ATP-binding Cassette Transporter A1 Contains a Novel C-terminal VFVNFA Motif That Is Required for Its Cholesterol Efflux and ApoA-I Binding Activities*

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The stimulation of cellular cholesterol and phospholipid efflux by apolipoprotein A-I is mediated by the activity of the ATP-binding cassette transporter A1 (ABCA1). Individuals with Tangier disease harbor loss-of-function mutations in this transporter that have proven useful in illuminating its activity. Here, we analyze a mutation that deletes the last 46 residues of the 2281 amino acid transporter (Δ46) and eliminates its lipid efflux. As the final four amino acids of the C terminus represent a putative PDZ-binding motif, we initially characterized deletion mutants lacking only these residues. Although a moderate decline in lipid efflux was detected, this decline was not as profound as that seen in the Δ46 mutant. Subsequent systematic analysis of the ABCA1 C terminus revealed a novel, highly conserved motif (VFVNFA) that was required for lipid efflux. Alteration of this motif, which is present in some but not all members of the ABCA family, did not prevent trafficking of the transporter to the plasma membrane but did eliminate its binding of apoA-I. Chimeric transporters, generated by substituting the C termini of either ABCA4 or ABCA7 for the endogenous terminus, demonstrated that ABCA1 could stimulate cholesterol efflux without its PDZ-binding motif but not without the VFVNFA motif. When a peptide containing the VFVNFA sequence was introduced into ABCA1-expressing cells, ABCA1-mediated lipid efflux was also markedly inhibited. These results indicate that the C-terminal VFVNFA motif of ABCA1 is essential for its lipid efflux activity. The data also suggest that this motif participates in novel protein-protein interactions that may be shared among members of the ABCA family.

The superfamily of ABC transporters is one of the largest and most ancient gene families with representatives in all extant phyla (1). In humans, this family is thought to comprise at least 49 members, 13 of which belong to the ABCA class of transporters (2). Of the A class members, ABCA1 and ABCA4 are best studied because of the fact that mutations in these two transporters are associated with Tangier disease and Stargardt’s macular degeneration, respectively (3–8). Tangier disease, loss of ABCA1 activity results in the near absence of circulating high density lipoproteins and the deposition of massive amounts of cholesterol esters in peripheral tissues. At the cellular level, loss of ABCA1 function eliminates the efflux of cholesterol and phospholipids in response to stimulation with the major apolipoprotein of the high density lipoproteins, apoA-I. At the molecular level, a variety of evidence indicates that ABCA1 forms a high affinity complex with apoA-I by binding amphipathic helices within the apolipoprotein (9–13). Although the formation of this complex appears central to efflux activity, the mechanism by which the formation and turnover of this complex results in the transfer of cholesterol and phospholipid to the apolipoprotein remains unclear (14–19).

Analysis of naturally occurring and engineered ABCA1 mutations has proven to be a fruitful approach in studying the efflux mechanism. We have previously elucidated the ABCA1 transporter’s topology and characterized some of its interactions with apoA-I using this approach (11, 12). In our ongoing effort to define the structure/function relationships that underlie the ABCA1 efflux mechanism, we focused on a truncation mutant that we had originally identified in a patient with Tangier disease (8). This mutation would be predicted to delete the last 46 amino acids of the transporter. The deleted sequences were of interest, as they included a cytoplasmic motif (ESYV) that conforms to the consensus sequence for binding to PDZ domain-containing proteins. PDZ proteins are named for the founding members of the group (PSD-95, Dlg, and ZO-1) and have recently reported the interaction of ABCA1 with three proteins containing PDZ domains (the α1 and β2 syntrophins and Lin7) and one non-PDZ protein (Fas-associated death domain protein) (21). Indeed, using yeast two-hybrid screens and either the last 165 or 120 amino acids of the ABCA1 C terminus, two groups have recently reported the interaction of ABCA1 with three proteins containing PDZ domains (the α1 and β2 syntrophins and Lin7) and one non-PDZ protein (Fas-associated death domain protein) (22–24).

