Potential role of ABCA7 in cellular lipid efflux to apoA-I

Patrick Linsel-Nitschke,[1,*] Andreas W. Jehle,[*] Jing Shan,[*] Guoqing Cao,[†] Desa Bacic,[§] Debin Lan,[*] Nan Wang,[*] and Alan R. Tall[*]

Division of Molecular Medicine,* College of Physicians and Surgeons, Columbia University, New York, NY; Lilly Research Laboratories,† Indianapolis, IN; and Institute of Physiology,§ University of Zurich-Irchel, Zurich, Switzerland

Abstract ABCA7 is homologous to ABCA1 and has recently been shown in cell culture to bind apolipoprotein A-I (apoA-I) and to promote the efflux of phospholipids. However, it is not known if ABCA7 promotes lipid efflux in vivo. When expressed in HEK293 cells, both human and mouse ABCA7 promoted phospholipid efflux to apoA-I but no detectable cholesterol efflux. However, genetic knockdown of ABCA7 in mouse peritoneal macrophages did not affect phospholipid or cholesterol efflux to apoA-I. Moreover, in ABCA1-knockout macrophages, there was no detectable apoA-I-stimulated phospholipid efflux, inconsistent with a residual role of ABCA7. In contrast to plasma membrane localization of ABCA7 in transfected embryonic kidney cells, immunofluorescence microscopy of endogenous ABCA7 in macrophages showed a predominantly intracellular localization of the protein. Strikingly, immunofluorescence studies of adult mouse kidney revealed an apical brush border membrane localization of ABCA7 in the proximal tubule, suggesting that ABCA7 may come in contact with apoA-I in the glomerular filtrate. Although ABCA7 does not contribute to apolipoprotein-mediated lipid efflux in resting macrophages, its cell surface location in the kidney suggests that it could serve such a role in tissue microenvironments.—Linsel-Nitschke, P., A. W. Jehle, J. Shan, G. Cao, D. Bacic, D. Lan, N. Wang, and A. R. Tall. **Potential role of ABCA7 in cellular lipid efflux to apoA-I.** ***J. Lipid Res.*** **2005.** 46: **86–92.**

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HDL-cholesterol levels are inversely correlated with the risk of coronary heart disease (1). HDL particles are capable of picking up excess cholesterol in peripheral tissues and thus promote the flux of excess cholesterol to the liver for excretion into the bile. This process has been termed "reverse cholesterol transport" and is considered the major reason for the antiatherogenic properties of HDL (2). Heart efflux of phospholipids and cholesterol to lipid-poor apolipoprotein A-I (apoA-I) constitutes the first crucial step in the formation of nascent HDL particles. Patients with Tangier disease show a defect in this lipidation of apoA-I, resulting in drastically reduced plasma HDL levels (3, 4). ABCA1 has been identified as the defective molecule in Tangier disease (5–7). ABCA1 is capable of binding lipid-poor apolipoproteins and promotes the efflux of phospholipids and free cholesterol (8).

ABCA7 and ABCA4, a retina-specific protein, are the closest homologs of ABCA1. ABCA7 is a 220 kDa protein that is widely expressed in a variety of tissues, including macrophages, erythrocytes, platelets, brain, lung, adrenal gland, kidney, spleen, thymus, lymph node, testis, keratinocytes, and pancreatic islets (9–12). We have recently shown that mouse ABCA7 binds apoA-I and promotes the efflux of phosphatidylcholine and sphingomyelin to apoA-I and apoE in ABCA7-transfected cells. In contrast to our findings, Abe-Dohmae and coworkers (13) reported increased efflux of both cholesterol and phospholipids to apoA-I and apoA-II after overexpressing the human ABCA7 protein and suggested that there might be a species difference in lipid efflux specificity. The in vivo role of ABCA7 remains poorly understood. Although expressed in macrophages, it is not known if ABCA7 contributes to lipid efflux in these cells. The goals of the present study were to compare the lipid efflux specificity of human versus mouse ABCA7 and to assess a potential role of ABCA7 in lipid efflux in macrophages and other cell types.

EXPERIMENTAL PROCEDURES

DNA construction and transfection

Full-length cDNA for mouse ABCA7 and mouse ABCA1 was cloned as described previously (12). Full-length cDNA for human ABCA7, transcript variant 1, was obtained by reverse tran-

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Abbreviations: apoA-I, apolipoprotein A-I; siRNA, small interfering RNA.

