Purification of ATP-binding Cassette Transporter A1 and Associated Binding Proteins Reveals the Importance of β1-Syntrophin in Cholesterol Efflux*

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ATP-binding cassette transporter A1 (ABCA1) plays a critical role in HDL cholesterol metabolism, but the mechanism by which it transports lipid across membranes is poorly understood. Because growing evidence implicates accessory proteins in this process, we developed a method by which proteins interacting with the intact transporter could be identified. cDNAs encoding wild-type ABCA1 and a mutant lacking the C-terminal PDZ binding motif of ABCA1 were transfected into 293 cells, and the expressed proteins were solubilized using detergent conditions (0.75% CHAPS, 1 mg/ml phosphatidylcholine) predicted to retain high affinity protein-protein interactions. Proteins that co-purified with ABCA1 on an antibody affinity column were identified by liquid chromatography-mass spectrometric analysis. A novel interaction with the PDZ domain of ABCA1 was identified using this approach, and this interaction was confirmed in human THP-1 macrophages and in mouse liver. Small interference RNA inhibition of β1-syntrophin expression reduced cholesterol efflux from primary skin fibroblasts by 50% while decreasing efflux 30% in bone marrow-derived macrophages. Inhibition of β1-syntrophin decreased ABCA1 protein levels, whereas overexpression of β1-syntrophin increased ABCA1 cell-surface expression and stimulated efflux to apolipoprotein A-I. These findings indicate that β1-syntrophin acts through a class-I PDZ interaction with the C terminus of ABCA1 to regulate the cellular distribution and activity of the transporter. The approach used to identify β1-syntrophin as an ABCA1-binding protein should prove useful in elucidating other protein interactions upon which ABCA1 function depends.

Maintenance of cellular cholesterol homeostasis is critical for normal human physiology, and, if disrupted, leads to a variety of pathological conditions, including cardiovascular disease (1, 2). The homeostatic mechanisms involved in regulating cellular cholesterol levels include an active efflux process in which cellular cholesterol is transferred to extracellular apolipoproteins, primarily apolipoprotein A-I (apoA-I). The physiological importance of this process is demonstrated in patients with Tangier disease, a rare genetic condition caused by loss-of-function mutations in the ABCA1 transporter that eliminate apoA-I-stimulated cholesterol efflux (3–6). Tangier disease patients have a near-absence of circulating HDL, prominent cholesterol-ester accumulations in tissue macrophages, and premature atherosclerotic vascular disease (7–11). Because HDL levels inversely correlate with cardiovascular disease, and cholesterol-engorged macrophages in the vessel wall are involved in the pathogenesis of atherosclerosis, a normally functioning ABCA1-mediated cholesterol efflux pathway is thought to play a critical role in preventing coronary artery disease (12, 13).

The ABCA1-mediated cholesterol efflux pathway appears to be tightly regulated via a complex set of mechanisms that regulate ABCA1 expression at the transcriptional and post-translational levels. In response to increased cellular cholesterol levels, ABCA1 gene transcription is activated, with the liver X receptor, a member of the nuclear hormone family of transcriptional factors, playing a key role in this induction (14, 15). Evidence for post-translational regulation includes the finding that binding of apoA-I to the transporter prolongs its half-life by inhibiting a calpain-mediated degradation pathway (16, 17). This process is associated with alterations in the phosphorylation status of ABCA1 (18, 19). Discordance between ABCA1 mRNA and protein levels, the very rapid turnover of the transporter protein, and the modulation of cholesterol efflux activity by a variety of protein kinases (including protein kinase A, protein kinase C, janus kinase 2, and protein kinase 2) all combine to suggest that the post-translational regulation of ABCA1 could play a central role in the regulation of the cellular cholesterol efflux pathway (19–25).

Our search for cellular proteins involved in regulating ABCA1 activity was initially stimulated by our identification of a Tangier patient carrying a 46-amino acid deletion of the ABCA1 C terminus (26). This mutation removes a putative PDZ protein-binding motif located in the terminal 3 residues of the cytoplasmic tail of the transporter. We originally speculated that this motif might be important for ABCA1 function, because PDZ proteins are involved in assembling protein complexes that contribute to the function of receptor and channel proteins (26). Namely for the founding members of the group (PSD-95, Dlg, ZO-1), PDZ proteins carry one or more copies of a 90-amino acid domain that binds proteins ending in several distinct consensus motifs (27). The ABCA1 C terminus (ESYG) conforms to the consensus motif (S/T-X-Y-COOH) bound by class-I PDZ proteins, and we have previously shown that the PDZ domains of both PSD-95 and Dlg-1 bind to this sequence (28). Recently, two other groups,

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‡‡1-Syntrophin in Cholesterol Efflux

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using yeast two-hybrid screens employing either the last 165 or 120 amino acids of the ABCA1 C terminus as bait, identified three PDZ-containing proteins (α1 and β2 syntrophins and Lin7) as putative interactors with the transporter (29, 30). The functional relevance of the β2-syntrophin and Lin7 interactions with ABCA1 are unclear, but α1-syntrophin was demonstrated to stabilize transporter expression and increase efflux activity in transfected cells (30).

Here we sought to develop a method in cultured human cell lines that would permit the identification of functionally important protein interactions with full-length human ABCA1. Once developed, we then planned to exploit this method to probe the structural domains within ABCA1 required for these protein interactions. Using a monoclonal antibody affinity column, we were able to purify ABCA1 from transfected 293-EBNA-T cells using detergent conditions previously used to purify a close homolog of ABCA1, ABCA4. Under these conditions, which preserved the biological activity of ABCA4, we found ABCA1 co-purified with additional proteins. To minimize the evaluation of non-specific, co-purifying proteins, and to expand our previous work showing the functional importance of the C terminus of the transporter, we also utilized a mutant ABCA1 protein lacking the C-terminal 40 amino acids. Liquid chromatography-mass spectrometry was used to identify the proteins that associated with wild-type and mutant ABCA1, permitting us to focus on the specific protein interactions lost when the last 40 amino acids of the transporter were deleted. This work led to the discovery of a novel interaction between ABCA1 and β-1 syntrophin. We further demonstrate that this interaction occurs under physiological conditions in murine liver and macrophages and that the interaction of β-1 syntrophin with ABCA1 is involved in regulating the cellular distribution and activity of the transporter.

