Naturally occurring mutations in ABCA1’s largest extracellular loops can disrupt its direct interaction with apolipoprotein A-I

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Running Title: Functional analysis of mutations in ABCA1
Summary
The ABCA1 transporter contains two large domains into which many of the genetic mutations in individuals with Tangier disease fall. To investigate the structural requirements for the cellular cholesterol efflux mediated by ABCA1, we have determined the topology of these two domains and generated transporters harboring five naturally occurring missense mutations in them. These mutants, unlike wild type ABCA1, produced little or no apo A-I stimulated cholesterol efflux when transfected into 293 cells, establishing their causality in Tangier disease. As all five mutant proteins were well expressed and detectable on the plasma membrane, their interaction with the ABCA1 ligand, apolipoprotein A-I, was measured using bifunctional cross-linking agents. Four of five mutants had a marked decline in cross-linking to apo A-I, while one (W590S) retained full cross-linking activity. Cross-linking of apo A-1 was temperature dependent, rapid in onset, and detectable with both lipid and water-soluble cross-linking agents. These results suggest that apo A-I stimulated cholesterol efflux cannot occur without a direct interaction between the apoprotein and critical residues in two extracellular loops of ABCA1. The behavior of the W590S mutant indicates that while binding of apoA-1 by ABCA1 may be necessary, it is not sufficient, for stimulation of cholesterol efflux.

Abbreviations used are: ABCA1, ATP-binding cassette transporter A1; AA, amino acid; apoA-I, apolipoprotein A-I; ORF, open reading frame; GFP, green fluorescent protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DSG, disuccinimidyl glutarate; DSP, dithiobis (succinimidylpropionate); DTSSP, dithiobis (sulfosuccinimidylpropionate); PCR, polymerase chain reaction.
Introduction

Cholesterol is an integral membrane constituent and a precursor in multiple metabolic pathways, including steroid hormone synthesis and bile acid production (1,2). As elevated levels of unesterified cholesterol are toxic to cells, intricate mechanisms have evolved to regulate both the endogenous synthesis of cholesterol and the uptake and metabolism of dietary cholesterol (3,4). More recently, it has been appreciated that an additional mechanism has evolved by which cells are able to actively extrude excess cholesterol (5). The ABC transporter, ABCA1, has been shown to play a rate-limiting role in this process (6-10). Many studies have now demonstrated that ABCA1 activity is associated with the transfer of cellular phospholipid and cholesterol to acceptor apolipoproteins, such as apo A-I (11-14). The absence of this activity in humans and mice leads to a profound reduction in plasma HDL cholesterol levels and the accumulation of lipid in macrophage-rich tissues (6-9,15,16).

Although it is clear that ABCA1 plays a critical role in the transfer of lipid to apoA-I, the structural determinants of this molecular interaction have not been elucidated. Both the topological disposition of ABCA1 within the lipid bilayer and the domains of the transporter that are required for the interaction within apoA-I remain unclear. We previously showed that the first hydrophobic domain of the transporter acts as signal sequence, leading to the extracellular positioning of the largest putative loop of the protein, comprising amino acids ~44 to 640 (17). Other laboratories subsequently confirmed this finding in ABCA1, as well as in another A class ABC transporter, ABCR (also called ABCA4) (18,19). While the disposition of this loop with respect to the extracellular space is firmly established, there remains a controversy over the tethering of this loop to the plasma membrane at its amino terminal end. Data from Tanaka et al.
suggested that the loop was not tethered, while our studies provided evidence for an uncleaved signal anchor sequence that would tether the loop to the plasma membrane at both ends (17,19).

In addition to this N-terminal extracellular loop, ABCA1 also contains an unusual, highly hydrophobic sequence in the central region of the transporter that was originally modeled as a hairpin structure embedded in, but not traversing, the lipid bilayer (20). Such a model has been challenged by recent experimental data on ABCR, a close homologue of ABCA1. Mutation of potential N-glycosylation sites in ABCR indicated that its highly hydrophobic domain traverses the lipid bilayer, producing another large extracellular domain in the central region of the protein (18). Whether the central highly hydrophobic domain of ABCA1 adopts a similar topology remains an unanswered question.

The topological arrangement of these two domains is particularly important, as approximately half of all described missense mutations associated with Tangier disease and familial hypoalphalipoproteinemia (FHA) fall within these two regions (6-10,21-26). If both regions are indeed extracellular, they provide a potential target for binding of the apolipoprotein ligands that stimulate ABCA1-mediated cholesterol efflux. In this study, we demonstrate that the highly hydrophobic domain of ABCA1 also traverses the plasma membrane, resulting in the extracellular positioning of amino acids ~1371-1650. With this data in hand, it was plausible then to test the hypothesis that mutations in these loops affect the interaction of ABCA1 with its apolipoprotein ligand, apoA-I. The results of these experiments indicate that both of the large extracellular loops contain residues that are critical to the direct interaction of apoA-1 and ABCA1 and that alterations in these residues can lead to loss of function of the transporter. This
work establishes the causality of these mutations in individuals with disrupted ABCA1 function and also clarifies the process by which apoA-I interacts with the transporter.

