Molecular and Functional Interaction of the ATP-binding Cassette Transporter A1 with Fas-associated Death Domain Protein*

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ATP-binding cassette transporter A1 (ABCA1) is a major regulator of cellular cholesterol and phospholipid homeostasis. Its function has not been fully characterized and may depend on the association with additional proteins. To identify ABCA1-interacting proteins a human liver yeast two-hybrid library was screened with the 144 C-terminal amino acids of ABCA1. Fas-associated death domain protein (FADD) was identified to bind to ABCA1, and this interaction was confirmed by pull-down assays and co-immunoprecipitations. Recombinant expression of a dominant negative form of FADD or the C terminus of ABCA1 in the human hepatoma cell line HepG2 markedly reduced the transfer of phospholipids to apoA-I. This indicates that the binding of additional proteins, one of them being full-length FADD, is required for ABCA1 function. The association of FADD with ABCA1 provides an unexpected link between high density lipoprotein metabolism and an adaptor molecule mainly described in death receptor signal transduction.

Patients with HDL deficiency suffer from massive accumulation of cholesteryl esters in many tissues and a variety of clinical phenotypes like hepatosplenomegaly, atherosclerosis, and peripheral neuropathy (1). Mutations in the ATP-binding cassette transporter A1 (ABCA1) are the underlying cause of Tangier disease (TD), and specific apolipoprotein A-I (apoA-I)-inducible phospholipid efflux is severely impaired in these patients (2–4). ABCA1 facilitates the transfer of phospholipids to apoA-I, thus generating an acceptor particle for reverse cholesterol transport (5). ABCA1 expressed in Spodoptera frugiperda 9 cells binds ATP but has no measurable ATPase activity, demonstrating that ABCA1 is not an active transporter but may function as a regulator protein analogous to the cystic fibrosis transmembrane conductance regulator and the sulfonlurea receptor 1 (6). Therefore ABCA1-mediated lipid efflux may depend on multiple, yet uncharacterized, associated proteins. Several proteins have been described to interact with ABCA1 (7), and three ABCA1-interacting proteins have been analyzed in more detail, namely apoA-I, Cdc42, and β2-syntrophin. ApoA-I was co-immunoprecipitated with ABCA1, and functional ABCA1 was required for the specific binding of apoA-I to the cell surface (5). In addition, ABCA1 was co-immunoprecipitated with Cdc42, a GTPase that binds to numerous effector proteins controlling cell polarity, cytoskeletal remodeling, and vesicular transport (8). The association of ABCA1 with Cdc42 may explain the altered actin cytoskeleton in cells overexpressing ABCA1 and suggests that ABCA1 regulates actin organization through Cdc42 (9, 10). Overexpression of Cdc42 increases apoA-I-dependent cholesterol efflux, whereas the expression of the dominant negative form of Cdc42 decreases lipid efflux (11). In addition, β2-syntrophin was found to interact with the C-terminal five amino acids of ABCA1, and utrophin, known to couple β2-syntrophin and its PDZ ligands to the F-actin cytoskeleton, was identified as a constituent of this complex (7).

To further elucidate the multifaceted and complex role of ABCA1, it is important to identify all interacting proteins. Therefore the C-terminal 144 amino acids of ABCA1 were used as bait to screen a human liver cDNA library by the yeast two-hybrid system. The C-terminal region of Fas-associated death domain protein (FADD) was identified to interact with ABCA1, and the association of FADD and ABCA1 was verified in mammalian cells by pull-down assays and co-immunoprecipitation. A dominant negative form of FADD markedly reduced apoA-I-dependent phospholipid efflux further supporting the biological relevance of this interaction.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Fibroblasts were cultivated in Dulbecco's modified Eagle's medium, 10% FCS in a 5% CO2 atmosphere. Meg-01 and HepG2 cells were grown in RPMI medium containing 10% FCS in a 5% CO2 atmosphere. Cells were transfected with the plasmids using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions, and stable cells were selected by G418.