To functionally characterize the importance of the putative PDZ-binding motif and to explore the impact of the loss of the C-terminal 46 amino acids in detail, we generated a series of ABCA1 C-terminal mutants. Initially, it was observed that the loss of the four amino acids composing the PDZ-binding motif, although detrimental to function, did not recapitulate the loss...
of all 46 amino acids at the C terminus. Further analysis demonstrated that the sequence between –41 and –46 (VFVNFA) was critical for efflux activity and that its loss abrogated apoA-I binding to ABCA1. This sequence is shared by some but not all members of the ABCA class of transporters. This finding was exploited to demonstrate that ABCA1 can efflux lipid normally when the VFVNFA-containing ABCA4 C terminus is substituted for that of ABCA1. That this activity is reconstituted despite the absence of the PDZ-binding motif in this chimeric protein indicates that the motif is not required for ABCA1 activity in all cell types. Substitution of the ABCA7 C terminus, which lacks the VVNFA sequence but retains the PDZ motif, did not produce an active transporter, further corroborating this conclusion. When these results are coupled with our finding that an intracellular peptide containing the VFVNFA motif was able to strongly inhibit ABCA1-dependent efflux, the data suggest that this motif in the C terminus of ABCA1 plays a critical role in facilitating its interactions with other proteins required for the transporter’s lipid efflux activity.

MATERIALS AND METHODS

Reagents—The following reagents were purchased from the indicated suppliers: LipofectAMINE 2000, the Anti-Xpress mouse monoclonal antibody, and Dulbecco’s modified Eagle’s medium (1 mg/ml BSA) for 2 h, washed twice with PBS, and incubated with Dulbecco’s modified Eagle’s medium (1 mg/ml BSA) for 24 h. The cells receiving [3H]cholesterol were then washed twice with warm PBS, incubated with Dulbecco’s modified Eagle’s medium (1 mg/ml BSA) with or without 10 μg/ml apoA-I for an additional 20 h. In parallel, the plates of unlabeled cells were chilled on ice for 10 min and cell surface expression of ABCA1 was detected by an M2 anti-FLAG antibody, while total ABCA1 expression was measured in GFP-ABCA1-transfected cells by flow cytometry (FACSCalibur System; BD Biosciences). The percentage of cholesterol efflux ([cpm media/cpm media + cell-associated cpm] × 100) was calculated by scintillation counting. Cell surface ABCA1 expression was calculated as cpm [3H]labeled secondary antibody bound per milligram of total cell protein (Bio-Rad protein assay), and total transporter expression was expressed as the average GFP-ABCA1 fluorescence intensity. Cholesterol efflux was normalized by subtracting the efflux activity of mock transfected cells and then dividing by the cell surface or total expression of ABCA1. All measurements were performed in triplicate. The S.D. of these measures was calculated and propagated after normalization using formulas for the subtraction and division of errors.

PDZ Domain Binding Assays—The interaction of PDZ domains with the ABCA1 C terminus was measured by overlay and oligopeptide binding assays (26). For overlay assays, His-tagged ABCA1 polypeptides containing amino acids 2076–2216 or 2076–2257 (Δ4) were generated. To express the PDZ domains 1 and 2 of PSD-95, a pRSETB plasmid (generous gift of Dr. Morgan Sheng, Massachusetts Institute of Technology, Cambridge, MA) containing a sequence corresponding to amino acids 84–398 of human PSD-95 was used. As a positive control for binding to PSD-95, glutathione S-transferase fused with amino acids 1453 to 1482 of the N-terminal α-asparrate receptor subunit NR2BC was used (26). His-tagged Rab6 and Rab11 polypeptides were used as negative controls for PDZ-95 binding (27). Polypeptides were subjected to 12% SDS-PAGE, transferred to nitrocellulose, and allowed to renature in 1× PBS (with 3% BSA) overnight. The transferred polypeptides were incubated with PSD-95 (1× 250 nm PBS and 3% BSA) for 2 h at room temperature, washed with 1× PBS (with 0.1% Tween 20), and incubated with anti-express mouse monoclonal antibody (1:5000 dilution). Membranes were washed, and bound antibody was detected with anti-mouse IgG-horseradish peroxidase antibody (1:30,000 dilution) and enhanced chemiluminescence (Super Signal; Pierce). Blots were stripped and sequentially re-probed with rabbit anti-ABCA1 antibody (25) and rabbit anti-Rab6 antibody.