**EXPERIMENTAL PROCEDURES**

*Reagents-* The following reagents were purchased from the indicated suppliers:

LipofectAMINE-2000 (Life Technologies, Inc), DSP, DSG, DSTTP (Pierce, Rockford, IL, USA), Gamma Bind Plus Sepharose (Amersham Pharmacia Biotech AB, Sweden), Anti-FLAG M2 monoclonal antibody, FLAG-bacterial alkaline phosphatase (Sigma, St. Louis MO, USA), N-glycosidase F (New England BioLabs, Beverly, MA, USA), Poly-D-Lysine coated tissue culture plates (BD Biosciences, Bedford MA, USA).

*DNA Constructs-* Five missense mutants of ABCA1 (R587W, W590S, Q597R, C1477R and S1506L) were generated using overlap polymerase chain reaction methods, as previously described (17). The regulatory domain construct (REG, AA 843 to 1650) and the highly hydrophobic sequence construct (HHS, AA 1311 to 1650) were generated by PCR using wt-ABCA1 cDNA as a template. The forward primers contained an NcoI site that generates a Kozak consensus sequence with a favorable initiator methionine, and the reverse primer encoded a 9-amino acid rhodopsin epitope (TETSQVAPA) at the COOH terminus of the constructs. The 8-amino acid FLAG tag (DYKDDDDK) was inserted after AA 207 and 1396 by restricting the ABCA1 cDNA with BstEII and Bsu36I, respectively, and ligating in duplex oligonucleotides encoding the FLAG tags. All constructs and mutations were verified by DNA sequencing, both to confirm the introduction of the desired changes as well as the absence of unintended PCR mutations.
Construct Expression and Immunological Methods-HEK293-EBNA-T (generous gift of Brian Seed, Mass. Gen. Hospital, Boston) cells were grown on poly-D-lysine coated plates and transfected with LipofectAMINE-2000 as previously described (17). Metabolic labeling, immuno-precipitations and immuno-blotting methods were performed as described (17). For immuno-precipitations of un-tagged ABCA1 polypeptides, a polyclonal rabbit anti-serum, directed against the last 200 AA of ABCA1, was used (17).

Cholesterol Efflux and ApoA-I/ABCA1 Cross-Linking Assays-Cholesterol efflux assays, cellular association of apoA-I and cross-linking of apoA-I to ABCA1 were carried out as described by Wang et al with the minor modifications noted below (13). For efflux assays, HEK293-EBNA-T cells plated in poly-D-lysine coated 24 well plates were transfected with empty vector or the appropriate ABCA1 construct (1 ug of DNA per well) and radiolabeled for 24 hours with [1,2-³H]-cholesterol (0.5 uCi/ml). The labeled cells were wash 2X with warm PBS, and allowed to incubate in DMEM (1mg/ml fatty acid free bovine serum albumin) at 37°C for 1h. The cells were transferred into fresh DMEM (1mg/ml fatty acid free bovine serum albumin) with or with delipidated apoA-I (10 ug/ml) and incubated at 37°C for an additional 4h. The media was removed, clarified of cellular material and the effluxed cholesterol was quantitated by scintillation counting. Cell layers were lysed in 0.1 N NaOH and cellular cholesterol quantitated by scintillation counting. Cholesterol efflux was expressed as percent effluxed [media counts/(media counts + cellular counts)*100]. Other inducers of ABCA1 expression (e.g. LXR agonists, cAMP mimetics, or cholesterol loading) were not included.
FLAG Cell Surface Expression Assay-ABCA1 constructs carrying the FLAG epitopes were transfected into HEK293-EBNA-T cells in 24 well plates. 24 h after transfection, cells were chilled on ice. To analyze cell surface FLAG tag expression, the media was directly replaced with blocking solution (1X PBS, 5% dry milk, 1% bovine serum albumin). To measure total FLAG tag expression, the cells were first fixed and permeabilized by incubation in 1X PBS/4% paraformaldehyde for 10 min, followed by 2 washes in 1X PBS, and a subsequent incubation in 1X PBS/0.2% Triton-X-100 for 10 min with a final 2 washes in 1X PBS before incubation in blocking solution. The Anti-FLAG M2 monoclonal antibody was then added to the blocking solution (4 ug/ml), cells were incubated for 1 h on ice, and washed 3X with cold PBS. Bound M2 antibody was subsequently detected by a 1h incubation on ice in blocking buffer containing 125I-goat-antimouse antibody (4 ug/ml). Cells were washed 3X with cold 1X PBS, lysed in 0.1 N NaOH, and bound counts were determined by gamma counting. Total cellular protein in the lysates was determined using a micro-BCA assay (Pierce) and bound counts were expressed per mg of total cellular protein.

RESULTS

Putative mutations in the ABCA1 transporter, identified by genetic studies of families with Tangier disease and familial hypoalphalipoproteinemia, have the potential to provide important insights into the structural requirements for transporter function (6-10,21-26). Before exploring structure/function relations using such mutants, their functional significance needs to be confirmed in order to eliminate the possibility that the putative mutations represent sequence polymorphisms in linkage dysequilibrium with another, as yet unidentified, causal mutation. In this work, we selected five missense mutations reported in TD and FHA families and generated
constructs that permitted the measurement of their functional activity. These mutations mapped to two domains in the transporter, one of which we had demonstrated to be extracellular (17). These loops were of interest, as approximately half of the missense mutations reported in TD and FHA cohorts fall within these two regions of the transporter. We hypothesized that both loops might be extracellular and could, therefore, mediate a direct interaction with the ABCA1 ligand, apoA-1. If this hypothesis were correct, the mutations might disrupt this interaction, providing both an explanation for their causality and, perhaps, novel insights into the mechanism by which apoA-1 stimulates ABCA1 mediated cholesterol efflux.