Assessment of ApoA-I-mediated Phospholipid Efflux—Cells were labeled with [3H]choline for 34 h and subsequently incubated in the presence of bovine serum albumin or bovine serum albumin plus apoA-I as described previously (12).

Pull-down Assays with FADD-Agarose—Cells were lysed in 1% Tri-
ton X-100 in phosphate-buffered saline with protease inhibitor mixture (Roche Molecular Biochemicals) for 30 min at 4°C followed by centrifuga-
tion at 14,000 rpm for 10 min. The supernatant was incubated overnight with 1 µl of FADD-agarose at 4°C (Upstate Biotechnology), washed three times with lysis buffer, and resolved in Laemmli buffer for subsequent analysis.

**Co-immunoprecipitation**—For co-immunoprecipitation cells were lysed in 1% Triton X-100 in phosphate-buffered saline with the protease inhibitor mixture for 30 min at 4°C followed by centrifugation at 14,000 rpm for 10 min. The supernatant was incubated overnight with ABCA1 antisera covalently linked to magnetic protein A beads at 4°C (protein A Dynabeads, Dynal, Hamburg, Germany). The beads were washed three times with the lysis buffer and were eluted with citric acid. The eluates were resolved by SDS-PAGE and subjected to Western blot analysis. Bands were visualized by chemiluminescence (Amersham Biosciences).

**Yeast Two-hybrid Library Screening**—The Matchmaker Two-Hybrid System from Clontech (Palo Alto, CA) was used following the instructions of the manufacturer. ABCA1 C terminus was cloned from bp 6292 to 6723 (European Molecular Biology Laboratory (EMBL) GenBank accession no. AB055982) in pAS-2.1, transformed to the yeast strain Y187 that had been pretransformed with a human liver cDNA library. Positive clones were isolated, and the plasmids were purified, transformed into *Escherichia coli*, and sequenced.

**Construction of Expression Plasmids for ABCA1 C Terminus and a Dominant Negative Form of FADD (FADD-DN)—**The C terminus of ABCA1 (bp 6292–6723, EMBL GenBank accession no. AB055982) and FADD-DN (bp 370–756, GenBank accession no. U24231) were cloned in-frame with green fluorescent protein (GFP) in pcDNA 3.1/NT-GFP (Invitrogen).

**RESULTS**

A fusion construct of the Gal4 DNA-binding domain with the C terminus of human ABCA1 (bp 6292–6723) was used as a bait for the screening of a human liver yeast two-hybrid cDNA library. One of the positive clones identified to interact with the ABCA1 C terminus encoded the 98 C-terminal amino acids of FADD (amino acid positions 111–208) as a Gal4 activation domain fusion protein. The identified ABCA1-interacting FADD fragment contains almost the complete C-terminal domain of FADD (amino acids 97–161). To further confirm the association of ABCA1 and FADD, His-tagged full-length human FADD bound to nickel-chelating resin was incubated with *in vitro* translated ABCA1 C terminus with an N-terminal Myc tag. Whereas incubation of FADD-agarose with Myc-tagged aldehyde oxidase as control gave no signal on Western blots, the ABCA1 C terminus was pulled down by FADD-agarose and detected on the immunoblot using a Myc antibody (Fig. 1A). In addition, FADD-agarose pulled down ABCA1 from cell lysates of the human hepatoma cell line HepG2. When FADD was eluted from the agarose with 100 mM imidazole before loading the cell lysate, ABCA1 was not detected in the precipitate (Fig. 1B).