To assess whether ABCA1-PDZ interactions have class I binding specificity, we used 20- and 16-mer biotinylated peptides representing the C termini of ABCA1 with and without the PDZ binding motif, propagated as streptavidin-purified biotinylated RNAs representing E-tag PDZ fusions of the first PDZ domain of PSD-95 (residues 41–161), the second PDZ domain of PSD-95 (residues 123–267), andDlg-1 (residues 209–332) or the first and second PDZ domains of synemin (residues 101–285) were translated in 500 μl of rabbit reticulocyte lysate translation reactions (Novagen) in the presence of 200 μCi of [35S]methionine (1000 Ci/mmol; PerkinElmer Life Sciences). The E-tag PDZ fusions were purified from the lysate using an anti-E tag column (Amersham Biosciences) and buffer was exchanged into 1× TntV (40 mM triethylamine, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Tween 20, and 100 μg/ml BSA) using NAP-10 columns (Amersham Biosciences). The purified PDZ domains were applied to 200 μl of the streptavidin-activated agarose and incubated at 4°C for 2 h. The bound fraction was eluted with free wild type ABCA1 peptide. The amount of input and eluted protein was quantitated by scintillation counting and expressed as percent bound.

Peptide Synthesis and Inhibition Assays—To test whether the VFVNFA motif alone could act in trans to modulate ABCA1 efflux activity, we generated peptides containing VFVNFA or six alanines fused to the C terminus of the third α-helix of the Antennapedia homeomain-domain (N-RQIKIWFQNRRMKWKKVFNFA-C and N-RQIKIWFQNRRMKWKKAAAAA-C) (28, 29). The peptides were purified to >95% homogeneity by high pressure liquid chromatography and analyzed by mass spectrometry. To measure the efficiency of cellular uptake, peptides were biotinylated and incubated with 293-EBNA-T cells for 2 h. The cells were washed extensively, trypsinized to remove cell surface-associated peptide, and lysed to determine the amount of internalized peptide. Equivalent amounts of cellular protein were spotted onto nitrocellulose and probed with streptavidin-conjugated horseradish peroxidase (Pierce). For efflux assays, 293-EBNA-T cells transfected with wild type ABCA1 or empty vector were loaded with [3H]cholesterol for 24 h, washed extensively, and treated with the Antennapedia peptide. Cells were incubated in the presence or absence of 2 μg/ml of peptide not exposed to apoA-I (10 μg/ml) for an additional 20 h, and the amount of cholesterol efflux was measured as described above. For acute inhibition assays, after incubation with the Antennapedia peptides for 2 h a wash step was added to remove free peptide before the addition of apoA-I. Cell viability was assessed by trypan blue uptake and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assays (American Type Culture Collection, Manassas, VA) to...
ensure that the peptides were not cytotoxic. All assays were performed on triplicate samples.

Pattern Searches for the VFVNFA Motif—A BLAST-P (2.2.9 release) search of the non-redundant translated data bases was done using the VFVNFA motif as a query sequence (30). Search parameters optimized for finding short, nearly exact matches were used (word size, 2; SEG filter, off; expect value, 20,000; composition based statistics, off, score matrix, PAM30). An additional search using a pattern-hit initiated BLAST using the entire ABCA1 protein sequence confirmed that the VFVNFA sequence was uniquely conserved in the ABCA class members A1, A2, and A4 from vertebrate organisms. Additional searches for ABCA transporters encoded by the genomes of Fugu rubripes and Ciona intestinalis were done through the web portal maintained by the Department of Energy Joint Genome Institute (www.jgi.doe.gov).