_Missense mutations in two putative extra-cellular loops of ABCA1 ablate efflux activity:_

Five missense mutations (R587W, W590S, Q597R, C1477R and S1506L) were introduced into a wild type ABCA1 cDNA using PCR mutagenesis techniques. Three of these mutations (R587W, W590S, Q597R) fall in a tight cluster within the large N-terminal loop at a point near the putative second transmembrane domain shown in Figure 3. The other two mutations (C1477R and S1506L) fall within the central loop of the protein. To characterize whether these single amino acid changes did indeed disrupt ABCA1 lipid transport, cholesterol efflux assays were performed comparing the mutants to wild type ABCA1. 24 hours after transfection and labeling with $[^{3}H]$-cholesterol, 293-EBNA-T cells were exposed to 10 ug/ml delipidated apoA-1 or not for four hours. For all five mutants, a profound deficit in efflux activity was measured (Fig. 1). These findings indicate that each of these mutations is causally involved in the disruption of cellular cholesterol efflux, which is a cell biologic hallmark of Tangier disease. To explore further the mechanism of this disruption, it was important to establish their topological location within the ABCA1 transporter.
The Central Highly Hydrophobic Domain of ABCA1 Traverses the Lipid Bilayer:

Although the three mutations clustered around position 590 in the N-terminal loop were clearly in the loop previously shown to be positioned in the extracellular space, the topological location of the other two mutations was unknown. ABCA1 contains an additional hydrophobic sequence (AA 1351 to 1370) that is unique to the A class of ABC transporters. Originally called the highly hydrophobic sequence (HHS), this loop has been modeled as a hairpin embedded in the lipid bilayer, but not traversing it ((20), and Fig. 2 left diagram). However, a recent study of another A class transporter (ABCR) indicated that its homologous HHS could indeed traverse the bilayer (18).

To determine the topology of the HHS and the adjacent central region of ABCA1, two approaches were employed. The first was aimed at testing whether the HHS segment could function as a transmembrane domain when tested in the context of a sub-domain of the full-length transporter. This approach permitted the use of glycosylation assays to establish the orientation of loops flanking the HHS domain. The second approach utilized the insertion of a nine amino acid epitope tag (FLAG) in the full-length cDNA, followed by analysis of anti-FLAG antibody binding to this FLAG-tagged construct. When HHS is expressed in the context of the entire central region of ABCA1 (AA 843 to 1650) as depicted in Fig. 2 (REG, left diagram, bottom), or as part of a smaller sub region (AA 1311 to 1650, HHS, right diagram bottom) the resulting polypeptides were found to be glycosylated, as evidenced by their increased migration in SDS-PAGE gels following treatment with glycosidases (Fig. 2, center panels). These results establish that the highly hydrophobic sequence, when expressed in these subdomain contexts, can function as a transmembrane domain and position amino acids 1371 to 1650 in the extracellular space (Fig. 2, right diagram, top).
To confirm that the HHS functioned as a transmembrane domain in the context of a full-length transporter, glycosylation assays could not be used. Instead, a FLAG epitope tag was inserted at AA 1396 (Fig. 3) and binding to anti-FLAG antibody measured in permeabilized and non-permeabilized cells. A control construct was created using the same FLAG tag inserted in the extracellular N-terminal loop (at AA 207, Fig. 3). The two FLAG tagged transporters effluxed as much cellular cholesterol to apoA-I as wild type, untagged ABCA1 (Fig. 4A, WT vs. N-FL and R2-FL, respectively), indicating that the FLAG insertions at these two positions did not disrupt functional activity. When transfected into cells, they also stimulated cellular binding to apoA-I. The stimulation of binding by the N-terminal FLAG construct was indistinguishable from that of the wild type protein, while the construct with the central tag evinced a modest reduction in binding (Fig.4B). The latter finding could reflect an influence of the central FLAG tag on the binding affinity of apoA-I for ABCA1. To further substantiate the lack of major effects of these tags on ABCA1 function, another assay of the apoA-I interaction with ABCA1 was utilized. This assay involves cross-linking apoA-I to ABCA1 through the use of a bifunctional cross-linking agent, followed by immuno-precipitation of the complex with an anti-ABCA1 antibody. In this assay, the central region FLAG tag construct performed similarly to the wild type protein (Fig. 4C). A modest increase in cross-linking was actually detected with the construct expressing the tag in the N-terminal loop (Fig. 4C). The absence of deleterious effects of the FLAG tags on ABCA1 efflux activity and apoA-I interactions strongly suggested that they had not caused a change in the topological orientation of the transporter with respect to the plasma membrane.
To determine the location of the centrally tagged (R2) loop, we then measured anti-FLAG antibody binding to cells that had been transfected with either of the FLAG constructs and then either permeabilized (Fig. 4E) or not (Fig. 4D) with detergent. Comparison of this antibody binding activity was made to cells transfected with an un-tagged ABCA1 (WT) or to a cytoplasmic FLAG tagged bacterial alkaline phosphatase peptide (FL-BAP). Non-permeabilized cells that had been transfected with the FLAG tagged transporters demonstrated much greater antibody binding than cells transfected with either the un-tagged ABCA1 or the FL-BAP (Fig. 4D, and Fig. 5B). The lack of binding to the FL-BAP transfected cells was indeed due to the inability of the antibody to penetrate the cells, as permeabilization with Triton-X-100 led to a marked increase in binding to that protein (Fig. 4E). Permeabilization of the cells transfected with either the N-terminal or the central region tagged ABCA1 transporters resulted in similar levels of anti-FLAG antibody binding. The glycosylation data shown in Figure 2, taken in conjunction with the FLAG antibody binding assays depicted in Figure 4, provide strong evidence that the highly hydrophobic domain traverses the plasma membrane and positions the residues immediately distal to AA 1340 in the extracellular space. These results, depicted in the topological model presented in Figure 3, are consistent with the ABCR studies of Bungert et al. and are likely representative of the A class as a whole (18). In order to maintain the intracellular localization of the ATP binding cassettes, two domains (H7 and H13), for which hydrophobicity plots predict a potential role as transmembrane domains, have been drawn as non-transmembrane segments in Figure 3. Their hydrophobicity indices are lower than that of other putative transmembrane segments, providing the rationale for this choice. This choice has not been experimentally verified, however, so the disposition of H7 and H13 relative to the lipid membrane must still be viewed as tentative (27). In contrast, the orientations of the N-terminal
and central loops are now established. Based on these findings, we have depicted the HHS segment as a transmembrane sequence termed H8 that now forms the seventh transmembrane spanning domain of the transporter.