[1] To whom correspondence should be addressed.
e-mail: pl2031@columbia.edu

scription PCR amplification of the 5′ end of human ABCA7 from human RNA isolated from Jurkat cells and linking of the PCR product to the 5′ end of a human expressed sequence tag clone (gi 21847676; Image-clone 6280653). Both full-length cDNAs were introduced into the pcDNA3.1Hygro+ vector (Invitrogen). The sequences of both inserts were confirmed by sequencing.

HEK293 cells were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen) under a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C.

For transient transfection of HEK293 cells, cells on 12- or 24-well collagen-coated plates were transfected with various plasmid constructs at the indicated DNA concentrations with Lipofectamine 2000 (Invitrogen) at 37°C overnight (~20 h). To estimate transfection efficiency, a construct expressing green fluorescent protein was routinely used in the experiment to visually monitor for transfection efficiency. The transfection efficiency of HEK293 cells was in the range of 50–80% of cells. Although transfection efficiency did vary from experiment to experiment, we found that the variation within the same experiment was small.

Cellular lipid efflux assays

The assays were carried out as described (8). Generally, HEK293 cells were labeled by culturing for 24 h in 10% FBS/Dulbecco's modified Eagle's medium containing either 2 μCi/ml [^{3}H]cholesterol for cholesterol efflux or 2 μCi/ml [^{3}H]choline for phospholipid efflux. The next day, cells were washed with fresh medium before or after treatment as indicated and then apoA-I was added as acceptor and incubated for the indicated period before the medium and cells were collected for analysis. Phospholipid and cholesterol efflux were expressed as the percentage of the radioactivity released from the cells into the medium relative to the total radioactivity in cells plus medium.

For cholesterol mass efflux, the collected media were extracted with hexane-isopropanol (3:2, v/v) with β-sitosterol (5 μg per sample) added as the internal standard. The recovered lipid fractions were dried under nitrogen gas, 100 μl of chloroform was added, and the samples were subjected to gas-liquid chromatographic analysis.

Immunoblot analysis

For immunoblot analysis of mouse ABCA7, ABCA1-Flag, human ABCA7-Flag, or mouse ABCA7-Flag, the transfected HEK293 cells were lysed in radioimmune precipitation assay buffer [10 mM Tris-HCl, pH 7.3, 1 mM $MgCl_2$, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, and 5 mM EDTA in the presence of protease inhibitors (0.5 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A); Roche Applied Science]. Postnuclear supernatants containing the indicated amounts of protein were subjected to Western analysis. Rabbit polyclonal antibody raised against the C terminus of mouse ABCA7 has been described previously (12). M2 anti-Flag mouse monoclonal antibody and mouse monoclonal anti-β-actin antibody were purchased from Sigma. Bands were detected by chemiluminescence, and the relative intensities were determined by densitometry (ImageQuaNT 2.2; Amersham Biosciences).

Small interfering RNA-mediated macrophage RNA interference

Small interfering RNA (siRNA) oligonucleotides derived from the mouse ABCA7 target sequence were obtained from Qiagen and used to suppress ABCA7 expression in thioglycolate-elicited mouse peritoneal macrophages. The target sequence was selected by using the program from Qiagen: 5′-CAGGGACTTGACCAAGGTTTA-3′ and 5′-AAGGCCGTGGTGCGTGAGAAA-3′ for ABCA7. Scrambled control RNA oligonucleotides were also obtained from Qiagen. Cells were transfected with siRNA using the Oligofectamine reagent (Invitrogen) at a final RNA concentration of 200 nM. Radioactive labeling of the cells was performed 24 h after transfection followed by the lipid efflux assay 24 h later.

ABCA1- and ABCA7-knockout mice

ABCA1-knockout (ABCA1$^{-/-}$) mice were kindly provided by O. Francone (Pfizer, Groton, CT), and macrophages isolated from wild-type mice and knockout littermates were used for the experiments. ABCA7 heterozygous mice were kindly provided by G. Cao (Lilly Research Laboratories, Indianapolis, IN). Targeting of the ABCA7-knockout allele resulting in the deletion of exon 21 was confirmed by Southern blotting and PCR and will be published in a separate article.

Immunofluorescence confocal microscopy

Cells were fixed with 3.7% formaldehyde for 10 min and then incubated with 0.1% Triton X-100 in phosphate-buffered saline for 2 min. After washing with phosphate-buffered saline, cells were incubated with primary antibody in 4 mg/ml normal goat globulin and 0.1% saponin in phosphate-buffered saline at room temperature for 30 min. Alexa 488-labeled goat anti-rabbit IgG was used as a secondary antibody. For immunohistochemistry, mouse kidney organs were perfusion-fixed with 3% paraformaldehyde. Peptide affinity-purified rabbit anti-ABCA7 antibody was used at a dilution of 1:25.