RESULTS

The ABCA1 C Terminus Is Highly Conserved and Is Bound by Class I PDZ Domains—To characterize the A46 Tangier mutation, we first determined whether the deleted sequences were conserved and, if so, whether they could be bounded by PDZ domains. The alignment of six ABCA1 orthologues showed that the sequences are strongly conserved (Fig. 1A). There is 91.3% identity between the C termini of human and chicken ABCA1 transporters, which are separated by 300 million years of evolution. This conservation is most striking relative to a comparably sized region in the variable first large extracellular loop of ABCA1 (40.4% identity between the sequences comprising codons 190 to 236). As the final four residues of the C terminus constituted a putative class I PDZ-binding motif, we examined the binding of several PDZ proteins to the ABCA1 C terminus using an overlay binding assay. We first tested the founding class I PDZ protein, PSD-95, in this assay (21, 31). The last 186 amino acids of ABCA1 were expressed as a His-tagged polypeptide encompassing the PDZ domains 1 and 2 of PSD-95 and was incubated with the transfected proteins, and the binding of the polypeptide was detected using an anti-epitope antibody. PSD-95 bound both the wild type ABCA1 polypeptide and the NR2B C terminus (Fig. 1B). Truncation of the ABCA1 ESYV motif largely ablated the PSD-95 interaction. In contrast, PSD-95 did not bind two other His-tagged proteins that did not contain consensus PDZ-binding motifs.

Because PSD-95 expression is largely confined to neuronal tissues whereas ABCA1 is ubiquitously expressed, we screened for additional class I PDZ proteins in a library constructed from cells known to express high levels of ABCA1. RNA for reverse transcription PCR reactions was prepared from human THP-1 macrophages treated with the phorbol ester phorbol 12-myristate 13-acetate, which up-regulates ABCA1 expression and cholesterol efflux (32). Reactions were run using primers designed to amplify class I PDZ proteins (PSD-95, Dlg-1, NE-dlg, and chapsyn-110) as well as the class II PDZ protein syntenin. In data not shown, we were able to amplify a small amount of a PSD-95 product. In contrast, Dlg-1 expression was easily detected whereas the expression of NE-dlg or chapsyn-110 was not. A product representing syntenin was also reliably amplified. To determine whether ABCA1-PDZ protein interactions have class I specificity, the PDZ domains from PSD-95, Dlg-1, and syntenin, were assessed for their ability to bind the ABCA1 C terminus. As with PSD-95, the second PDZ domain of Dlg-1 bound a C-terminal ABCA1 peptide, whereas the PDZ domains of syntenin did not (Fig. 1C). These results indicated that the highly conserved C terminus of ABCA1 can interact with proteins containing class I but not class II PDZ domains.

The ABCA1 C Terminus Is Essential for Efflux but the PDZ Protein-binding Motif Is Not—To test the functional importance of the C terminus, we engineered the 46-amino acid Tangier deletion (Q2215X, Δ46) as well as a larger deletion described by Clee et al. (R2144X, Δ117) (33) into our cDNA constructs. Both mutants showed no efflux activity (data not shown). The activity of a mutant with a deletion of the PDZ binding motif alone (elimination of only the last four amino acids of ABCA1) was expressed as a His-tagged polypeptide encompassing the PDZ domains 1 and 2 of PSD-95 and was incubated with the transfected proteins, and the binding of the polypeptide was detected using an anti-epitope antibody. PSD-95 bound both the wild type ABCA1 polypeptide and the NR2B C terminus (Fig. 1B). Truncation of the ABCA1 ESYV motif largely ablated the PSD-95 interaction. In contrast, PSD-95 did not bind two other His-tagged proteins that did not contain consensus PDZ-binding motifs.