The Missense Mutants Produce Full-Length Proteins that Reach the Cell Surface:

With the determination of the extracellular orientation of the two loops containing all five missense mutations, it was reasonable to explore their potential role in disrupting ABCA1 interactions with apoA-I. Before testing these interactions, it was first necessary to establish that the mutant proteins could be synthesized and traffic to the plasma membrane, where interactions with apoA-I are presumed to occur. Immuno-blotting with an antibody directed against the carboxyl-terminus of ABCA1 demonstrated that all of the mutant constructs, when transfected into 293 cells, produced full length ABCA1 at levels comparable to those produced by the wild type ABCA1 construct (Figure 5A). To assess alterations in transporter trafficking, the mutant constructs were further modified to contain the FLAG tag at AA 207, used in the experiments described in Figure 4. All of the mutants were expressed at the cell’s surface at levels ranging from 60 to 100% of that measured with the FLAG-tagged, wild type ABCA1. Thus, the marked reduction in cholesterol efflux activity of the mutants could not be accounted for by alterations in protein synthesis, degradation, or trafficking to the plasmalemma.

Characterization of the ApoA-I-ABCA1 Interaction as Determined by Cross-Linking:

As the loss of efflux activity of the missense mutants could not be explained by a loss in total or cell surface expression of ABCA1, we next tested their ability to interact with apoA-I. Binding studies were performed using radiolabeled apoA-I and 293 cells transfected with wild type and mutant constructs. As it has been difficult to produce permanently transfected cells
constitutively expressing high levels of ABCA1, these assays were done in transiently transfected cells and produced inconsistent results (data not shown). In addition, Figure 4B demonstrates that even in mock-transfected cells in which there is no evidence of any ABCA1 expression by immunoblot or immunoprecipitation, there is a substantial level of I\textsuperscript{125}-apoA-I cellular association that is competed by unlabeled ligand. Thus, we chose to measure the apoA-I interaction using a cross-linking assay in which we could not detect any evidence of apoA-I interaction with cells that have not been transfected with ABCA1 (Fig. 4C).

To establish the specificity of this interaction, cross-linking efficiency of apoA-I to ABCA1 was measured using another transmembrane receptor (scavenger receptor-A, SR-A) as a control cell surface protein. Cells were transfected with empty vector, ABCA1, or SR-A; both of which having been labeled at their carboxy termini with a rhodopsin epitope tag (17,28). The transfected cells were incubated with [\textsuperscript{125}I]-apoA-I either in the presence or absence of excess unlabeled apoA-I. Cross-linking was then executed using a thio-sensitive, bifunctional cross-linker, DSP (dithiobis-succinimidylpropionate). After immuno-precipitation with a monoclonal antibody against the rhodopsin epitope, the precipitated proteins were solubilized in SDS-loading buffer in the presence or absence of beta-mercaptoethanol. Following separation by SDS-PAGE, the bands were visualized by phosphorimaging (Fig. 6A). In the non-reduced ABCA1 sample, a band of apparent molecular mass of 240 kDa is detected that is not seen in the mock-transfected cells. This band is not detected when the binding of the radiolabeled apoA-I is conducted in the presence of competitive, unlabeled apoA-I. It is also not seen when lysates produced in the absence of competitor are electrophoresed under reducing conditions, eliminating the thio-sensitive bond between apoA-I and the proteins to which it cross-links. Under reducing
conditions, the radiolabeled apoA-I is detected at its native molecular mass of ~27 kDa, but only in lysates produced from cells transfected with ABCA1. In contrast, no cross-linking to SR-A was detected and no free apoA-I was visualized in lysates taken from cells transfected with SR-A and electrophoresed under reducing conditions. The lack of interaction between apoA-I and SR-A could not be explained by inefficient expression or immuno-precipitation of the latter, as immunoprecipitations performed using lysates from transfected cells labeled with [35S]-methionine and the anti-rhodopsin antibody, yielded as much or more SR-A protein compared to ABCA1 (Fig. 6B). The cross-linking interaction between ABCA1 and apoA-I was also not dependent on the presence of the rhodopsin epitope tag, nor on the use of the anti-rhodopsin antibody, as similar results were obtained when an anti-ABCA1 antibody was used in conjunction with an untagged ABCA1 construct (Fig 6A & B, right panels). Taken together, these results suggest the apoA-I cross-linking interaction with ABCA1 is specific and does not represent promiscuous cross-linking to cell surface receptors.