Increasing amounts of ABCA1 were pulled down by FADD-agarose when 10, 20, and 30 µg of cell lysate prepared from the megakaryoblastic leukemia cell line Meg-01 with a high expression of ABCA1 (7) were used (Fig. 1C). In addition, a faint ABCA1 signal was detected in the FADD precipitates from HepG2 cells, whereas the ABCA1 signal on immunoblots was much stronger when Meg-01 lysate was used (Fig. 1D). These experiments support our initial finding that ABCA1 interacts with FADD. Caspase 8, a protein described to interact with FADD, was also detected in the FADD-agarose precipitates by immunoblotting (Fig. 1, C and D). Although caspase 8 is not associated with FADD in non-apoptotic cells, caspase 8 was precipitated by FADD-agarose. With this approach potentially interacting proteins can be identified, but this alone does not prove that FADD and ABCA1 are really associated in these cell lysates. Therefore, to investigate the presumptive endogenous association of ABCA1 with FADD, ABCA1 antisera was covalently linked to magnetic beads. Upon incubation of the beads with cell lysates, the ABCA1 antibody was able to precipitate ABCA1 and endogenous FADD from lysates of the hepatoma cell line HepG2 and Meg-01 cells (Fig. 1E). Caspase 8, which is expressed in these cells (not shown), was not co-immunoprecipitated (Fig. 1E). ABCA1 and FADD but not caspase 8 were also precipitated from lysates of primary fibroblasts of two different donors (F1 and F2) using ABCA1 antibody (F1, IP-ABCA1; F2, IP-ABCA1). The lysate of F2 was also incubated with a control antibody (F2, IP-control). The supernatant of the lysate F2 after incubation with ABCA1 antibody was 5-fold concentrated (F3, lysate 5×). Immunoblots with the precipitates or the lysate were performed and probed with ABCA1, FADD, and caspase 8 antibodies.

![Fig. 1. Interaction of ABCA1 with FADD demonstrated by pull-down assays and immunoprecipitation.](image-url)

**A**, *in vitro* translated ABCA1 C terminus with an N-terminal Myc tag (*left*) and Myc-tagged aldehyde oxidase (*right*) as control were precipitated by FADD-agarose. The precipitates were analyzed on immunoblots using a Myc antibody. **B**, FADD-agarose (+ imidazole) and agarose where FADD has been eluted with 100 mM imidazole (+ imidazole) were incubated with cell lysates of the human hepatoma cell line HepG2. The precipitates were analyzed on immunoblots using ABCA1 antibody. **C**, FADD-agarose was incubated with 10, 20, and 30 µg of cell lysate prepared from the megakaryoblastic leukemia cell line Meg-01. The precipitates were analyzed on immunoblots with ABCA1 and caspase 8 antibodies. **D**, immunoblot of ABCA1 and caspase 8 precipitated from Meg-01 and HepG2 cell lysates using FADD-agarose. **E**, ABCA1 antibody was covalently linked to magnetic beads and incubated with lysates from Meg-01 and HepG2. The precipitates were analyzed on immunoblots with ABCA1, FADD, and caspase 8 using the respective antibodies. **F**, lysates of primary fibroblasts of two different donors (F1 and F2) were used for immunoprecipitation using ABCA1 antibody (**F1**, IP-ABCA1; **F2**, IP-ABCA1). The lysate of F2 was also incubated with a control antibody (**F2**, IP-control). The supernatant of the lysate F2 after incubation with ABCA1 antibody was 5-fold concentrated (**F3**, lysate 5×). Immunoblots with the precipitates or the lysate were performed and probed with ABCA1, FADD, and caspase 8 antibodies.

ABCA1 facilitates the transfer of phospholipids to apoA-I, and to further exclude an involvement of caspase activation in ABCA1-mediated phospholipid efflux, fibroblasts from three different donors were treated with the general caspase inhibitor zVAD-fmk (20 µM) that was added to the culture with apoA-I. Specific apoA-I-dependent efflux of choline phospho-
lipid was 16.2 ± 5.5% in untreated fibroblasts and 15.8 ± 5.2% in fibroblasts treated with the caspase inhibitor. This result demonstrates that caspase activation is not necessary for ABCA1-dependent lipid efflux.

FADD is mainly described as an adaptor molecule in death receptor-induced apoptosis, and the association of ABCA1 with FADD may imply an altered response of fibroblasts with mutated ABCA1 to apoptotic stimuli when compared with primary fibroblasts from controls. A characteristic feature of apoptosis is the release of histone-bound DNA fragments, which can be detected in the supernatant of these cells. Apoptosis was induced in primary fibroblasts from different donors by Fas cross-linking or the addition of TNF-α and cycloheximide (CHX). The release of histone-bound DNA fragments varied from donor to donor but was not consistently induced or suppressed in TD fibroblasts. Therefore, mutations of ABCA1 do not sensitize or protect fibroblasts from Fas- or TNF-α/CHX-induced apoptosis.