RESULTS

Dose-dependent phospholipid and cholesterol efflux to apoA-I

To compare the lipid efflux specificity of human ABCA7, mouse ABCA7, and mouse ABCA1, we performed a side-by-side comparison of both phospholipid and cholesterol efflux from cells transfected with the respective cDNA constructs. All three transporters caused a robust increase in phospholipid efflux to apoA-I. With increasing amounts of apoA-I, human and mouse ABCA7 displayed a similar progressive increase in phospholipid efflux. Mock-transfected HEK293 cells did not show significant apoA-I-mediated efflux of phospholipids or cholesterol and had no detectable endogenous expression of ABCA7 by Western blot using an antibody against human ABCA7 (data not shown). These observations are consistent with earlier reports (8, 12).

The results of the cholesterol efflux assays carried out in parallel were strikingly different (**Fig. 1B**). Although ABCA1 showed a marked stimulation of cholesterol efflux to apoA-I, human and mouse ABCA7 showed a slight increase in efflux only at the highest dose of apoA-I (20 μg/ml). This slight increase was also observed in mock-transfected control cells (data not shown) and most likely reflects background cholesterol efflux at high concentrations of apoA-I.

In addition to the isotopic cholesterol efflux assay, we also determined mass efflux of cholesterol by gas chromatography (Fig. 1C). The mass efflux data thus obtained confirmed the results obtained in the isotopic assay, with

