This model is consistent with many of the characteristic features observed for individuals with Stargardt disease and mice deficient in ABCA4. These include elevated levels of all-trans retinal and protonated N-retinylidene-PE in photoreceptor cells, and the progressive accumulation of A2E and other direct compounds as fluorescent lipofuscin deposits in RPE cells. In addition the accumulation of all-trans retinal in disc membranes can explain the delay in dark adaptation found in Stargardt patients and *abca4* mice.

Although there is considerable support for this model in which ABCA4 functions as N-retinylidene-PE transporter, a number of issues remain to be resolved. In particular, it is necessary to directly show that ABCA4 functions to transport or flip N-retinylidene-PE across disc membranes. The mechanism by which ATP hydrolysis is coupled to the transport of N-retinylidene-PE across membranes remains to be elucidated. Importantly, the direction of substrate transport needs to be clarified. In the present model, ABCA4 is envisioned to transport N-retinylidene-PE from the lumen to the cytoplasmic leaflet of disc membranes. This is the opposite direction of transport for many well studied mammalian ABC transporters. ABC transporters are known to be regulated through protein-protein interactions and/or phosphorylation. To date there is no information on factors which regulate the activity of ABCA4. Resolution of these issues awaits the further development of functional transport assays and the generation of high resolution structures of ABCA4.

9. Therapeutic Strategies for Stargardt Macular Degeneration and Related Diseases

Insight into the genetic and molecular basis for Stargardt and related ABCA4 retinal degenerative diseases has led to investigations into possible treatments for this set of disorders. Gene therapy offers a promising approach to prevent or slow the onset of ABCA4 related diseases [114]. For recessive diseases, this typically involves the delivery of the normal gene to cells harboring the defective gene. This strategy has proven successful in animal models for a number of retinal degenerative diseases caused by mutations in genes expressed in RPE or photoreceptor cells [115–117]. Most studies have employed recombinant adeno-associated viral (rAAV) vectors for gene delivery. These vectors have the advantage that they are nontoxic and relatively nonimmunogenic when administered by subretinal injections. In addition, rAAV vectors efficiently transduce non-dividing cells such as RPE and photoreceptors for long term gene expression. For example, rAAV containing the *RPE65* gene delivered to RPE cells restored vision in a canine model for Leber Congenital Amaurosis (LCA), a severe early onset retinal degenerative disease associated with complete loss in vision [118]. Success in preclinical animal studies for this disease has led to recent Phase I clinical trials in which partial recovery of vision has been demonstrated for individuals affected with LCA [119–121].

A potential disadvantage of rAAV for use in Stargardt disease is its limited gene packaging size of 4.7 kb. However, a recent study has shown that large genes including the 6.8 kb *ABCA4* gene can be stuffed into a chimeric rAAV (2/5) vector [122]. This vector has been used to deliver the mouse *Abca4* gene to photoreceptors of an *abca4* knockout mouse. In another study the human *ABCA4* gene has been delivered to photoreceptors of an *abca4* knockout mouse using lentiviral vector [123]. In both these studies, expression of ABCA4 in photoreceptors correlated with a reduction in A2E levels in RPE cells. These initial studies suggest that gene therapy has potential application as a treatment for Stargardt and other ABCA4 associated diseases.

Another approach has been to reduce the level of 11-*cis* retinal and consequently all-*trans* retinal in photoreceptors through the application of drugs that inhibit enzymes of the visual cycle [110]. In initial studies, isotretinoin or 13-*cis*-retinoic acid, also known as Accutane, a widely used agent for the treatment of acne, was used to inhibit 11-*cis*-RDH, the enzyme responsible for the formation of 11-*cis* retinal in RPE cell [124]. When *abca4* knockout mice were given isotretinoin, a marked reduction in the light-induced accumulation of A2E containing lipofuscin deposits was observed. Since isotretinoin is associated with side effects, other retinoid-based inhibitors have been developed and tested in animal studies. These include N-(4-hydroxyphenyl)retinamide known to reduce serum levels of vitamin A and retinylamine, an inhibitor of isomerase activity in RPE cells [125,126]. Both these retinoid compounds have been shown to reduce A2E accumulation in animal studies pointing to the therapeutic potential for these drugs.