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acids of ABCA1, Δ4) was then compared with the activity of the Δ46 mutant. Both mutants were similarly expressed, as determined by immunoblotting with an antibody directed against the N terminus of ABCA1 (Fig. 2A). Δ4, when expressed at moderate levels in 293 cells, consistently showed efflux deficits of up to 60% as compared with wild type ABCA1 activity. This defect, however, was much less apparent in 293-EBNA-T cells, which express our constructs at a much higher level than traditional 293 cells because of replication of the transfected DNA (Fig. 2, B and C). In contrast to Δ4, Δ46 showed a complete loss in efflux activity regardless of expression level. The failure of the deletion of the PDZ-binding motif sequence to recapitulate the loss of the terminal 46 amino acids of ABCA1 indicated that other sequences in the C terminus were playing a functionally important role. We next turned our attention to identifying those sequences.

C-terminal Deletion Mutants Reveal a Negative Regulatory Region between −30 and −40—To refine the sequences responsible for the loss of activity of Δ46, a series of 10 amino acid deletions in ABCA1 were constructed. Like Δ4, the Δ10, Δ20, and Δ30 transporters showed similar losses in efflux activity when expressed in 293 cells but were again largely indistinguishable from wild type ABCA1 when expressed in 293-EBNA-T cells (Fig. 3, A and B). Surprisingly, in both cell types Δ40 showed less functional impairment than the shorter truncations, suggesting that a negative regulatory element might be present between −30 and −40.

To explore the differences in the C-terminal deletion mutant series further, additional assays of transporter function were performed. Total transporter expression was found to be similar using both immunoblotting and fluorescence-activated cell sorter analysis of GFP expression of the mutants (Fig. 4A). In contrast, as the size of the deletion increased, the cell surface...
expression of the mutants decreased (Fig. 4B). Δ4, along with Δ10 and Δ20, were well expressed at the cell surface (particularly in 293-EBNA-T cells, data not shown), whereas Δ30, Δ40, and Δ46 reached the cell surface at 50, 40, and 30% of wild type levels, respectively. Because of the inherent variability in transient transfection assays, we normalized efflux to total ABCA1 expression (fluorescence-activated cell sorter analysis) or ABCA1 cell surface expression (radioimmuno-FLAG binding assays) (Fig. 5, A and B, respectively). Using these normalization methods, the intrinsic cholesterol efflux activity of the Δ40 mutant appears to be higher than that of wild type ABCA1, a finding that is clearly evident when the mutant is very highly expressed (see Figs. 3B and 5, A and B). To summarize, analysis of the deletion mutants indicated that the loss of the amino acids between −4 and −30 produced transporters whose reduced activity resembled that of the Δ4 mutant. However, loss of the region between −30 and −40 generated a transporter (Δ40) with enhanced intrinsic lipid efflux.

**FIG. 4.** Total and cell surface expression of the deletion series in 293 cells. A, total ABCA protein expression is similar in mutant and wild type (WT) constructs as shown by a Western blot of untagged versions (photograph) and fluorescence-activated cell sorter analysis of GFP versions of the mutants (graph). B, cell surface expression is impaired in larger deletion mutants as measured by antibody detection of the FLAG epitope in the extracellular loops of the transporters. Error bars represent S.D. of triplicate samples. Results are representative of two or more experiments.

**FIG. 5.** The gain-of-function phenotype of the Δ40 mutant persists when efflux is normalized to cell surface or total ABCA1 expression. A, percentage of cholesterol efflux in 293 cells normalized to cell surface ABCA1 expression (see “Materials and Methods”). B, percentage of cholesterol efflux in 293-EBNA-T cells normalized to total ABCA1 expression. Error bars represent S.D. of triplicate samples. Results are representative of two or more experiments. WT, wild type.
FIG. 6. Mutation of the VFVNFA motif disrupts efflux and apoA-I binding but not cell surface expression. A, percentage of cholesterol efflux in 293-EBNA-T cells of ABCA1 and the VFVFNA→AAAAAA mutant (AAAAAA). B, Apo-A-I complex formation as determined by cross-linking. C, cell surface expression. Error bars represent S.D. of triplicate samples. Results are representative of two or more experiments.