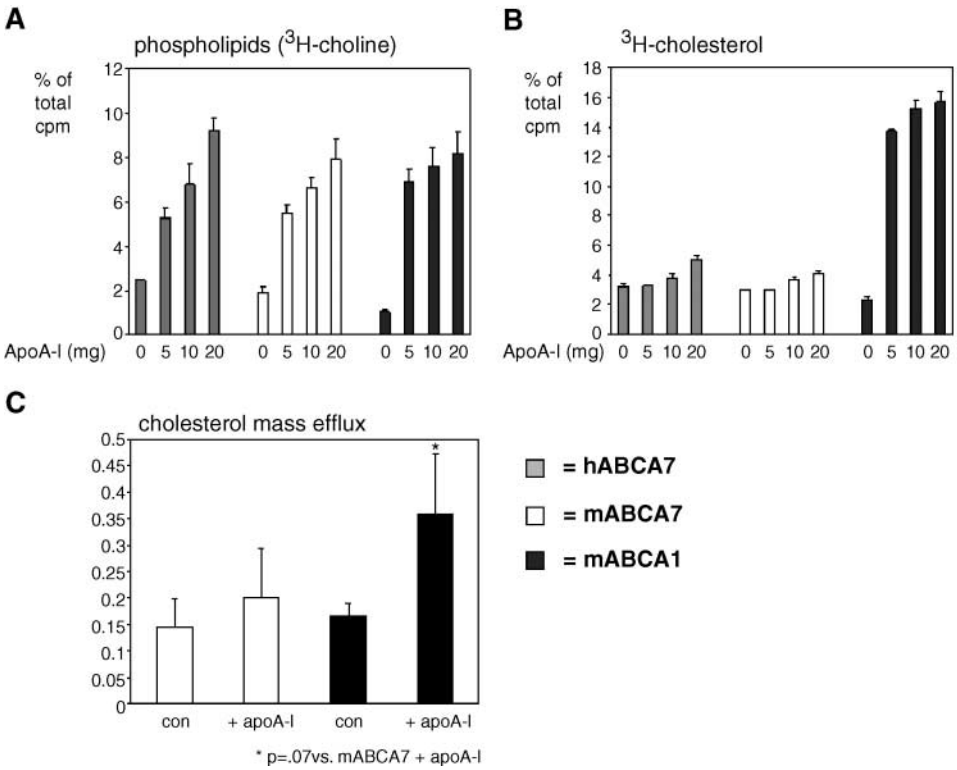

Fig. 1. Apolipoprotein A-I (apoA-I) dose-dependent efflux of phospholipids (A) and cholesterol (B) in transfected HEK293 cells. HEK293 cells plated on 24-well plates were radiolabeled with 1 μCi/ml [^{3}H]choline or 10 μCi/ml [^{3}H]cholesterol for 24 h and transfected with 0.2 μg/well pcDNA3.1 human ABCA7 (hABCA7), pcDNA3.1 mouse ABCA7 (mABCA7), or pcDNA3.1 mouse ABCA1 (mABCA1). Cells were incubated with 0, 5, 10, or 20 μg/ml apoA-I for 24 h. The medium was collected, and ^{3}H counts in the lipid phase of the medium and of total cell lysates were determined. The efflux of phospholipids or cholesterol to apoA-I was calculated as a percentage of total cellular counts released into the medium. Each bar represents duplicate determinations. Results from a representative experiment are shown. C: Mass efflux of free cholesterol into the culture media of transfected HEK293 cells. Cells were transfected with 0.5 mg/well mouse ABCA7-Flag or mouse ABCA1-Flag plasmid DNA and incubated with or without apoA-I for 24 h. Each bar represents quadruplicate determinations. The *y* axis shows the cholesterol mass in the medium relative to the internal standard (arbitrary units). Error bars represent SD. **P* = 0.07 (Student's *t*-test).

only the ABCA1-transfected cells showing significant mass efflux of cholesterol to apoA-I.

Time course of cholesterol efflux to apoA-I

Abe-Dohmae and colleagues (13) reported significant cholesterol efflux mediated by ABCA7 after 16 and 24 h of incubation with apoA-I. In our previously published study, we examined shorter incubation periods and could not detect significant ABCA7-mediated cholesterol efflux (12). To address this difference in experimental design, we carried out a time course of ABCA1- and ABCA7-mediated cholesterol efflux from 4 to 24 h (**Fig. 2A**). In contrast to the progressive increase in cholesterol efflux to apoA-I displayed by ABCA1, no significant efflux of cholesterol to apoA-I was observed for either human or mouse ABCA7 transfected cells at any time point. For all three transporters, there was a modest increase in cholesterol radioactivity in the medium at 24 h, but for human and mouse ABCA7 this was not further increased by the addition of apoA-I. These findings indicate nonspecific accumulation of cholesterol radioactivity in the medium. Therefore, neither human nor mouse ABCA7 promoted cholesterol efflux to apoA-I.

Comparison of mouse ABCA1 and mouse ABCA7 efflux activity after normalization for protein expression

The previous studies compared ABCA7 and ABCA1 without controlling for protein expression levels (3, 12, 13). Using Flag-tagged constructs, we noted that ABCA7 protein was expressed at higher levels than ABCA1 when comparable amounts of plasmid were transfected, raising the issue of artifactual properties related to high levels of overexpression. Cell surface protein concentration of both transporters was measured by biotinylation assay and immunoprecipitation showing that the plasma membrane concentrations of both ABCA1 and ABCA7 were proportionate to total protein concentrations (data not shown). At a plasmid DNA concentration of 0.1 μg/well for mouse ABCA7-Flag and 0.5 μg/well for mouse ABCA1-Flag, comparable expression levels for both proteins were observed by Western immunoblot using anti-Flag antibody (Fig. 2B). When the corresponding phospholipid efflux at these plas-

