A PEST Deletion Mutant of ABCA1 Shows Impaired Internalization and Defective Cholesterol Efflux from Late Endosomes*

Wengen Chen, Nan Wang, and Alan R. Tall‡

From the Division of Molecular Medicine, Department of Medicine, Columbia University, New York, New York 10032

ATP-binding cassette transporter A1 (ABCA1) promotes the efflux of cellular cholesterol and phospholipids to apoA-I. We described previously a cytoplasmic PEST sequence in ABCA1 and showed that deletion of the PEST sequence results in a prominent increase in the cell surface concentration of ABCA1. In the current study we evaluated the hypothesis that the PEST sequence-deleted ABCA1 might display defective internalization and trafficking to the late endosomes/lysosomes. As assessed by monensin treatment and cell surface biotinylation, the internalization rate of PEST sequence-deleted ABCA1 (ABCA1-dPEST) was markedly decreased compared with wild-type ABCA1 (ABCA1-wt). Immunofluorescence confocal microscopy of ABCA1-wt showed both plasma membrane localization and substantial co-localization with LAMP2 in late endosomes. In contrast, ABCA1-dPEST showed more prominent plasma membrane localization but little co-localization with LAMP2. To assess cholesterol efflux from late endosomes, HEK293 cells were transiently co-transfected with scavenger receptor A (SR-A) and incubated with [3H]cholesterol/acetyl low density lipoprotein (acLDL). Although ABCA1-dPEST showed higher cholesterol efflux than did ABCA1-wt following cell surface labeling ([3H]cholesterol/acLDL in the absence of SR-A co-transfection), it showed impaired cholesterol efflux after late endosomal labeling ([3H]cholesterol/acLDL in the presence of SR-A). Thus, deletion of the PEST sequence leads to a decrease in the internalization of ABCA1 and decreased cholesterol efflux from late endosomal cholesterol pools, providing evidence that the internalization and trafficking of ABCA1 is functionally important in mediating cholesterol efflux from intracellular cholesterol pools.

In Tangier disease, mutations in ATP-binding cassette transporter A1 (ABCA1) lead to low retention of high density lipoprotein and cholesterol in macrophage foam cells (1–4). ABCA1 mediates cholesterol efflux from cells to apolipoprotein A-I (apoA-I), giving rise to nascent high density lipoprotein (5, 6). However, the mechanisms of cellular cholesterol efflux are not completely understood. It is generally thought that ABCA1 functions on the cell surface as suggested by its plasma membrane localization and apoA-I binding (7–9). However, markedly defective cholesterol efflux to apoA-I in Niemann-Pick C1 macrophages, which are defective in lysosomal cholesterol trafficking, suggested that ABCA1 stimulates cholesterol efflux from a late endosomal/lysosomal pool (10). Neufeld et al. (11) showed that ABCA1 is also present in late endosomes and lysosomes and traffics between late endosomes and the cell surface. Together these data suggested that trafficking of ABCA1 between the cell surface and late endosomes/lysosomes could be involved in cholesterol efflux. However, direct evidence that internalization of ABCA1 facilitates cholesterol efflux from intracellular sites is lacking.

We recently described a cytoplasmic domain PEST sequence in ABCA1 and showed that the PEST sequence has a role in regulating ABCA1 protein turnover at least in part by promoting calpain degradation of ABCA1 (12). In cells expressing a mutant form of ABCA1 in which the PEST sequence was deleted (ABCA1-dPEST) there was a striking increase in the cell surface concentration of ABCA1 without an increase in the overall level of ABCA1 in cell lysates. This suggested the possibility of a defect in the internalization of ABCA1-dPEST. In the current study we have confirmed the defective internalization of ABCA1-dPEST and have shown that the mutant has a parallel defect in mediating cholesterol efflux from cells after cholesterol is introduced into late endosomes/lysosomes via scavenger receptor A (SR-A). These studies provide evidence that ABCA1 internalization and trafficking to late endosomes is functionally important in mediating cholesterol efflux from this intracellular location.