Having confirmed the specificity of the cross-linking reaction, we next tested the ability of cross-linkers of varying lengths (DSG, disuccinimidyl glutarate, 7 angstroms vs. DSP, dithiobis-succinimidylpropionate, 12 angstroms) and water-solubility (DSTTP, dithiobis-sulfo succinimidylpropionate) to cross-link apoA-I to ABCA1. DSTTP (unlike DSG and DSP) is not known to penetrate cell membranes, so if active in the assay, it would constitute evidence that the apo A-I and ABCA1 interaction occurs at an aqueous interface, rather than within the lipid membrane. It was found that the shorter DSG, whose cross-linking activity is not thiol sensitive, was at least as efficient as the longer DSP in cross-linking apoA-I to ABCA1 (Fig 7A). DSTTP also cross-linked apoA-I to ABCA1, although the total amount of cross-linked protein was slightly reduced using this water-soluble agent (Fig. 7A). These findings indicate that the
interaction between apoA-I and ABCA1 occurs over a distance no greater than 7 angstroms and can take place between domains that are, at least in part, in an aqueous environment.

To further explore the interaction of apoA-I with ABCA1, the kinetics and temperature dependence of the cross-linking assay were tested. Cross-linking efficiency was measured after incubating apoA-I with ABCA1 transfected cells for one hour at 37°C, 25°C and 4°C (Fig. 7B). To isolate the effect of temperature during the incubation period, from the temperature effects of the actual cross-linking step, all samples were chilled to 4°C after the initial incubation was carried out at the same or higher temperatures. The DSP cross-linker was then added and cross-linking was allowed to occur at 4°C. It was found that cross-linking efficiency dropped off dramatically if the initial incubation of apoA-I with the cells was conducted at 4°C. The intermediate efficiency seen at incubations at 25°C suggests that the apoA-I/ABCA1 interaction has a linear dependence on temperature (Fig. 7B, graph). The kinetics of the association at 37°C were probed by varying the incubation period from 5 min to 4 h and comparing this to a sample that had been incubated at 4°C for 4 hours (Fig. 7C). Within 5 min, a significant amount of cross-linking was observed at 37°C that did not increase substantially after 45 min of incubation. In contrast, the 4h incubation at 4°C produced little, if any, cross-links between apoA-I and ABCA1 (Fig. 7C).

_Mutations in the Large Extracellular Loops Differentially Affect the ABCA1/apoA-I Interaction:_

To assess whether the loss in efflux activity exhibited by the ABCA1 mutants (Fig. 1) was paralleled by a loss in their ability to directly interact with apoA-I, we determined their cross-linking efficiency at 37°C (Fig. 8). Three of the mutants (Q597R, C1477R and S1506L) had dramatic reductions in their cross-linking efficiency to apoA-I, relative to the wild type.
transporter (90 % or greater reduction). The R587W mutant showed an intermediate phenotype, with cross-linking efficiency reduced approximately 50%. Surprisingly, the W590S mutant consistently cross-linked to apo A-I at an efficiency that was equal to or greater than that of wild type ABCA1. Thus, while we generated no mutants that could efflux in the absence of cross-linking to apo A-I, it was possible to find a mutant that loses efflux activity but retains cross-linking.
DISCUSSION

In this study, we have established that several naturally occurring missense mutations in ABCA1 (R587W, W590S, Q597R, C1477R and S1506L), located in the two largest loop domains of the protein (comprising amino acids ~44-640 and ~1371-1649, respectively) are, in fact, loss-of-function mutations. We have confirmed our earlier work showing the extracellular disposition of a large N-terminal loop and provided new evidence that the central loop of ABCA1 is similarly oriented in the exocytoplasmic space (17). Thus, the mutants in these two loops are presented on the exofacial leaflet of the plasma membrane. The introduction, into a wild type ABCA1 cDNA, of each of these five single amino acid substitutions provides unequivocal proof of their causality in Tangier disease or familial hypoalphalipoproteinemia. This proof facilitates their utility in population studies searching for loss-of-function mutations in ABCA1. These mutants also provided new insights into the structural requirements for ABCA1 function.