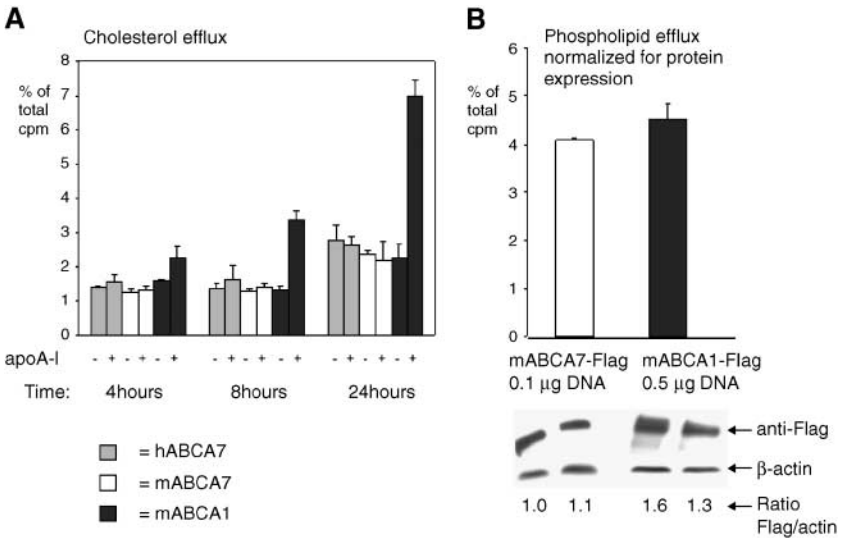

Fig. 2. A: Time course of apoA-I-mediated cholesterol efflux in transfected HEK293 cells. Cells were radiolabeled with 10 μCi/ml [^{3}H]cholesterol for 24 h and transfected with 0.2 μg/well pcDNA3.1 human ABCA7 (hABCA7), pcDNA3.1 mouse ABCA7 (mABCA7), or pcDNA3.1 mouse ABCA1 (mABCA1). Twenty-four hours after transfection, cells were incubated with 10 μg of apoA-I for the various lengths of time indicated. Efflux of [^{3}H]cholesterol into the medium was determined and is shown as a percentage of total cell counts. Each bar represents four wells and is the mean of two experiments. B: Comparison of mouse ABCA1 and mouse ABCA7 phospholipid efflux after normalization for protein expression. HEK293 cells were transfected with various concentrations (0, 0.1, 0.25, and 0.5 mg) of mouse ABCA1-Flag or mouse ABCA7-Flag DNA, and phospholipid efflux to apoA-I was determined. A small aliquot of the total cell lysates was subjected to Western immunoblotting using monoclonal antibodies against the Flag epitope and β-actin. For a better comparison, the ratio of Flag versus β-actin in each lane was determined by densitometric analysis. Bars show the apoA-I-specific phospholipid efflux at plasmid DNA concentrations resulting in similar protein expression normalized for the Flag/β-actin ratio. Error bars represent SD.

mid DNA concentrations was normalized to protein expression using the Flag/β-actin ratio, similar phospholipid efflux activity was observed for both ABCA1 and ABCA7 (Fig. 2B). Our results show that at similar protein concentrations, both ABCA7 and ABCA1 are capable of mediating significant phospholipid efflux.

Knockdown of ABCA7 in mouse peritoneal macrophages

To investigate the physiological role of ABCA7-mediated phospholipid efflux in macrophages, we next carried out genetic knockdown experiments of ABCA7 in mouse peritoneal macrophages. Using a siRNA construct complementary to mouse ABCA7, we observed an average reduction of protein expression of 40% (compared with the scrambled siRNA construct used as a control) (**Fig. 3A**). This knockdown amount was consistently observed in several separate experiments. Despite the reduction in ABCA7 protein expression, there was no effect on either phospholipid or cholesterol efflux to apoA-I.

We have recently obtained mice with a targeted deletion of exon 21 of the mouse ABCA7 gene and confirmed correct targeting of the knockout vector by Southern blot (data not shown). Homozygous ABCA7-knockout mice showed markedly decreased viability/embryonic lethality and will be presented in a separate publication. Because no homozygous ABCA7-knockout mice have been obtained to date, we chose to use heterozygous ABCA7-knockout mice to obtain macrophages. These were compared with macrophages from wild-type littermate control mice in efflux and protein expression studies. Peritoneal macrophages from ABCA7-heterozygous mice showed an average reduction of 55% in ABCA7 protein levels compared with the littermate controls (Fig. 3B). Consistent with the results obtained by siRNA interference, there was no reduction in phospholipid or cholesterol efflux to apoA-I in the ABCA7 heterozygous macrophages compared with ABCA7 homozygous macrophages (Fig. 3B).

As a further approach to measuring a potential role of ABCA7 in apolipoprotein-mediated lipid efflux in macrophages, we characterized ABCA1$^{-/-}$ cells to determine if there was any residual lipid efflux that could be attributed to ABCA7. We have reported previously that no significant residual cholesterol efflux to apoA-I is detectable in peritoneal macrophages from ABCA1$^{-/-}$ mice (14) (Fig. 3B). No residual phospholipid efflux activity could be detected in ABCA1$^{-/-}$ macrophages, confirming that ABCA7 does not contribute to apoA-I-mediated lipid efflux in resting macrophages.

Immunofluorescence microscopy and immunohistochemistry

To understand why ABCA7 does not contribute to macrophage lipid efflux to apoA-I, we next examined the tissue distribution and subcellular localization of ABCA7 in mouse peritoneal macrophages and some other selected tissues. As previously described (12), ABCA7 can be readily