Experimental Procedures

Materials—All of the cell culture reagents were from Invitrogen. HEK293 cells were purchased from ATCC (Manassas, VA). Lipofectamine 2000 was from Invitrogen. Human apoA-I was commercially obtained (BioDesign). Acetyl low density lipoprotein (acLDL) was from Biomedical Technologies Inc. (Stoughton, MA). Fatty acid-free bovine serum albumin (BSA) and monoclonal M2 anti-FLAG antibody were from Sigma. Mouse anti-human LAMP2 antibody, developed by Dr. J. T. August, was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Iowa City, IA). Alexa-labeled secondary antibodies were obtained from Molecular Probes (Eugene, OR).

Plasmid Constructs and Internalization of Cell Surface ABCA1—C-terminally FLAG-tagged murine wild-type ABCA1 (ABCA1-wt) and ABCA1-dPEST plasmids were constructed as described previously (12, 13). Internalization of cell surface ABCA1 was monitored using a modification of the protocol described previously (14). Briefly HEK293 cells transiently transfected with ABCA1-wt or ABCA1-dPEST were incubated with 10 μM monensin to prevent recycling of the internalized protein back to the plasma membrane. After the treatment, cells were cooled down on ice and then biotinylated with 0.5 mg/ml EZ-Link™ sulfo-NHS-LC-biotin (Pierce) at 4 °C for 50 min. After washing, cells were lysed with radiomune precipitation assay buffer (10 mM Tris-HCl, pH 7.3, 1 mM MgCl₂, 0.5 M monensin to prevent recycling of the internalized protein back to the plasma membrane. After the treatment, cells were cooled down on ice and then biotinylated with 0.5 mg/ml EZ-Link™ sulfo-NHS-LC-biotin (Pierce) at 4 °C for 50 min. After washing, cells were lysed with radiomune precipitation assay buffer (10 mM Tris-HCl, pH 7.3, 1 mM MgCl₂, 

This paper is available on line at http://www.jbc.org

Received for publication, May 20, 2005, and in revised form, June 9, 2005
Published, JBC Papers in Press, June 10, 2005, DOI 10.1074/jbc.M505566200

© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Wild-type ABCA1.

BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s me-

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 280, No. 32, Issue of August 12, pp. 29277–29281, 2005

This work was supported by National Institutes of Health Grant HL22682. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Div. of Molecular Medicine, Department of Medicine, Columbia University, New York, New York 10032

§ The abbreviations used are: ABCA1, ATP-binding cassette transporter A1; acLDL, acetyl low density lipoprotein; apoA-I, apolipoprotein A-I; ABCA1-dPEST, ABCA1 in which the PEST sequence is deleted; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; SR-A, scavenger receptor A; ABCA1-wt, wild-type ABCA1.
were transfected with 0.5 g/ml apoA-I in 0.1% BSA/DMEM for 4 h. After efflux, medium was collected and centrifuged at 6000 × g for 10 min. Radioactivity in supernatant medium was determined by liquid scintillation counting. The counts were in 0.5 ml of 0.1 M sodium hydroxide and 0.1% SDS, and the radioactivity was determined. Cholesterol efflux was expressed as the percentage of the radioactivity released from cells into the medium relative to the total radioactivity in cells and medium. Results were shown as net efflux (efflux with apoA-I minus efflux with 0.1% BSA alone).

ApoA-I Cellular Association and Internalization—HEK293 cells transiently transfected with ABCA1-wt or ABCA1-dPEST were incubated for 30 min at 37 °C with 1 μg/ml [125I]apoA-I (1750 cpm/ng) with or without 100 μg/ml apoA-I in 0.1% BSA/DMEM. The cells then were washed five times with phosphate-buffered saline containing 1 mM calcium chloride and 0.1% BSA. Cell association of [125I]apoA-I was measured as the cpm in total cell lysates. In parallel plates, following the 30-min incubation and washings, cells were returned to 37 °C and chased for 1 h in 0.1% BSA/DMEM. At the completion of the chase period, cells were washed and lysed. Residual [125I]apoA-I was measured as the cpm in the cell lysates. Internalization ratio (%) of [125I]apoA-I was calculated as the percentage of residual [125I]apoA-I/cell-associated [125I]apoA-I.