MATERIALS AND METHODS

Reagents—M2 anti-FLAG mouse monoclonal agarose-conjugated antibody (Sigma), anti-syntrophin mouse monoclonal antibody (Clone 1351, Affinity BioReagents), anti-utrophin mouse monoclonal antibody (Vector Laboratories), and rabbit anti-cyclophilin B antibody (Santa Cruz Biotechnology) were used. The rabbit anti-ABCA1 antibody has been previously described (31), and the rabbit anti-β1-syntrophin antibody (syn251) was generously provided by Dr. Stanley Froehner (University of Washington, Seattle, WA). SMARTPool siRNA duplexes were from Dharmacon (Lafayette, CO). Radionucleotides were from PerkinElmer Life Sciences.

Cell Transfection—Full-length and C-terminally truncated (Δ40) FLAG-tagged ABCA1 cDNAs were used as previously described (28, 32). Following trypsinization, a single cell suspension of HEK293-EBNA-T cells (5.6 × 10^6 cells) was transfected with cDNAs or empty vector using Lipofectamine DNA complexes (750 μg of DNA/1.875 ml of LF-2000) in Optimem serum-free media (4.98 × 10^6 cells/ml) with gentle agitation for 20 min at 37°C. After transfection, the cells were plated into fifteen 150 mm tissue culture plates and incubated for 24 h prior to use.

Affinity Purification of FLAG-ABCA1—A 15,000 × g post-nuclear membrane pellet that contains the majority of ABCA1 was isolated from transfected cells as previously described (31). In brief, chilled cells were lifted by scraping in a hypotonic sucrose buffer (250 mM sucrose, 10 mM HEPES, pH 7.5) supplemented with a protease inhibitor mixture (Sigma), and disrupted in a Dounce with 20 strokes with a tight fitting pestle. Non-lysed cells and nuclei were removed by centrifugation (800 × g, 5 min), and the supernatant was further centrifuged at 15,000 × g for 10 min. The 15,000 × g pellet was solubilized under either stringent (1% IGEPAL CA-630, 0.25% sodium deoxycholate, 50 mM Tris pH 7.5, 150 mM NaCl) or mild detergent (0.75% CHAPS, 50 mM HEPES, pH 7.0, 140 mM NaCl, 1 mg/ml phosphatidylycholine, 10% glycerol, 3 mM MgCl2, 5 mM β-mercaptoethanol) conditions as previously described by Sun et al. (33). After clarification, the soluble material was incubated on an anti-FLAG antibody affinity column, washed extensively (40 column volumes), and eluted with a 100 μg/ml solution of competing FLAG peptide. The column eluate from FLAG-ABCA1 or mock-transfected cells was concentrated by ultrafiltration, separated on 4–20% SDS-PAGE gels and Coomassie stained to visualize eluted proteins. The amount of ABCA1 eluting from the column was assessed by Western blotting as previously described (31).

Mass Spectrometry Analysis of Proteins Co-purified with Wild-type and Δ40 ABCA1—Wild-type and Δ40 FLAG-ABCA1-expressing cells were lysed using CHAPS/PC buffer and affinity-purified as described above. Determination of protein concentration in the 4–20% SDS-PAGE gel samples was performed by staining with Sypro Orange (Molecular Probes) and quantification of fluorescent intensity of unknowns and bovine serum albumin standards using an Alpha Innotech FlourChem 8800 Imager. The amount of purified FLAG-ABCA1 was quantitated by immunoblotting using an anti-ABCA1 antibody that recognizes the N-terminal extracellular loop and was compared with a standard curve of the purified recombiant extracellular loop. Manipulations of the proteins were performed in a dual isolation BioSafety Cabinet to minimize exogenous keratin contamination. Stained gels were divided into five regions based on molecular mass (<200, 200–100, 100–65, and 50–30 kDa). Regions of the gel below 30 kDa were not analyzed due to potential contamination by the FLAG peptide. Gel pieces were placed in 1.5-ml Axygen tubes, destained with three washes of 50% methanol, 5% acetic acid, and rinsed with three alternating washes of ammonium bicarbonate (100 mM) and acetonitrile. Gel slices were dried and hydrated on ice in Promega Sequencing grade trypsin (5.5 μg/ml, 25 μl) in 50 mM ammonium bicarbonate for 15 min, an equal volume of 50 mM ammonium bicarbonate was added, and the tubes were incubated for 16 h at 37°C. The peptide solutions were extracted with two 50 mM ammonium bicarbonate washes (75 μl), two 50% acetonitrile, 0.1% formic acid washes (75 μl), and then lyophilized. The lyophilate was dissolved in 25 μl of 5% acetonitrile 0.1% formic acid. The samples were analyzed on a LCQ DECA XP plus mass spectrometer equipped with a Famos Autosampler, and Surveyor MS Pump (Thermo Electron) with split flow to deliver ~200 nL/min to the in-house packed C18 column (75 μm inner diameter × 18 cm). For each run, 10 μl of reconstituted samples was injected and separated using a gradient of 5–60% buffer B (Buffer A was water with 0.1% formic acid, Buffer B was acetonitrile with 0.1% formic acid) over the course of 90 min. In between each set of samples, standards (a mix of five Angiotensin peptides, Michrom BioResources) were analyzed to ascertain column performance and to determine any potential carryover. The LCQ was operated in the data-dependent mode with a top five configuration (one full MS scan followed by five MS/MS scans). Dynamic exclusion was set to 1 with a limit of 30 s. Peptides were identified using the Sequest software package through the Bioworks Browser 3.1. Sequential data base searches were made using the NCBI RefSeqHuman Data base using differential carbamidomethyl-modified cysteines and oxidized methionines. Peptide score cutoff values were chosen at a cross-correlation of 1.8 for singly charged ions, 2.5 for doubly charged ions, and 3.0 for triply charged ions, along with ΔCN values of 0.1 or greater, and RSP values of 1. The cross correlation values chosen for each peptide assure a high confidence match for the different charge states, whereas the ΔCN cutoff insures the uniqueness of the peptide hit. The rank score preliminary (RSP) value of 1 insures that the peptide matched the top hit in the preliminary scoring.
Immunoprecipitations and Overlay Assays—ABCA1/B1-syntrophin complexes were co-precipitated from differentiated (100 nM phorbol 12-myristate 13-acetate for 72 h) human THP-1 macrophage protein lysates or from mouse liver lysates using the anti-syntrophin monoclonal antibody. 15,000 × g pellets were isolated as described above and solubilized in TX100 buffer (1% Triton X-100, 140 mM NaCl, 3 mM MgCl₂, 10% glycerol, 50 mM HEPES, pH 7.0, and protease inhibitor mixture). Clarified supernatants were incubated with 50 μg of anti-pan-syntrophin Ab or mouse IgG overnight at 4 °C. Immunocomplexes were captured on protein G-Sepharose for 1 h at room temperature and washed three times with TX100 buffer, and precipitated proteins were analyzed by SDS-PAGE and immunoblotting using the rabbit anti-ABCA1 and anti-B1-syntrophin antibodies.