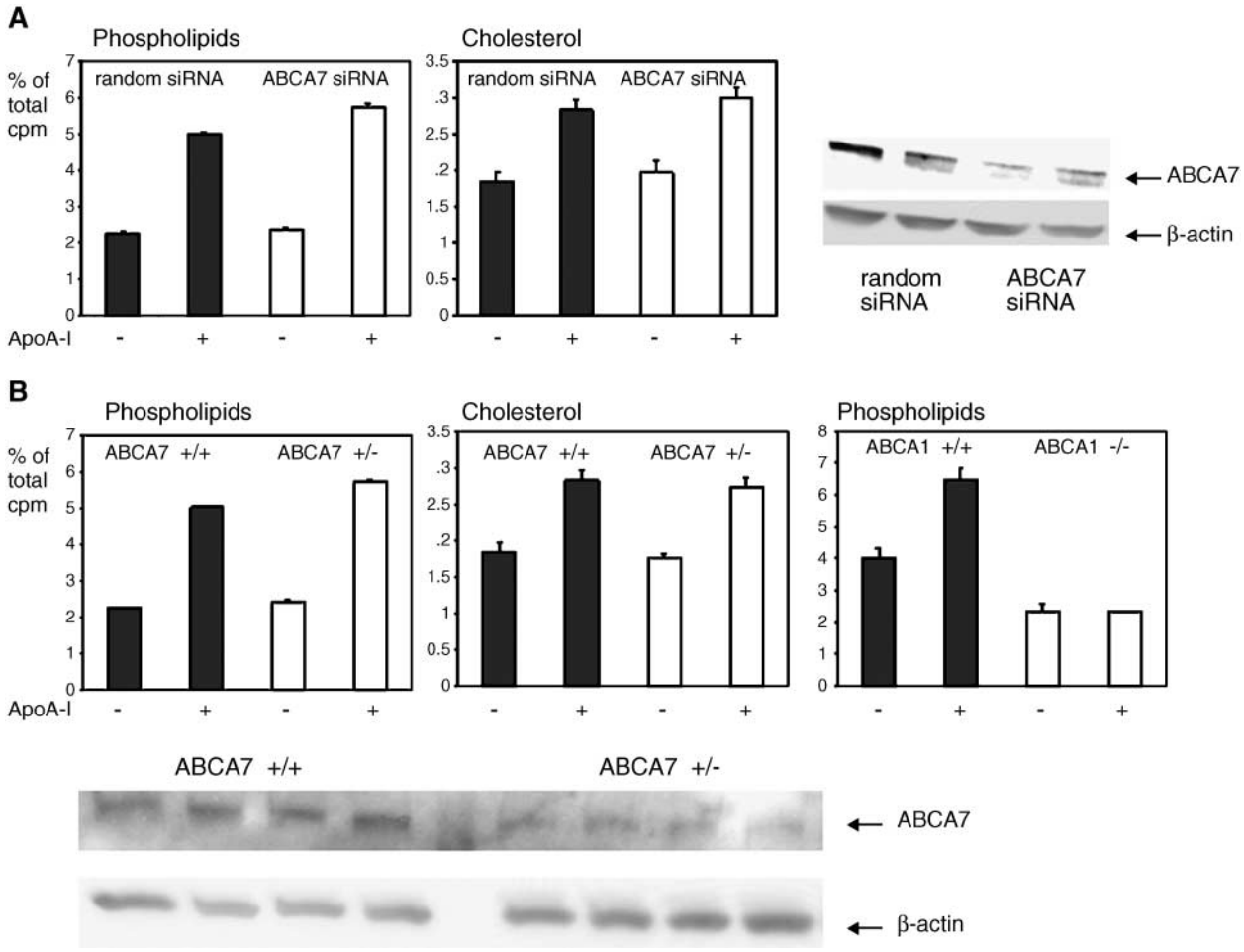

Fig. 3. A: Small interfering RNA (siRNA) knockdown of endogenous ABCA7 in mouse peritoneal macrophages. Thioglycolate-elicited mouse peritoneal macrophages were transfected with either random siRNA or ABCA7 siRNA. Isotopic cholesterol or phospholipid efflux to apoA-I was determined 48 h after transfection. Each bar represents quadruplicate determinations. A small aliquot of the total cell lysates was subjected to Western immunoblotting using antibodies against mouse ABCA7 and β-actin. B: Comparison of phospholipid and cholesterol efflux from wild-type, ABCA7 heterozygous ($ABCA7^{+/-}$), and ABCA1-knockout ($ABCA1^{-/-}$) mouse peritoneal macrophages. Thioglycolate-elicited mouse peritoneal macrophages were harvested from the respective knockout mice or control littermates. Isotopic efflux of [^{3}H]choline or [^{3}H]cholesterol was determined after 24 h of incubation with or without apoA-I. Each bar represents quadruplicate determinations. A small fraction of the total cell lysates was subjected to Western immunoblotting. Error bars represent SD.

detected on the plasma membrane by immunofluorescence microscopy when overexpressed in HEK293 cells (**Fig. 4A**). In contrast, when we examined mouse peritoneal macrophages under the same conditions, we could not detect an ABCA7 signal at the plasma membrane and found the protein to be predominantly localized in the intracellular space (Fig. 4B).