The loss in efflux activity of the mutants employed in these studies could not be accounted for by changes in protein production or stability, as all of the mutants were expressed at the cell surface at levels ranging from 60 to 100% of wild type ABCA1. Studies in ABCR have provided evidence that the symmetrical halves of A class transporters may be linked by a disulfide bridge and both of the large extracellular domains of ABCR and ABCA1 contain multiple cysteines that could serve as the residues that form such bridges (18). Only one cysteine mutant was utilized in this study (C1477R) and work is currently underway to determine if this mutation affects the formation of a disulfide linkage in ABCA1. Most interestingly, the five mutants used in this work displayed varying abilities to interact with apoA-I, as determined by a
ligand cross-linking assay. While three of the mutations (Q597R, C1477R and S1506L) showed no appreciable cross-linking to apoA-I, the R587W mutant had an intermediate activity and the W590S mutant retained full, if not enhanced, cross-linking to the apoprotein.

These findings contribute to our evolving understanding of the molecular interaction between ABCA1 and one of its apolipoprotein ligands, apoA-I. Several competing models have been proposed for this interaction. Chambenoit et al. have reported that even though ABCA1 expression increases the amount of membrane bound apoA-I, its association with cellular membranes exhibits diffusional properties that are consistent with apoA-I binding to membrane lipid rather than an integral membrane protein (29). These authors suggested that efflux activity is not dependent upon a direct protein-protein interaction between apoA-I and ABCA1, but rather upon the ABCA1-dependent generation of more phosphatidylserine in the exofacial leaflet of the plasma membrane (29). Recently, Smith et al. challenged this interpretation by showing that while ABCA1 expression is associated with an increase in cell surface phosphatidylserine presentation, the cellular association of apoA-I is not competed by annexin V, a phosphatidylserine binding protein (30). While this result suggests a less direct apoA-I/phosphatidylserine interaction, it is also possible that annexin V and apoA-I bind to non-competing sites. The same authors also reported a photo-bleaching assay in which cell associated fluorescent apoA-I was found to diffuse much more slowly than a fluorescent phospholipid analog. The slower diffusion of apoA-I was hypothesized to result from tethering to an integral membrane. Whether this postulated tethering was due to a direct association with ABCA1 was not established.
Wang et al have shown that glybenclamide inhibits, in a parallel manner, cholesterol efflux activity, cell association of apoA-I, and cross-linking of apo A-I to ABCA1 (14). These data suggest a more direct interaction of the two proteins. Like Chambenoit et al, however, Wang et al found that an ABCA1 construct carrying a mutation designed to abrogate ATPase activity failed to cross-link to apoA-I (14,29). These investigators favored the possibility that ATPase activity might be required for ABCA1 to adopt a conformation that enables direct apoA-I binding. In this regard, our observations on the kinetics and temperature dependence of the apoA-I/ABCA1 cross-linking interaction are of interest. At 37°C, apoA-I could cross-link to ABCA1 within 5 minutes. However, at 4°C, even a 4h incubation led to little or no observable cross-linking. This temperature dependence of the apoA-I/ABCA1 interaction could result from the loss of an energy dependent step that mimics the behavior of the ATPase mutant. Alternatively, changes in membrane fluidity at the lower temperature might influence the conformational insertion of apoA-I into the lipid bilayer, preventing the contacts required for cross-linking to ABCA1. While several labs have reported binding studies with apoA-I conducted at 4°C, the results have been somewhat inconsistent (14,29,31-33). Thus, it is unclear if ABCA1 can directly bind to apoA-I at low temperatures.

A model that we currently favor is that apoA-I can bind to plasma membranes at both physiologic and colder temperatures, but it may only become closely associated with ABCA1 at higher temperatures. At physiologic temperatures, the association between apoA-I is very close (less than 7 angstroms) and, we believe, is most consistent with a direct protein-protein interaction. This contact likely involves specific residues in the two extracellular loops of the protein that we have shown in this work to be critical to the interaction. Some of the contact
points between apoA-I and ABCA1 are in an aqueous environment, as they can be demonstrated using the water-soluble cross-linking agent, DSTTP. It is possible that some subsequent maturation step in the interaction between apoA-I and ABCA1 must take place at 37°C (e.g., endocytosis or a conformational shift in the protein) explaining the dependence on temperature and ATP hydrolysis for cross-linking. Recent data from Silver et al may be pertinent to this hypothesis, in that they have provided evidence that another HDL receptor, called SR-BI, can internalize its ligand and then separate the lipid from the lipoprotein in internal vesicles (34,35). The apoprotein then is recycled back to the plasma membrane to which it originally bound, yielding a net result called selective cholesterol uptake. Previous studies suggesting that cholesterol efflux to apoA-I depends on retroendocytosis are consistent with ABCA1 playing a role that reverses the process described for SR-BI (36). That is, selective lipid acquisition by apoA-I might occur in an internalized vesicle and the close association between apoA-I and ABCA1 that we have demonstrated in our cross-linking experiments might require this endocytotic step. If this model is correct, the endocytosis process will likely require other protein interactions with ABCA1 that remain to be identified. This model, however, remains speculative and there is no definitive evidence that lipid transfer to apoA-I requires internalization of the apoprotein. What is clear is that a direct interaction between apoA-I and ABCA1 is not sufficient for the movement of cholesterol out of the cell, as the W590S mutant retained full cross-linking activity and yet could not efflux cholesterol. Thus, the simple insertion of apoA-I into the membrane in close enough proximity to ABCA1 to permit cross-linking may be necessary, but it is not sufficient, to generate cholesterol efflux.
In summary, in this work we have shown that naturally occurring single amino acid changes in the two largest extracellular loops of ABCA1 can disrupt cholesterol efflux to apolipoprotein A-I. These finding assign a causal role to the mutations studied and explain their association with the low HDL levels seen in Tangier and FHA patients. While none of the mutants were functionally active in the cholesterol efflux assay, it was possible to identify a functionally inactive mutant that retained its ability to interact directly with apo A-I. This finding indicates that apoA-I insertion into the plasma membrane, in close proximity to ABCA1, is insufficient to stimulate cholesterol efflux. Further characterization of the interaction between the transporter and its apoprotein ligands is needed to elucidate the molecular mechanisms of lipid efflux and HDL homeostasis.