Binding of B1-syntrophin to the ABCA1 C terminus was assayed using bacterial His-tagged polypeptides representing the terminal 185 amino acids of wild-type ABCA1 or ABCA1 lacking the PDZ protein binding motif (Δ4). These polypeptides were separated by SDS-PAGE then transferred to nitrocellulose, and the membrane was then incubated with a total cell lysate (TX100 buffer) of 293-EBNA-T cells transfected with either empty vector or with the B1-syntrophin cDNA for 1 h at room temperature (280 mg of total cell protein diluted in 20 ml of blocking solution, 1× phosphate-buffered saline, 5% milk protein, 1% bovine serum albumin). After washing (1× phosphate-buffered saline, 0.1% Tween-20), binding of B1-syntrophin was detected with the anti-B1-syntrophin antibody, and the membrane was stripped and reprobed with the anti-C-terminal ABCA1 antibody to demonstrate equal loading of the two His-tagged polypeptides. The interaction of the ABCA1 C terminus with the PDZ domain of B1-syntrophin was further analyzed using a biotinylated peptide representing the final 20 amino acids of the ABCA1 C terminus. The ABCA1 peptide was used to probe an array of 34 purified GST-PDZ domain protein fusions from 24 proteins, including B1-syntrophin (Panomics TranSignal™ PDZ Domain Array IV). 15 μl of the biotin-ABCA1 peptide (50 μM) was mixed with 15 μl of avidin-horseradish peroxidase (1 mg/ml) and incubated for 30 min at 4 °C. The peptide/avidin mix was added into 5 ml of 1× blocking buffer (final ABCA1 peptide concentration 15 nM) and incubated with the array for 2 h at room temperature. After washing, binding of the peptide was detected using enhanced chemiluminescence and quantitated using the Alpha Innotech FlourChem 8800 Imager.

Determination of ABCA1 Turnover and Synthesis—Fibroblasts treated with B1-syntrophin siRNA were incubated in methionine/cysteine-free DMEM (2 mg/ml fatty acid-free bovine serum albumin) for 1 h, pulsed with a 35S-protein labeling mix for 2 h, washed twice with phosphate-buffered saline, and incubated in complete media for the indicated times. ABCA1 protein was determined by immunoprecipitation with a protein-A purified anti-ABCA1 antibody. The synthesis rate of ABCA1 was assayed in HEK293 cells transfected with the ABCA1 cDNA and empty vector, or with the ABCA1 and B1-syntrophin cDNAs incubated as above in the 35S-protein labeling mix for increasing periods of time. ABCA1 was directly quantitated by immunoprecipitation followed by phosphor imaging of the 35S-labeled ABCA1 on a STORM 860 scanner (GE Healthcare).

The measurement of ABCA1 cell-surface expression by the radioimmunodetection of the FLAG epitope and assessment of the distribution of GFP-ABCA1 by confocal microscopy were performed as previously described (31, 32).

Cholesterol Efflux Assay in siRNA-transfected 1056 Human Fibroblasts and Mouse Bone Marrow-derived Macrophages—Pools of four siRNAs for each targeted gene, as well as a control pool of four duplexes with no significant homology to any known human transcripts were designed by a SMART selection algorithm (see supplemental Table II). Primary 1056 human fibroblasts (ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum. For transfection, cells were treated with 50 pmol of siRNA and 2 μl of Dharmafect 4 (Dharmacon). 24 h after transfection, cholesterol efflux was measured as we previously described (31). In brief, cells were incubated with DMEM (10% fetal bovine serum) containing 10 μg/ml cholesterol, 0.5 μCi/ml [3H]cholesterol for 24 h, and the medium was replaced with DMEM containing 2 mg/ml fatty acid-free bovine serum albumin for another 24 h. Cells were then incubated with DMEM (2 mg/ml fatty acid-free bovine serum albumin) with or without 10 μg/ml apoA-I for 20 h. Media was collected, cleared of cellular debris by an 800 × g spin for 10 min, and the cell layers were dissolved in 0.1 N NaOH, and percent cholesterol efflux (media cpm/(cpm of media + cell associated cpm) × 100) was calculated by scintillation counting. The effectiveness of siRNA inhibition was measured by immunoblotting on a parallel set of unlabeled cells. For mouse bone marrow macrophages, marrow cells were isolated from C57/BL6 mice and cultured for 6 days in L29-conditioned medium as described previously (34). BMDM in 24-well plates were treated with 5 pmol of siRNA and 1 μl of Dharmafect 4 in each well. Following 24 h of transfection, cholesterol efflux was measured as described above.

RNA Extraction and Quantitative Real-time PCR—Total RNA was prepared from siRNA transfected fibroblasts using TRIzol reagent (Invitrogen). RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase and random primers (Promega), and cDNAs were used as the template in quantitative real-time amplifications using the Bio-Rad iCycler. Each reaction (50 μl) contained 0.3 μM primers, 25 μl of 2× Bio-Rad SYBR green SuperMix and 2 μl of template, and was amplified by 40 cycles of denaturation (94 °C and 30 s), annealing (60 °C and 30 s), and extension (72 °C and 30 s). Standards were prepared from full-length cDNAs serially diluted from 5 ng/μl to 5 fg/μl. Transcript levels were normalized to the amount of β-actin transcript and were expressed as a percentage of the control siRNA sample.