To identify other tissues or cell types in which ABCA7 might physically interact with apoA-I, we carried out immunohistochemistry of various tissues expressing ABCA7. A variety of tissues were found to express ABCA7 protein, including kidney, thymus, spleen, lymph node, brain, lung, and pancreatic islets. Because lipid-poor apoA-I is present in the glomerular filtrate (15), we considered the possibility that ABCA7 might be present in the renal tubules. Strikingly, in the polarized epithelia of the proximal tubule, ABCA7 was localized to the apical brush border membrane; the preimmune serum did not yield any signal (Fig. 4C). This result shows that ABCA7 can be localized at the cell surface in particular cell types under physiological conditions and might mediate phospholipid efflux to apoA-I at these locations. In an attempt to assess a lipid efflux role of ABCA7 in the proximal tubule, primary cultures of tubular cells were prepared. However, ABCA7 expression was markedly reduced in the primary cultures, so this was not possible.

DISCUSSION

We previously reported that mouse ABCA7 promotes phospholipid but not cholesterol efflux to apoA-I in transfected cells, contrasting with the ability of the related transporter ABCA1 to promote the efflux of both cholesterol and phospholipids. We now show that this lipid efflux specificity is conserved between human and mouse

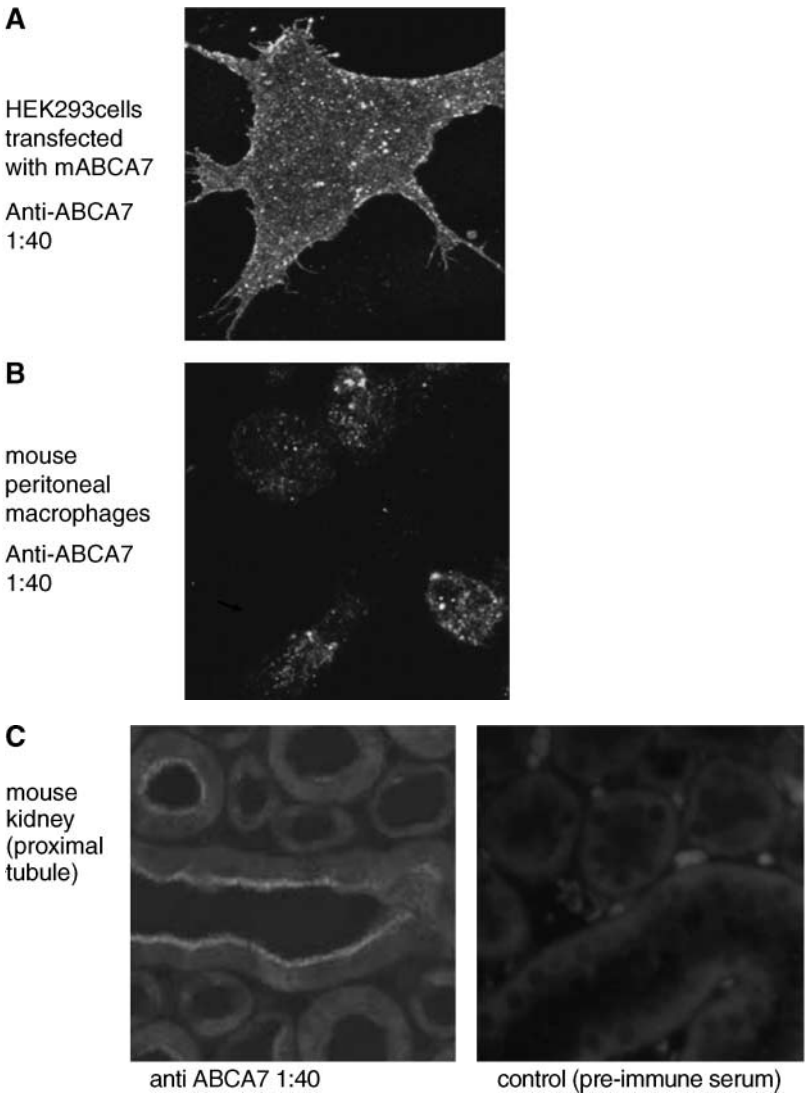

Fig. 4. A: Fluorescence confocal microscopy of mouse ABCA7 (mABCA7)-transfected HEK293 cells (A) and mouse peritoneal macrophages (B). Peptide affinity-purified rabbit anti-ABCA7 antibody (dilution, 1:40) was used as a primary antibody. C: Immunohistochemistry of mouse kidney using rabbit anti-ABCA7 antibody (dilution, 1:25; left) or preimmune serum (dilution, 1:25; right). A proximal tubule is cut longitudinally in both panels, showing positive staining of the brush border membrane with the ABCA7 antibody.