other proteins that contained the motif. A BLAST-P search of translated data bases retrieved 50 exact matches, 88% of which were to various homologs and orthologs in the ABCA class of transporters. The remaining 12% were matches to five bacterial proteins and a microtubule-associated protein from Dictyostelium discoideum. Of the bacterial proteins that contain the VFVNFA sequence, one was annotated as an ABC permease and one had limited homology to putative ABC transporters. Homologs and orthologs of these proteins, however, did not conserve the motif. Significantly, not only was the VFVNFA motif uniquely conserved in the ABCA subclass, it further divided the ABCA transporters into those that maintained the motif (ABCA1, A2, and A4), and those that have related but not identical sequences (ABCA7, as well as the other ABCA class members). Comparison of the C termini of ABCA4 and A7 was particularly interesting, because ABCA4 contains a VFVNFA motif but lacks the PDZ-binding motif found in ABCA1, whereas ABCA7 contains a putative PDZ-binding motif but lacks the VFVNFA sequence. Thus, we speculated that an ABCA1/A4 chimera, which retained the VFVNFA sequence but lost the PDZ-binding motif, would remain functionally active whereas an analogous ABCA1/A7 chimera would not. To test this hypothesis, the last 183 amino acids of the ABCA1 C terminus were exchanged for the corresponding sequences from the C termini of ABCA4 and A7. As predicted, the A1/A4 chimera, when expressed in 293 cells, demonstrated wild type or higher activity regardless of the cell line used in the transfection (Fig. 8, A and B). ABCA7 possesses a sequence (i.e. VFLYFS) that resembles VFVNFA and is found in the same location. To test if this sequence could replace VFVNFA in the context of the wild type ABCA1 C terminus, we replaced the ABCA1 residues with those from ABCA7. This mutant (VFLYFS) had a severe efflux deficit in both 293 and 293-EBNA-T cells, similar to that seen in A6 and the A1/A7 chimeras (Fig. 9A). Because VFLYFS could not functionally replace the VFVNFA motif, we attempted a more subtle change in which the previously identified critical residue in the latter motif (V2217) was replaced by the leucine found in the analogous position in the VFLYFS sequence. The substitution of leucine for valine is a very conservative change, as it extends the side chain by only one methyl group. The V2217L transporter showed little activity when transfected into 293-EBNA-T cells (Fig. 9B). Thus, the efflux activity of the chimeras and the additional VFVNFA mutants confirmed the importance of the VFVNFA motif and indicated that even closely related sequences could not recapitulate its function.

VFVNFA Cell-penetrating Peptides Inhibit ABCA1-dependent Efflux—To further explore the functional role of the VFVNFA sequence, we tested the effect of introducing a peptide mimic of this sequence into cells transfected with wild type ABCA1. Because the C terminus of ABCA1, including its VFVNFA motif, reside on a cytoplasmic domain of the transporter, it was necessary to generate a peptide that could be internalized by cells. To accomplish this, two peptides were synthesized containing either the VFVNFA sequence or six alamines fused to the C terminus of the third a-helix of the Antennapedia homeodomain. The Antennapedia sequences are able to penetrate cellular membranes and have been used to study the molecular interactions of various cytoplasmic and membrane complexes. These membrane-permeable peptides have the advantage that they enter the cell through a non-degradative pathway with an efficiency approaching 100% (28, 29). Initial experiments demonstrated that the cellular uptake of the two peptides was similar. Biotinylated peptides were incubated with 293-EBNA-T cells for 2 h, after which the cells were washed extensively, trypsinized to remove any cell surface-associated peptide, and washed again. The cells were lysed, and the amount of internalized peptide was determined by spotting equivalent amounts of cellular protein onto nitrocellulose and probing for the biotinylated peptides with streptavidin-conjugated horseradish peroxidase. Similar amounts of the two peptides were internalized by the cells as analyzed by these dot blots (data not shown). ABCA1 efflux activity was then measured in 293-EBNA-T cells transfected with wild type ABCA1 or empty vector, loaded with radiolabeled cholesterol for 24 h, and then treated with the VFVNFA or AAAAAA Antennapedia peptides (50 μM) for 2 h. Cells were exposed or exposed not to apoA-I (10 μg/ml) for an additional 20 h, and the amount of labeled cholesterol efflux in the media was measured. The VFVNFA-containing peptide significantly inhibited cholesterol efflux in this assay (~70% decline; Fig. 10), whereas the AAAAAA-containing peptide was without significant effect (p = 0.002 versus 0.4; paired Student’s t test). In these initial experiments, the inhibition of ABCA1 efflux by the VFVNFA peptide was associated with a slight cellular toxicity, as indicated by a viability assay, when the peptide was present for 22 h. To address this finding, we conducted acute inhibition studies in which cells were treated with the peptides as described previously for 2 h, washed to remove any free peptide, and incubated with apoA-I for 6 h. As seen in the previous experiment, the VFVNFA peptide caused a 56% reduction in activity (p = 0.036; paired Student’s t test), whereas the 13%
reduction in activity seen with the AAAAAA peptide was not significant ($p = 0.55$). Under these assay conditions, the VFVNFA peptide showed no cellular toxicity. Thus, when presented in trans as an isolated peptide sequence, the VFVNFA motif acts to inhibit ABCA1 efflux activity. This finding provides evidence that the VFVNFA motif may be acting to bind additional proteins required for ABCA1 function and that its interaction can be competed by peptide inhibitors of this binding.