Statistical Analysis—An analysis of variance was used to determine the significance of siRNA knockdown on apoA-I-dependent efflux activity. The data set consisted of four independent efflux experiments with triplicate sampling comparing the negative control siRNA (n = 12), the duplex pool targeting utrophin (n = 9), the duplex pool targeting B1-syntrophin (n = 12), and the duplex pool targeting B2-syntrophin (n = 9). A factorial comparison was done comparing apoA-I-dependent efflux versus the siRNA treatment groups.

RESULTS

Affinity Purification of ABCA1—To purify adequate amounts of protein for subsequent liquid chromatography-MS analysis, multiple steps in the transfection and purification protocol required optimization. We found that the use of lipophilic transfection reagents immediately after lifting cells with trypsin, when combined with a ratio of 3.2 μg of DNA per 2.4 × 10⁶ cells, reliably produced transfection efficiencies of ~90%, while minimizing cellular toxicity (Fig. 1A). This transfection efficiency induced a correspondingly robust cholesterol efflux activity in 293-EBNA-T cells, with 18.18 ± 1.74% of cellular cholesterol transferred to apoA-I after 20 h of incubation (Fig. 1B). These findings also demonstrated that the FLAG-tagged ABCA1 retained wild-type cholesterol efflux activity, as we have previously reported (32).

As our highest affinity polyclonal antibody to ABCA1 recognizes the C terminus of the protein, and we intended to delete that region to identify proteins interacting with it, we chose not to make an immunoaffinity column with antibodies directed at wild-type ABCA1. Instead, the FLAG-tagged ABCA1 permitted us to utilize an anti-FLAG mono-
clonal antibody, high affinity purification column. Two methods were used to solubilize the proteins from transfected cells prior to application on the affinity column. The first method employed stringent detergent conditions (1% IGEPAL CA-630, 0.25% sodium deoxycholate, 50 mM Tris, pH 7.5, 150 mM NaCl) intended to disrupt any protein interactions and provide an assessment of the ABCA1 purification achieved with the column. Fig. 2A shows that under these conditions, microgram quantities of transporter protein can be isolated. Although small amounts of

FIGURE 1. Optimization of ABCA1 transfection in 293-EBNA-T cells induces a robust efflux activity. A, transfection of 293-EBNA-T cells with empty vector or a GFP-ABCA1 cDNA after trypsinization resulted in 90% transfection efficiency as analyzed by FACS analysis. This optimization of transfection resulted in the induction of a robust cholesterol efflux activity in 293-EBNA-T cells transfected with the FLAG-ABCA1 cDNA (B). Error bars represent standard deviations of triplicate samples, open bars (media), closed bars (apoA-I).
contaminating protein may be present at levels below the limit of detection by Coomassie staining, this solubilization procedure clearly yielded highly purified ABCA1 protein. When less stringent detergent conditions (0.75% CHAPS, 50 mM HEPES, 140 mM NaCl, 1 mg/ml phosphatidylcholine, and 10% glycerol) that had previously been used to purify biochemically active ABCA4 were employed (33), a large number of additional proteins eluted from the column (Fig. 2B).

Although the elution of ABCA1 and the proteins bound to it was accomplished using a competitive FLAG peptide, the number and diversity of proteins detected by the Coomassie stain (Fig. 2B) suggested that some of these co-eluting proteins might not represent specific interactions with ABCA1. To help distinguish specific from nonspecific interactions, we chose to employ a mutant form of ABCA1 lacking the C-terminal 40 amino acids (Δ40). This mutant was chosen because our previous work had shown it was the largest C-terminal deletion we could create that retained cell-surface expression and functional activity in transfected cells (28). We reasoned that nonspecific interactions with ABCA1 would be retained using this mutant while those protein interactions specifically dependent on the C terminus would be lost. Because the last three amino acids of the ABCA1 C terminus represent a putative PDZ binding motif, the use of this mutant was expected to specifically disrupt the interactions of the transporter with PDZ domain-containing proteins, some of which have been described by other investigators using yeast two-hybrid technology (29, 30).

**Mass Spectrometry Analysis of Proteins That Co-purify with ABCA1 and the Δ40 Mutant**—After elution of proteins retained on the affinity column and their subsequent SDS-PAGE separation, the amount of total eluted protein was quantitated using fluorescent imaging (Fig. 2C) and their subsequent SDS-PAGE separation, the amount of total eluted protein was quantitated. Using quantitative immunoblotting we estimated that there was ~30 μg each of ABCA1 and the Δ40 mutant present in the samples (data not shown). The stained gels were divided into five regions based on molecular mass (<200, 200–100, 100–65, and 50–30 kDa). Gel pieces were fixed, trypsinized, and extracted, and the resulting samples were analyzed on a LCQ DECA XP plus mass spectrometer as described under “Materials and Methods.” The proteins identified in this experiment were analyzed by sorting them according to functional annotations. Redundant identifications of a single protein from the samples of different molecular weight regions of the gel were consolidated. Next, trypsin, albumin, and keratin identifications, likely representing both exogenous and endogenous nonspecific contaminants, were deleted from the set of protein identities. Following these filtering steps, 275 and 279 unique proteins were identified for wild-type ABCA1 and Δ40 mutant, respectively. 76 proteins found in the wild-type sample were selectively lost in the Δ40 mutant (supplemental Table I). TABLE ONE shows the PDZ proteins which were found to be differentially bound to the wild-type ABCA1 and Δ40 mutant, as well as examples of proteins that bound both proteins equally. Although the proteins that bound similarly to the wild-type and mutant transporters could represent physiologically relevant protein interactions that are not dependent on the C terminus of the protein, they could also reflect nonspecific interactions. In the studies reported herein, we chose to concentrate on exploring the functional importance of the PDZ protein interactions identified by the column chromatography work.