In addition, we investigated the expression of FADD by immunoblotting in whole cell lysates from primary fibroblasts of healthy probands and of patients with Tangier disease (Fig. 2A). The two TD patients from pedigree TD2 are homozygous for a 1,500-nucleotide deletion at the 3'-end of the ABCA1 mRNA, and the patients from the pedigrees TD4 and TD5 are compound heterozygous for missense mutations (2). Full-length ABCA1 was detected in fibroblasts from TD4 and TD5, whereas the TD2 fibroblasts expressed no ABCA1 protein (Fig. 2A). FADD was found to be reduced in fibroblasts of five different TD patients when compared with four controls. The protein levels of caspase 8 and FLIP_L, known to directly interact with FADD, varied within different fibroblasts but did not exhibit a consistent down- or up-regulation in TD cells (Fig. 2A). Decreased expression of FADD may be a direct consequence of disturbed lipid homeostasis in these fibroblasts. Therefore we investigated the protein expression of FADD in fibroblast from controls incubated with enzymatically modified low density lipoprotein (24 h, not shown) in serum-starved cells (3 days) and in cells incubated with HDL₃ (24 h) (Fig. 2B) to promote lipid efflux. FADD expression was not altered under these culture conditions indicating that FADD expression is not sensitive to lipid loading/derepotentiation but may be compensatorily down-regulated in cells with mutated ABCA1.

As described elsewhere (13, 14) the recombinant expression of the amino acids 80–208 moiety of FADD (FADD-DN) acts as a dominant negative form. To analyze whether inhibition of FADD may modulate ABCA1 function, FADD-DN was cloned into pcDNA3.1/NT-GFP and expressed with a N-terminal GFP fusion in HepG2 cells. In addition, a GFP fusion with the 144-amino acid C terminus of ABCA1 was generated to analyze a possible inhibitory function of the ABCA1 C terminus. Stable clones were selected, and expression of GFP was monitored by fluorescence microscopy and flow cytomtery. Nearly 95% of the transiently transfected HepG2 cells were GFP-positive with both methods. Immunoblot analysis with GFP antisem revealed that the ABCA1 C-terminal construct was weakly expressed, whereas FADD-DN was expressed at a much higher level (Fig. 3A). In the transfected cells expression of ABCA1, FADD, and caspase 8 was investigated by immunoblots. Recombinant FADD-DN was much more abundant than native FADD, which was similarly expressed in the transfected cells. Furthermore native ABCA1 and caspase 8 levels were comparable in these cells (Fig. 3A). ABCA1 antisemum, which was generated using the 20 C-terminal amino acids of ABCA1, was capable of precipitating FADD-DN, confirming that FADD-DN really binds to ABCA1 (Fig. 3B).

The transfected cells were further analyzed for choline phospholipid efflux to apoA-I. For each assay five to seven different stable clones were combined to exclude differences due to clonal variability. Four independent assays with triplicate determinations were performed, and [³H]choline-derived radioactivity incorporation was found to be similar in the transfected cells. Expression of the ABCA1 C terminus inhibited apoA-I-induci-
ble phospholipid efflux 4-fold (Fig. 3C), and the expression of FADD-DN reduced efflux by 50% thereby confirming that full-length FADD has a relevance for ABCA1 function (Fig. 3C). FADD-DN protected HepG2 cells from TNF-α/CHX-induced apoptosis, whereas HepG2 cells expressing GFP or the ABCA1 C terminus revealed a similar response as detected by the release of histone-bound DNA fragments and trypan blue staining of dead cells.