DISCUSSION

In this study, we analyzed a naturally occurring ABCA1 mutation that deleted the last 46 amino acids of the transporter. The deletion causes a severe loss-of-function phenotype, explaining its association with Tangier disease. The deleted amino acids reside in the cytoplasm and are highly conserved, as expected for a functionally important domain. Initially, we hypothesized that any deleterious effects resulting from this mutation would be a consequence of disrupting the PDZ-binding motif represented in the penultimate four amino acids of the C terminus. This hypothesis appeared tenable, as it was possible to demonstrate that the C terminus of native ABCA1 was specifically bound by PDZ domains from PSD-95 and Dlg-1, two class I PDZ proteins, whereas this binding was lost when the last four amino acids were removed. Furthermore, during the course of our studies, two other groups used yeast two-hybrid screens to isolate four proteins that bind the ABCA1 C terminus. Three of these proteins contain PDZ domains ($\alpha$- and $\beta$-syntrophin and Lin7) and one does not (22–24). The interaction of $\alpha$-syntrophin and ABCA1 was shown to require the final three amino acids of ABCA1, and, when co-transfected with ABCA1 in 293 cells, $\alpha$-syntrophin stabilized ABCA1 expression and increased efflux activity. To test whether the loss of such PDZ interactions were of functional importance, we created a mutant (44) that disrupts the ability of class I PDZ domains ($\alpha$1- and $\beta$2-syntrophin and Lin7) and one does not (22–24). The interaction of $\alpha$-syntrophin and ABCA1 was shown to require the final three amino acids of ABCA1, and, when co-transfected with ABCA1 in 293 cells, $\alpha$-syntrophin stabilized ABCA1 expression and increased efflux activity. 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cells. These observations indicate that the PDZ interactions mediated by the C terminus of the protein are not essential for efflux activity. Other mutants used in our studies (e.g., Δ40 mutant and the ABCA1/A4 chimera) corroborate this finding, as they lack the PDZ-binding motif but show wild type or greater efflux activity. Although our studies have not directly assessed the in vivo role of PDZ protein interactions with the C terminus of ABCA1, Neufeld et al. have shown that an ABCA1 chimera with GFP fused at its C terminus was able to fully correct the efflux deficit in fibroblasts from a Tangier patient (34). Furthermore, the same ABCA1-GFP chimera, when expressed in the mouse liver by adenoviral infection, was able to appropriately localize to basolateral membranes of hepatocytes, efflux phospholipids, and cholesterol and increase circulating high density lipoprotein levels (35). Because classical PDZ interactions are strongly dependent upon hydrogen bonds between amino acids of the PDZ domain and the carboxylate group of the C-terminal hydrophobic group of the PDZ binding partner, such interactions are likely disrupted in the ABCA1-GFP chimera (21, 36). Thus, our in vitro data, along with the mouse expression studies, indicate that PDZ protein interactions with the C terminus of ABCA1 are not essential for the efflux function of the transporter.