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References


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Figure Legends

Figure 1. Missense mutations in the two large extracellular loops ablate ABCA1 efflux activity. Cells were transfected with either empty vector (mock), wild type ABCA1 (WT) or ABCA1 constructs carrying the indicated point mutations (R587W, W590S, Q597R, C1477R and S1506L). After labeling for 24 h with H\(^3\)-cholesterol the cells were exposed to apoA-I (10 ug/ml) at 37°C for 4h, or to media alone. Counts of H\(^3\)-cholesterol were determined for the media and cells and expressed as apoA-I dependent efflux (apo-A1 dependent efflux minus media only dependent efflux). Absolute apoA-I and media efflux values, respectively, are as follows: mock, 1.59±0.04% vs. 1.21±0.39%; WT, 3.92±0.13% vs. 1.9±0.08%; R587W, 1.78±0.11% vs. 1.61±0.24%; W590S, 1.92±0.24% vs. 1.63±0.08%; Q597R, 1.5±0.14% vs. 1.49±0.03%; C1477R, 1.67±0.18% vs. 1.52±0.15%; and S1506L, 1.66±0.28% vs. 1.6±0.13%. Results are representative of two or more experiments with measurements performed in triplicate and error bars representing standard deviations.

Figure 2. Polypeptides expressing the central region of ABCA1 are glycosylated indicating the 8th hydrophobic domain spans the lipid bilayer. The highly hydrophobic sequence (HHS), previously modeled as not crossing the plasma membrane (left diagram, top) was expressed in the context of the entire central region of ABCA1 (AA 843-1650, REG, left diagram, bottom; potential N-glycosylation sites in the constructs are indicated by “N”) or a subdomain of that central region (AA 1311 to 1650, HHS, right diagram, bottom). Cells were transfected with empty vector, or with the REG and HHS constructs, and subsequently metabolically labeled with
S\textsuperscript{35}-methionine. The expressed polypeptides were immuno-precipitated, treated with the glycoamidase, PGNase, separated on 12 \% SDS-PAGE gels, and phosphorimaged. The central panels illustrate that de-glycosylation of these polypeptides results in faster migration of the proteins (double arrow heads identify glycosylated and de-glycosylated polypeptides), indicating that both the larger central region protein and its subdomain are glycosylated peptides. These results are consistent with the topologies for the polypeptides illustrated in the top right diagram.

Figure 3. **Topological model of ABCA1 showing the two large extracellular loops.** Presented is a topological model that incorporates H1 and H8 as membrane spanning domains based upon the results in Figs. 1 & 2 and reference (17), H7 and H13 are shown as not spanning the lipid bilayer, as they have lower hydrophobic indices than the other TM segments. The topology is constrained by the expectation that the ATP binding domains will be cytoplasmic and the experimental evidence that places the R2 and N-terminal loops in the extracellular space. Shown are the positions of the FLAG tags and missense mutations in the two large extracellular loops from AA 44 to 640 and AA 1371 to 1650, as well as the cytoplasmic localization of the control FLAG tagged bacterial alkaline phosphatase.

Figure 4. **FLAG epitopes at AA 207 and 1396 are accessible in non-permeabilized cells, confirming an extracellular disposition of AA 44 to 640 and AA 1371 to 1650.** The eight amino acid FLAG epitope (DYKDDDDK) was inserted either after AA 207 or after 1396 in the open reading frame of ABCA1. A, B, C; The resulting FLAG-ABCA1 constructs (N-FL, R2-FL, respectively) were expressed and found to be fully functional for efflux activity (A), stimulation of cellular apoA-1 association (B), and the ability to interact with apoA-I as determined by cross-
linking (C). D, E: Accessibility of the FLAG epitopes in N-FL and R2-FL was compared to that of un-tagged ABCA1 (WT) or a control cytoplasmic protein (FLAG-bacterial alkaline phosphatase, FL-BAP) by transfecting cells and assessing anti-FLAG antibody binding characteristics to the cells after permeabilization with Triton-X-100 (E), or not (D). Anti-FLAG antibody binding was detected with an \( ^{125}\text{I} \)-goat anti-mouse antibody and gamma counting. The results are expressed as bound counts per mg of total cellular protein. Results are representative of two or more experiments with measurements for A, B, D, & E performed in triplicate and error bars representing standard deviations.