DISCUSSION

The identification and molecular understanding of ABCA1-associated proteins will provide important insights into ABCA1 function. ApoA-I, as a primary acceptor of phospholipids, associates with ABCA1 (5) as does Cde42 (9) and β2-syntrophin (7). Here we demonstrate that FADD directly interacts with ABCA1 in the hepatoma cell line HepG2, in the megakaryoblastic leukemia cell line Meg-01, and in primary human fibroblasts indicating that the association of ABCA1 with FADD is not cell type-specific. The FADD-ABCA1 interaction is a surprising finding that links HDL metabolism and reverse lipid efflux with a protein mainly described in the context of death receptor-induced apoptosis (15). Death domain-containing proteins like FADD mostly interact with death domains. However, no death domain was identified in the ABCA1 C terminus, and the positively interacting FADD-derived clone in the yeast two-hybrid system did not encode the complete death domain of FADD. Similar observations have been made in the BAG-4/TNFRI system. The N terminus of BAG-4 (SODD (silencer of death domains)) binds the death domain of TNFRI preventing cell death signaling, although no death domain is found in this protein (16). Recombinant expression of the ABCA1 C terminus and FADD-DN significantly reduced cellular efflux of choline phospholipid to apoA-I. This underlines the importance of proteins associated with the C terminus of ABCA1 for ABCA1 function and indicates that full-length FADD is one of these accessory proteins. The death effector domain of FADD is known to interact with the death effector domain of caspase 8 (17). However, caspase 8 was not found in ABCA1-FADD complexes, and activation of caspases was not necessary for apoA-I-dependent phospholipid efflux.

The expression of FADD was found to be reduced in primary fibroblasts from five TD patients compared with fibroblast from four controls. FADD expression was not altered in cholesterol-loaded or serum-starved cells nor influenced by stimulating reverse lipid efflux, and therefore the reduced expression of FADD in TD fibroblasts is not a secondary response to disturbed cholesterol homeostasis. FADD expression was reduced in fibroblasts from patients with mutated ABCA1 but was not down-regulated in HepG2 cells stably expressing ABCA1 C terminus or FADD-DN with reduced apolipoprotein A-I-dependent phospholipid efflux. Therefore the reduced expression of FADD may be a consequence of absent or defective ABCA1 protein. However, additional work is necessary to fully explain the details of this observation. Recombinant expression of the ABCA1 C terminus did not alter the cellular response to death receptor-induced apoptosis; a similar result was observed in TD fibroblasts when compared with controls indicating that neither the inhibition of apoA-I-dependent lipid efflux nor the reduced expression of FADD in TD fibroblasts alters the cellular response to TNF-α/CHX- or Fas-induced apoptosis.

ABCA1 was suggested to be a phosphatidylserine translocase that facilitates phosphatidylserine exofacial flipping (18). The transient local exposure of anionic phospholipids in the outer membrane leaflet enhances the engulfment of apoptotic cells (19), endocytosis (19), and binding of apoA-I (20). Furthermore, the phosphatidylserine transmembrane redistribution at the cell surface is one of the early characteristics of cells undergoing apoptosis (21) and also occurs in cells fulfilling a more specialized function, such as the phosphatidylserine-dependent procoagulant response of platelets after appropriate activation (21). The association of ABCA1 with FADD shown here and the high expression of ABCA1 on platelets (7) may reflect an ABCA1-related phosphatidylserine translocase activity. Alternatively the FADD/ABCA1 interaction may indicate an antiapoptotic ABCA1 function independent from phosphatidylserine translocase activity.

Besides being a signal transducer in apoptosis, the dominant negative form of FADD reduces the proliferation of T-cells and fibroblasts (13) and is involved in the regulation of the cell cycle (22). Growth and cell cycle abnormalities of fibroblasts from Tangier disease patients have been described suggesting that similar pathways seem to be involved in the disturbances of lipid transport and growth retardation (22). An inverse relation between proliferation and apoA-I-mediated choline phospholipid efflux was described recently (23). Our finding of the molecular and functional association of ABCA1 with FADD may contribute to a better understanding of the processes of cholesterol homeostasis, proliferation, and apoptosis with the emphasis on the involvement of ABCA1.

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