Within the set of proteins that differentially bound to wild-type ABCA1 were both α1- and β2-syntrophin, PDZ proteins that have been shown to interact with the ABCA1 C terminus by yeast two-hybrid screens (29, 30). The confirmation of these previously reported interactions provided evidence for the utility of our purification method. Utrophin, a large scaffolding molecule that interacts with the syntrophins and links macromolecular complexes to the actin cytoskeleton, was also selectively lost in the Δ40 preparation, consistent with the previous finding of Buechler et al. (29) that utrophin could be immunoprecipitated from macrophages in a complex containing ABCA1. In addition to confirming the previously reported PDZ interactions with ABCA1, we also found a novel interaction involving another member of the syntrophin family, β1-syntrophin. To confirm the mass spectrometry identification of the PDZ interactors, immunoblots of the proteins co-eluting with wild-type and Δ40 ABCA1 proteins were performed. Full-length utrophin and β1-syntrophin were clearly present in the eluate from the wild-type ABCA1 column (Fig. 2C). Neither protein was detected by immunoblot in the Δ40 mutant sample, despite equivalent amounts of the mutant and wild-type transporter eluting from the column (Fig. 2C, right lanes). An antibody recognizing multiple members of the syntrophin family (pan-syntrophin) further confirmed the presence of syntrophins only in eluate taken from the wild-type ABCA1 column (Fig. 2C). Because the pan-syntrophin antibody does not detect human β1-syntrophin in Western blots the signal from this antibody represents a combination of the α1- and β2-syntrophins. These results confirmed the accuracy of the mass spectrometry screen to identify PDZ protein interactions of potential functional importance.

**β1-Syntrophin Interacts with ABCA1 in Vivo and the Interaction Is Dependent on the PDZ Binding Motif of ABCA1 C Terminus**—The interaction of β1-syntrophin with ABCA1 identified in our screen was of interest for several reasons. First, it had not previously been identified in published yeast two-hybrid screens using the ABCA1 C terminus as bait, and, secondly, among the syntrophins, its expression is uniquely high in the liver, a tissue where ABCA1 efflux function is critical for the maintenance of circulating HDL levels (37). Tissue macrophages represent another important cell type where ABCA1-mediated cholesterol

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**TABLE ONE**

| Co-purified protein        | No. of peptide matches WT | No. of peptide matches Δ40 | Calculated molecular mass kDa | Coverage % WT | Coverage % Δ40 |
|---------------------------|----------------------------|----------------------------|-------------------------------|---------------|---------------|
| Utrophin                  | 47                         | 394.5                      | 18.6                          |
| Basic β2-syntrophin isoform a | 13                        | 57.9                       | 27.0                          |
| Basic β1-syntrophin       | 4                          | 58.1                       | 13.2                          |
| Dystrobrevin, β isoform 1  | 3                          | 71.3                       | 10.5                          |
| Acidic α1-syntrophin      | 1                          | 58.1                       | 3.2                           |
| ATP synthase β subunit    | 41                         | 56.6                       | 71.1                          |
| Calnexin                  | 25                         | 67.6                       | 34.1                          |

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K-I. Okuhira, M. L. Fitzgerald, and M. W. Freeman, unpublished data.
Proteomic Analysis of ABCA1 Interactions

efflux is thought to play a critical role in lipid homeostasis, but the expression of β1-syntrophin in macrophages has not been reported. Therefore, to explore β1-syntrophin-ABCA1 interactions at physiological expression levels of both proteins in liver and macrophages, we performed immunoprecipitations using the pan-syntrophin antibody. Although this antibody is an ineffective tool for recognizing β1-syntrophin by Western blotting, it is capable of immunoprecipitating both human and mouse β1-syntrophin (36) (and data not shown). Lysates of human PMA differentiated THP-1 macrophages and mouse liver were generated and incubated with this antibody. After separation by SDS-PAGE and transfer to nitrocellulose, immunoblots of the macrophage (Fig. 2D) and liver (Fig. 2E) immunoprecipitates clearly demonstrated the presence of ABCA1. β1-Syntrophin was also detected in the human macrophage samples (Fig. 2D), but the lack of cross-reactivity of the anti-human β1-syntrophin antibody to the murine homolog precluded detection of the latter protein. However, it is likely that co-precipitation of ABCA1 from murine liver using the pan-syntrophin antibody was due to the pan-syntrophin antibody’s recognition of murine β1-syntrophin, because α1-syntrophins are expressed at very low levels in this tissue and β1-syntrophin protein expression strongly predominates over that of β2-syntrophin (36). A more definitive result for murine liver awaits the generation of species-specific immunological reagents to the syntrophin proteins. These results indicate that ABCA1 and β1-syntrophin form a complex at physiological concentrations of each protein in the cells and tissues where ABCA1 efflux activity has been shown to be critical for the maintenance of HDL levels and the prevention of atherosclerosis.

Having demonstrated a physiological interaction between ABCA1 and β1-syntrophin, we next sought to determine if this interaction was dependent on the presence of the PDZ binding motif encoded in the last four amino acids of the ABCA1 C terminus. We performed an overlay assay, using purified His-tagged bacterial polypeptides representing the last 20 amino acids of the wild-type ABCA1 C terminus (wt) or the C terminus lacking the PDZ binding motif (Δ4) (Fig. 3A, top). After separating the polypeptides by SDS-PAGE and transferring them to nitrocellulose, the resulting membranes were incubated with a lysate from 293-EBNA-T cells that had been transfected with a human β1-syntrophin cDNA or a lysate from cells transfected with empty vector. As shown in Fig. 3A (middle), the expressed β1-syntrophin bound to the wild-type ABCA1 polypeptide but not to the polypeptide lacking the PDZ binding motif. This result indicated that the interaction of β1-syntrophin with ABCA1 was dependent on the C-terminal PDZ binding motif. However, these data did not establish that the interaction between β1-syntrophin and ABCA1 was direct, as other proteins present in the lysate could mediate the binding interaction. We therefore sought further evidence that β1-syntrophin alone could directly bind the ABCA1 C terminus.

A biotinylated peptide, representing the last 20 amino acids of the ABCA1 C terminus, was generated and used to probe a protein array of 34 PDZ domains from 24 PDZ proteins, including the PDZ domain of β1-syntrophin alone can bind the ABCA1 C terminus. A biotinylated 20-mer peptide representing the ABCA1 C terminus was used to probe an array of 34 purified PDZ domains from 24 proteins spotted in duplicate. Bound peptide was detected with avidin-horseradish peroxidase. C, a schematic of the array indicating which protein (top line), and for proteins with multiple PDZ domains which PDZ domain (bottom line) were spotted in the quadrants. D5 and 6 (SNB1) contains the PDZ domain of β1-syntrophin. TABLE TWO lists the fluorescent intensities of the bound ABCA1 peptide for each quadrant.