10. Conclusions

ABCA4 is an ABC transporter that is expressed in vertebrate rod and cone photoreceptors and localized to the rim regions of outer segment disc membranes. Mutations in ABCA4 are responsible for Stargardt macular degeneration and related retinal degenerative diseases that cause significant loss in vision. Functional properties of the purified protein together with biochemical analysis of *abca4* knockout mice have implicated ABCA4 in the transport of N-retinylidene-PE across the disc membranes following photoexcitation an essential step in the complete removal of all-*trans* retinal and N-retinylidene-PE from photoreceptor membranes following photoexcitation. Loss in the lipid transport function of ABCA4 due to disease-causing mutations leads to the accumulation of all-*trans* retinal and N-retinylidene-PE in disc membranes and the formation of potentially toxic diretinal compounds. Upon phagocytosis of outer segments, the diretinal compounds including A2E progressively accumulate in RPE cells as lipofuscin deposits. A2E and related diretinal compounds compromise the function and viability of RPE cells ultimately resulting in photoreceptor degeneration and a loss in vision.

Although considerable progress has been made in understanding the role of ABCA4 in the visual cycle and the pathogenesis of ABCA4 associated diseases, a number of issues remain to be resolved. These include generation of a high resolution structure of ABCA4, development of a transport assay to measure the direction of substrate transport, identification of mechanisms that regulate the activity of ABCA4, elucidation of the molecular mechanisms underlying the coupling of transport to ATP binding and hydrolysis, and further development of genetic and drug based therapies for Stargardt and other ABCA4 associated diseases.

Acknowledgement

This work was supported by grants from the Canadian Institutes of Health Research (MT 5822), National Eye Institute (EY02422) and a grant from the Macula Vision Research Foundation.

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Figure 1. Structural features of the ABCA4 transporter. (a) A linear diagram showing the full transporter arranged as two tandem halves. Each half consists of six membrane spanning segments which together make up the transmembrane domain (TMD). A large exocytosomal (extracellular/lumen) domain (ECD) separates the first membrane spanning segment from the remaining five membrane spanning segments in each half. The nucleotide binding domain (NBD) lies downstream of the multiple membrane spanning segments. (b) Topological model showing the organization of ABCA4 in the membrane. Each ECD contains multiple N-linked oligosaccharide chains (hexagons) exposed to the lumen side of the disc membrane [14,15]. A

six amino acid motif (VFVNFA) present in both ABCA4 and ABCA1 is close to the C-terminus [38,44]. Other ABCA family members have a similar topological organization.

	A-loop	Walker A	
NBD1	-----VPG-----VCVKNLVKI F EPGCRPAVDRLNITFYENQITAF LGHNGAGKT TTL		973
NBD2	-----IITGGNKTDILRLHELT KI YPGTSSPAVDRLCV G VRPGECFGL LGVNGAGKT TTT		1982
	Q-loop		
NBD1	SILTGLLPPTSGTVLVGGRDIETSLDAVR Q SLGMCPOHNILFHHLTVAEHMLFYAQLK GK		1033
NBD2	KMLTGDTT V TSGDATVAGKSILTNISEV HQ NMGYCPQFDAID E LLTGREHL Y LYA RL RGV		2042
	ABC signature	Walker B	D-loop
NBD1	SQEE AQ LEMEAMLED T GLHHRNEEAQD L SGGM Q R K L S VAIAFVGDAKVV I L D EPTSGVD		1093
NBD2	PAEEIEKVANWSIKSLGLTVYADCLAG T YSGGN K R KLSTAIALIGCPPLV L LDEPTTGMD		2102
	H-loop		
NBD1	PYSRRSIWDL L L-KY R SGRTIIMST HH MDEADLLGD-----		1128
NBD2	PQA RR MLWNVIVSIIREGRAVVLT S HSMECEA-----		2135

Figure 2a,b

Figure 2.