Figure 5. **Missense mutants express full-length ABCA1 proteins that reach the cell surface.** A, Cells were transfected as described in the methods and total cellular ABCA1 protein was determined after 24 h by immuno-blotting. Top panel shows a representative blot (15 ug of total protein from a post-nuclear supernatant). Graphed below are relative ABCA1 levels in duplicate samples as determined by densitometry using a Kodak 1000 imager. B, Cells were transfected as in A. Constructs used for the transfection included an empty vector (mock), untagged ABCA1 (WT-ABCA1), FLAG-tagged wild type ABCA1 (FLAG-ABCA1), and the numbered missense mutant constructs incorporating a FLAG epitope tag at AA 207. Anti-FLAG antibody binding was determined in non-permeabilized cells as described in Fig. 4D.

Figure 6. **The apoA-I/ABCA1 interaction as determined by cross-linking is specific.** A, Cells were transfected with ABCA1 and SR-A constructs carrying a rhodopsin tag at the COOH termini of the open reading frames (left panel), or with WT un-tagged ABCA1 (right panel). 24 h after transfection, cells were incubated for 1h at 37\(^0\)C with \(^{125}\text{I}\)-ApoA-I (1ug/ml), with or without
50 ng/ml cold apoA-I as indicated. Cells were washed 2X with cold PBS on ice and exposed to the DSP cross-linker for 1h at RT. Expressed polypeptides were immuno-precipitated with an anti-Rhodopsin antibody (left panel), or with an anti-ABCA1 antibody (right panel) and run on 6% SDS-PAGE gels after treatment or not with beta-mercaptoethanol as indicated. Shown is a phosphorimage of the resulting gels. B, Cells were transfected as in A and metabolically labeled with S\textsuperscript{35}-methionine followed by immuno-precipitation with the anti-rhodopsin antibody (left panel) or the anti-ABCA1 antibody (right panel). Immuno-precipitated proteins were resolved by SDS-PAGE and detected by phosphor imaging.

Figure 7. **The apoA-I/ABCA1 interaction is spatially close, temperature dependent and occurs rapidly.** A, Cells were transfected with wt-ABCA1 or empty vector as indicated and incubated with I\textsuperscript{125}-apoA-I as in Fig. 6. Cross-linking was carried out with the indicated cross-linkers (DSP, DSTTP and DSG) and ABCA1 was immuno-precipitated with a polyclonal-antibody directed to the last 200 AA of ABCA1. All samples were treated with beta-mercaptoethanol before SDS-PAGE and phosphor imaging. Note that both the DSP and DSTTP cross-linkers contain thiol sensitive bonds whereas the DSG cross-linker does not. Thus, apoA-I remains attached to ABCA1 when electrophoresed under reducing conditions when the DSG cross-linker is employed. Quantitation of duplicated samples indicated the DSG and DSTTP cross-linking efficiency was 269±81% and 80±5 respectively vs 100±24% for DSP (duplicate samples ±SD with DSP cross-linking normalized to 100%). B, Cells were transfected and incubated with apoA-I (1 ng/ml) for 1h at the indicated temperatures. All cells were then chilled on ice, washed, and exposed to the DSP cross-linker for 2h on ice. The resulting immuno-precipitated apoA-I is shown in the top panel and the amount of apoA-I is graphed below vs.
temperature. Cells were transfected with ABCA1 or empty vector and incubated with apoA-I (1 ug/ml) for increasing periods of time at 4°C or 37°C as indicated. The top panel shows the immuno-precipitated apoA-I and the amount of apoA-I is graphed below vs. time.

Figure 8. **The missense mutants display a variable ability to interact with apoA-I as determined by cross-linking.** Cells were transfected with empty vector or the indicated constructs and incubated with apoA-I (1 ug/ml) 24h later for 1h at 37°C. Cross-linking was carried out with the DSP cross-linker. Immuno-precipitated apoA-I is shown in the top panel and the amount of precipitated apoA-I from duplicated samples is graphed below. Results are representative of 2 or more experiments.
Fig. 1
Fig. 2
Fig. 3
**Fig. 4**

A. % cholesterol efflux

- **media**
- **apoA-I**

B. bound ApoA-I (ng/mg cell protein)

- **I^{125}_{apoA-I}**
- **I^{125}_{apoA-I} + apoA-I**

C. apoA-I cross-linking

- 29 kDa
- 21 kDa

D. non-permeabilized

  - bound anti-FLAG Ab (counts/mg cell protein)

  - WT
  - N-FL
  - R2-FL
  - FL-BAP

E. permeabilized

  - bound anti-FLAG Ab (counts/mg cell protein)

  - WT
  - N-FL
  - R2-FL
  - FL-BAP
Fig. 5
ApoA-I X-linking

**A**

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217 kDa --
119 kDa --
48 kDa --
29 kDa --
21 kDa --

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**B**

S\(^{35}\)-labeling & IP

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Fig. 6
Fig. 7
Fig. 8

X-linking (phosphor pixels)

WT
R587W
W590S
Q597R
S1506L

mock
WT
R587W
W590S
Q597R
S1506L

C1477R

mock
WT
C1477R

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