FIGURE 3. β1-Syntrophin binding of the ABCA1 C terminus depends on the Class I PDZ motif and the PDZ domain of β1-syntrophin can directly interact with the ABCA1 C terminus. A, an overlay assay shows the β1-syntrophin interaction requires only the PDZ motif encoded in the last four amino acids of the ABCA1 C terminus. Purified His-tagged polypeptides representing the wild-type ABCA1 C terminus with (WT) and without the PDZ motif (Δ4) were separated by SDS-PAGE (top) and transferred to nitrocellulose. The membrane was incubated with lysates from 293-EBNA-T cells transfected with empty vector (mock) or with a β1-syntrophin cDNA. β1-Syntrophin binding was detected by immunoblotting (middle) and the membrane was stripped and reprobed for ABCA1 demonstrating equivalent levels of the two peptides on the membrane (bottom). B, the PDZ domain of β1-syntrophin alone can bind the ABCA1 C terminus. A biotinylated 20-mer peptide representing the ABCA1 C terminus was used to probe an array of 34 purified PDZ domains from 24 proteins spotted in duplicate. Bound peptide was detected with avidin-horseradish peroxidase. C, a schematic of the array indicating which protein (top line), and for proteins with multiple PDZ domains which PDZ domain (bottom line) were spotted in the quadrants. D5 and 6 (SNB1) contains the PDZ domain of β1-syntrophin. TABLE TWO lists the fluorescent intensities of the bound ABCA1 peptide for each quadrant.
It was further established that the fibroblasts expressed utrophin, β1-syntrophin, and a significant amount of β2-syntrophin and that we could significantly reduce the expression of each of these proteins with siRNA duplexes targeting their respective mRNAs (Fig. 4, B and C) (supplemental Table II shows the sequences of the siRNA duplexes). Knockdown of β1-syntrophin induced a small increase in pan-syntrophin signal, likely representing increased β2-syntrophin protein, because β2-syntrophin mRNA levels were elevated in cells transfected with the β1-syntrophin siRNAs (Fig. 4C). Targeting utrophin expression produced a more complex phenotype that involve repressing β1-syntrophin protein and message levels, as well as total syntrophin levels. Although complex, the effect of utrophin siRNA on syntrophin expression appears specific, because β-actin protein and mRNA levels were unchanged.

Having found that fibroblasts express the syntrophins and utrophin and confirming the efficacy of siRNA duplexes to reduce their cognate protein expression, we next tested the impact of the siRNAs on apoA-I-stimulated, ABCA1-dependent cholesterol efflux. Cells receiving duplexes targeting β1-syntrophin reduced efflux by 50%, compared with cells transfected with a non-targeted control pool of siRNAs (Fig. 4D). The siRNAs targeting utrophin reduced efflux by 40%, whereas the β2-syntrophin duplexes reduced efflux by a more modest 20% (Fig.

### Table Two

**PDZ domains that bound the ABCA1 C-terminal peptide**

| Position | Average fluorescence intensity ($\times 10^5$) | Protein                                                                 | Accession no. |
|----------|---------------------------------------------|------------------------------------------------------------------------|---------------|
| A9, A10  | 201.6                                       | Multiple PDZ domain protein, domain 13                                 | NP_003820     |
| D5, D6   | 197.8                                       | β1-Syntrophin; tax interaction protein 43; dystrophin-associated protein A1, 59 kDa, basic component 1 | NP_066301     |
| A13, A14 | 197.4                                       | Synapse-associated protein 102; neuroendocrine-dlg; discs, large homolog 3, domain 2 | NP_066943     |
| C13, C14 | 197.0                                       | Rho guanine exchange factor (GEF) 11; glutamate transporter EAAT4-associated protein 48; KIAA0380 protein | NP_055599     |
| D7, D8   | 196.9                                       | Acidic 1α-syntrophin; dystrophin-associated protein A1, 59 kDa, acidic component; pro-TGF-α cytoplasmic domain-interacting protein 1 | NP_003089     |
| C15, C16 | 173.0                                       | Rho guanine exchange factor (GEF) 12; leukemia-associated GEF; similar to mouse Lsc oncogene | NP_056128     |
| A15, A16 | 161.5                                       | Synapse-associated protein 102; neuroendocrine-dlg; discs, large homolog 3, domain 3 | NP_066943     |
| C7, C8   | 156.0                                       | Lin-7 homolog C                                                        | NP_060832     |
| B9, B10  | 136.2                                       | Channel-associated protein of synapse-110 (Chapsyn-110), domain 3       | NP_001355     |
| A5, A6   | 132.6                                       | Multiple PDZ domain protein, domain 2                                   | NP_003820     |
| C9, C10  | 50.3                                        | Lin-7 homolog B                                                        | NP_071448     |
| C11, C12 | 14.8                                        | Lin-7 homolog A                                                        | NP_004655     |
| A7, A8   | 4.0                                          | Multiple PDZ domain protein, domain 3                                   | NP_003820     |
| A11, A12 | 2.9                                          | Multiple PDZ domain protein, domain 1                                   | NP_003820     |
| D9, D10  | 2.4                                          | Somatostatin receptor-interacting protein; SH3 and multiple ankyrin repeat domains 1 | NP_057232     |
| B11, B12 | 1.6                                          | Partitioning defective-6 homolog β, domain 3                            | BAB40756      |
| B7, B8   | 1.4                                          | Discs, large homolog 5, domain 2                                        | NP_004738     |
| D3, D4   | 1.1                                          | PDZ domain containing 1, domain 2                                       | NP_002605     |
| B13, B14 | 1.0                                          | LIM domain kinase 1 isoform 1; LIM motif-containing protein kinase      | NP_002305     |
| D13, D14 | 1.0                                          | Golgi-associated and coiled-coil motif-containing protein; CFTR-associated PDZ/ coiled-coil domain binding partner for the Rho family GTPase TC10; fused in glloblastoma; Golgi-associated PDZ and coiled-coil motif-containing protein | NP_065132     |
| B15, B16 | 0.9                                          | LIM domain only 7 isoform α; KIAA0858 protein                           | NP_005349     |
| C1, C2   | 0.8                                          | LIM protein RIL (Reversion-induced LIM protein)                          | NP_003678     |
| C5, C6   | 0.7                                          | T-cell lymphoma invasion and metastasis 1                               | NP_003244     |
| D15, D16 | 0.6                                          | Rap guanine nucleotide exchange factor; PDZ domain-containing guanine nucleotide exchange factor 1 | NP_057424     |
| B5, B6   | 0.5                                          | Discs, large homolog 5, domain 3                                        | NP_004738     |
| E1, E2   | 0.4                                          | Regulating synaptic membrane exocytosis 2; RAB3-interacting protein 3; KIAA0751 protein | NP_055492     |
| A3, A4   | 0.4                                          | Multiple PDZ domain protein, domain 12                                  | NP_003820     |
| D11, D12 | 0.4                                          | Membrane protein, palmitoylated 6; protein associated with Lin7 2; VELI-associated MAGUK 1; MAGUK protein p25T | NP_057531     |
| C3, C4   | 0.3                                          | α-Actinin-2-associated LIM protein; enigma homolog                      | NP_055291     |
| B3, B4   | 0.2                                          | Discs, large homolog 5, domain 4                                        | NP_004738     |
| D1, D2   | 0.1                                          | PDZ domain containing 1, domain 1                                       | NP_002605     |
| A1, A2   | 0.1                                          | Multiple PDZ domain protein, domain 6                                   | NP_003820     |
| B1, B2   | 0.1                                          | Discs, large homolog 5, domain 1                                        | NP_004738     |
| E3, E4   | 204.5                                        | PDZ domain-positive control for Kvl 4 ligand                            | N/A           |
| E5, E6   | 0.7                                          | Glutathione S-transferase                                               | N/A           |
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4D). An analysis of variance of four independent experiments showed the reductions in efflux caused by the siRNAs targeting β1-syntrophin and utrophin to be statistically significant (p = 0.0032 and p = 0.017, respectively). The β2-syntrophin knockdown effect, although modest, may also be significant but failed to meet our threshold p value (p = 0.055). We next sought to confirm these results in macrophages. Preliminary studies showed siRNAs were poorly transfected into THP-1 cells, but bone marrow-derived mouse macrophages were efficiently transfected. Therefore, we tested the effect of β1-syntrophin and utrophin knockdown in these cells. Again, knockdown of β1-syntrophin expression significantly reduced apoA-I-stimulated cholesterol efflux by 30% (p = 0.05). Utrophin inhibition decreased macrophage cholesterol efflux by only 20%, but this effect was not statistically significant (Fig. 4E). Thus, at physiological levels of ABCA1 and β1-syntrophin expression, loss of β1-syntrophin expression has a significant impact on cholesterol efflux in both fibroblasts and macrophages.