ABCA7 and can be demonstrated under a variety of dose-response and time-course conditions. Although failing to confirm a proposed species difference between mouse and human ABCA7 (13), our results provide further evidence that ABCA7 may function as a lipid transporter with efflux specificity for phospholipids over cholesterol. However, the in vivo role of ABCA7 remains enigmatic, and our results indicate that although ABCA7 is unlikely to contribute significantly to lipid efflux in macrophages, it could play this role in specific tissue microenvironments such as the renal tubule.

The reasons for the apparent discrepancy between our findings and those of Abe-Dohmae et al. (13) are unclear. Even though not noted, the initial results reported by this group suggest a preferential efflux of phospholipids compared with cholesterol in transiently transfected cells. Under comparable conditions, the ratio of cholesterol-to-phospholipid efflux was ~1:4 for ABCA1 and 1:8 for ABCA7 (see Fig. 1 in 13). In later experiments, these workers selected high-expressing clones of cells with stable expression by fluorescent-activated cell sorting analysis and found efflux of both cholesterol and phospholipids to apoA-I (13). It is possible that the different results are related to clonal cell differences or very high expression levels. Also, their analysis used enzymatic assays of cholesterol and phospholipid mass, whereas we used isotopic tracers or measured mass cholesterol efflux by gas chromatography in our experiments. This should not be a source of differences, because isotopic efflux to lipid-poor apolipoproteins reflects net mass efflux of lipids.

Although our studies suggest that ABCA7 has a role as a phospholipid transporter and demonstrate an interaction with lipid-poor apolipoproteins, the in vivo functions of ABCA7 remain unknown. Genetic knockdown of ABCA7 in mouse peritoneal macrophages by two different approaches failed to show any effect on phospholipid or

cholesterol to apoA-I. The knockdown approaches only reduced ABCA7 protein levels by 40–55%, so it is still conceivable that the remaining expression was sufficient to support lipid efflux. However, we also found no cholesterol or phospholipid efflux to apoA-I in macrophages from ABCA1$^{-/-}$ mice, inconsistent with a residual role of ABCA7. Formally, it remains possible that the activity of ABCA7 requires ABCA1 and that ABCA7 is present in excess in macrophages. However, cell transfection in HEK293 cells in the absence of ABCA1 resulted in abundant lipid efflux activity, making this possibility unlikely. Therefore, ABCA7 is unlikely to play a role in macrophage lipid efflux, at least in the resting state. The lack of an effect on macrophage lipid efflux could be explained by the absence of ABCA7 from the plasma membranes of peritoneal macrophages, so that no direct physical interaction with apoA-I is possible.

It is possible that ABCA7 has a more basic cellular function related to membrane phospholipid translocation. This property is detected in cellular overexpression experiments as a result of the addition of lipid-poor apolipoproteins to the cells. However, under physiological conditions, the contact between ABCA7 and apoA-I may be limited and restricted to specific cellular conditions or microenviroments. In the case of macrophages or other white blood cells that express ABCA7, the transporter could be translocated to the plasma membrane under special circumstances such as cell migration or phagocytosis and could then physically interact with apoA-I. Because high-level expression of ABCA7 has been detected in erythrocytes (12) and platelets (9) and both types of blood cells contain only a single membrane, ABCA7 could potentially come into contact with apoA-I and mediate phospholipid efflux in these cell types. Furthermore, by immunofluorescence, we identified the apical brush border membrane in the proximal tubule of the kidney as another location where ABCA7 is highly concentrated at the cell surface. Earlier studies have shown that apoA-I can be filtered at the glomerulus and appears in the glomerular filtrate (15), so that a physical interaction between ABCA7 and apoA-I in the lumen of the proximal tubule seems likely. Moreover, the kidney represents a major site of apoA-I catabolism, although the cellular and molecular mechanisms are poorly understood. A variety of ABC transporters, including ABCA1, ABCG1, and ABCA7 are expressed in the kidney (12, 16, 17), suggesting that there may be major modifications of HDL and apolipoproteins by these transporters in the kidney.

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