(a). Alignment of the nucleotide binding domains of ABCA4. Conserved motifs including the A-loop, Walker A, signature, Walker B, Q-loop, and the H-loop are in bold. Disease mutations, which are substituted in Stargardt disease, are shown in red italics – NBD1 (N965S, T971N, A1038V, S1071V, E1087K, R1108C); NBD2 (G1961E, L1971R, G1977S, L2027F, R2038W, R2077W, R2106C, R2107H). (b) Homology model of the NBD1 of ABCA4 shows Walker A (purple) and B (blue) sites with the Q-loop (yellow) and signature motif (orange). The α/β domain and α -helical domain are demarcated by an arbitrary line representing Arms I and II. This model was generated on the basis of the coordinates from the crystal structure of an ATP binding domain from an ATP binding domain (35% identity; 55% similarity; PDB: 1VPL)

using Modeller 7v7 [Sali and Blundell, 1993] by making a structural alignment of NBD1, NBD2, and 1VPL. 100 models of NBD1 were generated. Five models with the lowest objective function value were selected for energy minimization in GROMACS 3.3. Energy minimization was done by steepest gradient descent, with an initial step size of 0.01 and a maximum of 1000 steps; each of the models converged and the energy minimized models were evaluated for stereochemical quality by PROCHECK and ranked according to score. For the purpose of demonstration, the highest-ranking model was used.

Figure 3.

Role of ABCA4 in the visual cycle. Light isomerizes 11-*cis* retinal to all-*trans* retinal in rhodopsin. All-*trans* retinal dissociates from opsin and is reduced to all-*trans* retinol by all-*trans* retinol dehydrogenase (RDH8). All-*trans* retinol is esterified by lecithin:retinol acyl transferase (LRAT), isomerized to 11-*cis* retinol by Rpe65-isomerase, and oxidized to 11-*cis* retinal by 11 *cis* retinol dehydrogenases. 11-*cis* retinal is transported back to the outer segment where it can recombine with opsin to produce rhodopsin. All-*trans* retinal released from rhodopsin can also react with phosphatidylethanolamine (PE) to produce N-retinylidene-PE which is in equilibrium with its protonated form. In this model ABCA4 transports N-retinylidene-PE from the lumen to the cytoplasmic side of the disc membrane where all-*trans* retinal can be reduced to all-*trans* retinol by RDH8 as part of the visual cycle.

Figure 4.

Reactions involved in the formation of A2E due to loss in ABCA4 activity. All-*trans* retinal reacts with phosphatidylethanolamine (PE) to form N-retinylidene-PE which can condense with all-*trans* retinal to produce the di-retinoid pyridinium-PE compound known as A2PE in outer segments. Upon phagocytosis, A2PE can be hydrolyzed by lysosomal enzymes in the RPE cells to A2E and phosphatidic acid (PA). The reduction of N-retinylidene-PE to N-retinyl-PE by sodium borohydride is also shown.

Figure 5.

A simplified model for the transport of N-retinylidene-PE across the disc membrane by ABCA4. In the absence of ATP, N-retinylidene-PE binds to a high affinity site within the transmembrane domain (TMD) of ABCA4 altering the affinity of the nucleotide binding domains (NBDs) for ATP. The binding of ATP to one or both NBDs causes a dimerization of the NBDs and a conformation change in the TMD that converts the high affinity N-retinylidene-PE substrate binding site to a low affinity site and access to the cytoplasmic side of the disc membrane. Dissociation of N-retinylidene-PE from ABCA4 on the cytoplasmic side of the lipid bilayer together with ATP hydrolysis and dissociation of ADP returns ABCA4 to its initial state .

Figure 6.

Model for the ABCA4-mediated photoreceptor degeneration in Stargardt disease. Partial or complete loss of ABCA4 function due to disease-causing mutations (ABCA4 – star) results in the accumulation of all-*trans* retinal and N-retinylidene-PE (N-Ret-PE) in outer segment disc membrane following the light activation of rhodopsin. All-*trans* retinal condenses with N-retinylidene-PE to produce A2PE. Outer segments undergo phagocytosis by adjacent RPE cells and degradation by lysosomal enzymes. As part of this process A2PE is hydrolyzed to A2E which then accumulates as lipofuscin deposits in the RPE cells. A2E can act as a detergent or alternatively it can be converted to toxic epoxides by the action of short wavelength light. These compounds can compromise RPE cell function leading to apoptosis. Loss in RPE cells will result in photoreceptor cell death and a loss in vision.