Inhibiting β1-Syntrophin Expression Decreases ABCA1 Levels by Accelerating Degradation of Newly Synthesized Protein—To explore the mechanism by which loss of β1-syntrophin expression affects ABCA1 efflux activity, ABCA1 expression levels were assayed. Immunoblots of ABCA1 protein were performed on lysates of fibroblasts in which expression of utrophin and the syntrophins had been inhibited by siRNAs. These experiments demonstrated that inhibition of β1-syntrophin reduced ABCA1 expression by >50%, whereas utrophin or β2-syntrophin inhibition reduced ABCA1 levels by ~20% (Fig. 5A). Reverse transcription-PCR analysis of ABCA1 mRNA levels under these conditions showed the reduction in ABCA1 protein caused by β1-syntrophin knockdown was not explained by a transcriptional effect (Fig. 5B). To determine whether loss of ABCA1-β1-syntrophin interactions might stimulate degradation of the transporter, the half-life of ABCA1 protein was measured in fibroblasts transfected with β1-syntrophin siRNA or the non-targeting, control siRNA. After a 2-h pulse of [35S]methionine, the transfected cells were washed and further incubated in media without label for the indicated times. The amount of radioactivity incorporated into ABCA1 was then determined by immunoprecipitation. As expected, the amount of labeled ABCA1 recovered in the precipitates from the β1-syntrophin siRNA-treated cells was consistently less than that of the control treated cells, confirming that the loss of β1-syntrophin expression reduced ABCA1 protein levels (Fig. 5C). Quantification of the immunoprecipitates indicated that the loss of β1-syntrophin had the largest impact on accelerating the decay of ABCA1 protein at the early time points, when the pool of newly synthesized protein was the greatest.
Co-expression of β1-Syntrophin Clusters ABCA1, Protecting It from Degradation and Increases Cell-surface Expression and Efflux Activity—To further explore the impact of β1-syntrophin interactions with ABCA1, we tested the effect of co-expression of the two proteins on ABCA1 protein levels and efflux activity in transfected 293 cells. Co-transfection of β1-syntrophin with wild-type ABCA1, but not with the ABCA1-Δ4 mutant, significantly increased efflux activity (Fig. 6A). Consistent with the siRNA results, co-transfection of β1-syntrophin increased the total level of ABCA1 protein as well as cell surface ABCA1 (Fig. 6, B and C). Because the pulse-chase experiment in fibroblasts had indicated that loss of β1-syntrophin activity affected the stability of newly synthesized ABCA1, we sought to determine if β1-syntrophin co-expression could influence the translation rate or degradation of newly synthesized protein. 293 cells expressing ABCA1 alone or ABCA1 and β1-syntrophin were pulsed for increasing times with [35S]methionine and the amount of synthesized ABCA1 was assessed by immunoprecipitation (Fig. 6D). The initial synthesis rate of ABCA1 appeared to be independent of the β1-syntrophin transfection status of the cells. In cells not transfected with β1-syntrophin, however, the amount of ABCA1 rapidly reached a plateau, whereas newly synthesized ABCA1 continued to accumulate for over 180 min in the cells transfected with the syntrophin, indicating the transporter was being protected from degradation. As PDZ proteins can alter the trafficking of membrane proteins by clustering them into macromolecular complexes, confocal microscopy was used to compare the localization of GFP-ABCA1 in the presence or absence of co-expressed β1-syntrophin (Fig. 7). Co-expression of β1-syntrophin increased the overall fluorescence of the transporter and enhanced its expression at the cell surface. This effect was not seen in cells transfected with β1-syntrophin and either the GFP-ABCA1-Δ4 or Δ40 mutants. Moreover, β1-syntrophin appeared to induce a clustering of GFP-ABCA1 proteins, as evidenced by an increased number of fluorescent puncta of larger size and intensity at or
In additional to β1-syntrophin, α1- and β2-syntrophin were also identified in the mass spectrometry screen as ABCA1-interacting proteins. Thus, our approach has confirmed recently published studies that employed yeast two-hybrid screens using either the last 165 or 120 amino acids of the ABCA1 C terminus as bait (29, 30, 35). These screens isolated three proteins containing PDZ domains (α1- and β2-syntrophins, and Lin7) and one non-PDZ protein, the Fas-associated death domain protein from human liver and bone marrow libraries. Although the interaction with α1-syntrophin was demonstrated to have functional significance in transfected 293 cells, the Lin7 interaction was found not to influence ABCA1 activity (30). No functional data on ABCA1 activity was reported for β2-syntrophin. The use of siRNAs in normal human skin fibroblasts permitted us to examine whether loss of β1- and β2-syntrophin interactions would have a functional impact on ABCA1 activity in a cellular environment in which the proteins were expressed at physiological levels. This work demonstrated that siRNA inhibition of β1-syntrophin significantly reduced ABCA1 activity >50%, whereas inhibition of β2-syntrophin reduced ABCA1 activity to a lesser extent. Because macrophages must ingest large amounts of lipid and cholesterol, ABCA1 efflux activity is critical for their ability to maintain cholesterol homeostasis. Importantly, we were able to demonstrate the existence of ABCA1-β1-syntrophin complexes in human THP-1 macrophages. Moreover, siRNA inhibition of β1-syntrophin significantly reduced ABCA1 efflux in mouse bone marrow-derived macrophages. Although these findings are consistent with a greater functional role for β1-syntrophin in cholesterol efflux, it is possible that small differences in the effectiveness of the inhibition of protein activity could account for the lesser effects seen with the loss of β2-syntrophin activity. Furthermore, it may be that isoform specific syntrophin interactions with ABCA1 may vary in their importance depending upon the cellular environment. This is suggested by the distinct tissue distribution displayed by the various syntrophins. α1-Syntrophin expression is largely restricted to heart, brain, and muscle, whereas β1-syntrophin is uniquely expressed at high levels in the liver. Expression of β2-syntrophin is highest in the intestine. Thus, in different tissues, these syntrophin interactions may vary in importance. Given the importance of hepatic ABCA1 expression in maintaining circulating HDL levels (15, 37, 40), the β1 syntrophin activity in this tissue is of considerable interest. Future studies will be required to further elucidate the role of β1-syntrophin in maintaining ABCA1 expression and efflux activity in hepatic tissue.