The failure of the PDZ-binding motif to account for the loss of activity of the Δ46 mutant led to our discovery of a novel protein motif located 40 amino acids upstream of the C terminus. Because the Δ4 mutation did not recapitulate the Δ46 Tangier mutation, additional deletion mutants were analyzed. These studies showed that mutation of the sequences between −41 and −46 (VFVNFA) recapitulated the Δ46 efflux deficit. A search of translated databases revealed that the VFVNFA motif was strongly conserved in a subset of vertebrate ABCA class transporters. This conservation extended to the ABCA1 orthologs of both the freshwater puffer fish Tetraodon nigroviridis and the saltwater puffer fish F. rubripes. In contrast, the motif was not found in any ABC transporters from Drosophila melanogaster, Caenorhabditis elegans, Arabidopsis thaliana, and Ciona intestinalis. Thus, the lack of the motif in transporters from the urochordate C. intestinalis and lower invertebrate organisms and its presence in transporters of fish, birds, and mammals indicate that the motif likely arose early in the divergence of the vertebrate lineage. This occurred ~450 million years ago, at a time when complex closed circulatory systems first evolved (37–39). Unlike the PDZ binding motif, theVFVNFA sequence is required for ABCA1 to form a complex with apoA-I and to transfer cholesterol to the apolipoprotein. Significantly, the VFVNFA motif could not be replaced by closely homologous sequences from ABCA7. In addition, when presented on an intracellular peptide, this motif was able to inhibit ABCA1-mediated efflux to apoA-I.

Why might the VFVNFA motif, which resides on a cytoplasmic domain of ABCA1, be critical for the transporters ability to bind apoA-I and stimulate cholesterol efflux? A trivial possibility is that the motif forms a structural element that is important in the global folding of the transporter. This appears not to be the case, as all of the VFVNFA mutants were stably expressed and reached the cell surface at wild type levels, a result that would be unexpected for a misfolded protein targeted for degradation by chaperones. The possibility of a folding defect also seems remote given our observation that a single V2217L mutation inhibits ABCA1-mediated efflux to apoA-I.

A Novel ABCA1 VFVNFA Motif

FIG. 9. The ABCA1 VFVNFA motif cannot be replaced by the homologous sequences from ABCA7. Percentage of cholesterol efflux activity in 293-EBNA-T cells of the VFVNFA-VFLYFS mutant (A) and the V2217L mutant (B). Error bars represent S.D. of triplicate samples. Results are representative of two or more experiments.

FIG. 10. An intracellular peptide containing the VFVNFA motif inhibits ABCA1 efflux activity. 293-EBNA-T cells were transfected with the ABCA1 cDNA or an empty vector DNA and then labeled with [3H]cholesterol. These cells were treated with vehicle (1% ethanol) or the indicated cell-penetrating peptides (50 μM) containing the VFVNFA sequence or six alanines (AAAAAA) for 2 h prior to exposure to media containing apoA-I (10 μg/ml) or not (media). After 20 h, the percent cholesterol efflux was calculated as described in the Fig. 2 legend. Error bars represent S.D. of triplicate samples. Results are representative of two or more experiments.
interaction domain that recruits additional factors into an efflux complex. This possibility is the one we currently favor. Our finding that a peptide mimic of the VFVNFA motif inhibits ABCA-mediated cholesterol efflux, coupled with observations that other ABC transporters rely on their C termini to recruit proteins that regulate their function (41, 42), make it attractive to hypothesize that this novel motif is involved in a similar protein recruitment activity. As the same motif is present in other members of the ABCA class of transporters, we speculate that these transporters may share common protein interactors that are required for their function. Current work in our laboratory is focused on the identification of such proteins.

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ATP-binding Cassette Transporter A1 Contains a Novel C-terminal VFVNFA Motif That Is Required for Its Cholesterol Efflux and ApoA-I Binding Activities
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