**DISCUSSION**

In this report, we report an affinity purification method for ABCA1. By varying detergent conditions, it was possible to isolate the transporter either as a relatively pure protein or in conjunction with co-purifying proteins. Using liquid chromatography coupled to mass spectrometry, co-purifying proteins were identified whose interaction with ABCA1 was dependent on the presence of the C-terminal 40 amino acids of the transporter. This screen identified a novel interaction between ABCA1 and β1-syntrophin, a PDZ protein. ABCA1-β1-syntrophin complexes were found in human THP-1 macrophages and in the mouse liver. The PDZ domain of β1-syntrophin can directly bind the ABCA1 C terminus, and the interaction of ABCA1 and β1-syntrophin requires the conserved terminal amino acids of ABCA1 that encode a class-I PDZ protein binding motif. Knockdown of β1-syntrophin expression by siRNAs in primary human fibroblasts or bone marrow-derived mouse macrophages significantly inhibited ABCA1-dependent efflux. Conversely, increased expression of β1-syntrophin-stimulated efflux, altered the cellular distribution of ABCA1, and protected the transporter from degradation. In composite these results indicate the ABCA1/β1-syntrophin interaction plays an important role in regulating the apoA-I-dependent efflux activity of the transporter in tissues critical to its physiological function.
Our mass spectrometry screen also identified utrophin and β-dystrobrevin as ABCA1-interacting proteins whose binding depended upon the C terminus of the transporter. These are dystrophin-related proteins that can bind the syntrophins and whose activity has been studied largely in the context of muscle cell function. At the neuromuscular junction, utrophin, and β-dystrobrevin, along with dystrophin, form a large macromolecular complex that scaffolds membrane proteins and links them to the cytoskeleton. However, utrophin and β-dystrobrevin are more widely expressed than dystrophin and they have been suggested to have additional functions, including the organization of signaling complexes. Indeed, fibroblasts express utrophin and when we inhibited its expression, ABCA1 efflux was significantly reduced. The mechanism of this effect was complicated, as the loss of utrophin expression also significantly reduced expression of both β1- and β2-syntrophin. Why knockdown of utrophin in bone marrow-derived macrophages had a less significant impact on the syntrophins is an issue presently under investigation. It may be that macrophages express other proteins, like β-dystrobrevin, that can compensate for the loss of utrophin expression.

Why are β1-syntrophin-ABCA1 interactions important for efflux? We found that blocking these interactions reduced ABCA1 expression and accelerated its degradation, whereas increasing β1-syntrophin levels increased ABCA1 protein by stabilizing newly translated protein. This effect was associated with an increased clustering of the transporter. These clusters were found at internal sites and at or near the cell surface. These results suggest a model, presented in Fig. 8, where interactions of ABCA1, the syntrophins, and likely utrophin result in the assembly of the transporter into a multiprotein complex that affects ABCA1 localization and lipid transfer activity. The highly conserved C terminus of ABCA1 is shown as binding the syntrophins based on the data presented in this study and others cited above. ABCA1-syntrophin-utrophin interactions allow for linkage to the actin cytoskeleton. Recent work from our laboratory showing that another C-terminal conserved motif, the VFVNF motif, is required for ABCA1 function suggests that another, as yet unidentified protein, may also participate in forming this C-terminal complex (28). As the combined deletion of both the PDZ binding motif and the VFVNF motif block the trafficking of ABCA1 to the plasma membrane, it is likely that the protein interactions at the transporter’s C terminus are critical for the appropriate localization of ABCA1 within the cell.

The ability to purify microgram quantities of ABCA1 and analyze the transporter’s structural requirements for interactions with co-purifying proteins by mass spectrometry should help in further elucidating the complex cell biology that underlies the efflux mechanism of ABCA1. Because other members of the ABCA family of proteins may share molecular mechanisms of lipid transport, we think the approach outlined in this report may be of more general value in clarifying the structure-function relationships of this important class of ABC transporters.

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