RESULTS

We showed previously a prominent increase in the cell surface concentration of ABCA1-dPEST compared with ABCA1-wt (12). Here we measured the internalization rate of ABCA1 by monitoring the disappearance of cell surface ABCA1 in transiently transfected HEK293 cells treated with monensin to block recycling of internalized protein back to the plasma membrane, as described under “Experimental Procedures.” In A, cells were treated with monensin for different time periods followed by cell surface biotinylation. Cell surface ABCA1 (Surface) represents biotinylated ABCA1, and Total represents ABCA1 in whole cell lysates. The quantification of the surface protein immunoblots normalized by the total protein is shown in the graph (lower panel). B, mean ± S.E. of cell surface ABCA1-wt and ABCA1-dPEST proteins from four independent experiments with and without 2 h of monensin treatment is shown (*, p < 0.001, t test). C, HEK293 cells transiently transfected with ABCA1-wt were incubated with or without 10 μg/ml apoA-I and with or without 10 μm monensin (Mon) for 2 h, and cell surface protein levels were measured by biotinylation.

Cholesterol Labeling and Efflux Assays—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. One day before transfection, the cells were plated on 24-well plates. The next day, cells at about 85% confluence were transfected with 0.5 μg of ABCA1-wt or ABCA1-dPEST Lipofectamine 2000. To label the late endosomal/lysosomal pool, cells were incubated with 5% milk in phosphate-buffered saline, fixed with 3.7% formaldehyde for 10 min, and then incubated with 0.1% Triton X-100 in phosphate-buffered saline for 2 min at room temperature. After blocking with 5% milk in phosphate-buffered saline, cells were incubated with primary anti-FLAG and anti-LAMP2 antibodies (each at 1:200 dilution) in 2% milk/phosphate-buffered saline at room temperature for 30 min. Alexa568- or -486-labeled secondary antibodies were used at 1:200 dilution. After washing and fixing, cells were examined by Zeiss LSM 510 META scanning confocal microscope.

Immunofluorescence Confocal Microscopy—HEK293 cells transiently transfected with ABCA1-wt or ABCA1-dPEST in glass chamber slides were washed in phosphate-buffered saline, fixed with 3.7% formaldehyde for 10 min, and then incubated with 0.1% Triton X-100 in phosphate-buffered saline for 2 min at room temperature. After blocking with 5% milk in phosphate-buffered saline, cells were incubated with primary anti-FLAG and anti-LAMP2 antibodies (each at 1:200 dilution) in 2% milk/phosphate-buffered saline at room temperature for 30 min. Alexa568- or -486-labeled secondary antibodies were used at 1:200 dilution. After washing and fixing, cells were examined by Zeiss LSM 510 META scanning confocal microscope.

ABC1 Promotes Cholesterol Efflux from Late Endosomes

Fig. 1. Internalization of cell surface ABCA1-wt and ABCA1-dPEST. HEK293 cells were transiently transfected with ABCA1-wt or ABCA1-dPEST. The internalization rate of ABCA1 was estimated by cell surface biotinylation after treatment of cells with 10 μM monensin to block recycling of internalized protein back to the plasma membrane, as described under “Experimental Procedures.” In A, cells were treated with monensin for different time periods followed by cell surface biotinylation. Cell surface ABCA1 (Surface) represents biotinylated ABCA1, and Total represents ABCA1 in whole cell lysates. The quantification of the surface protein immunoblots normalized by the total protein is shown in the graph (lower panel). B, mean ± S.E. of cell surface ABCA1-wt and ABCA1-dPEST proteins from four independent experiments with and without 2 h of monensin treatment is shown (*, p < 0.001, t test). C, HEK293 cells transiently transfected with ABCA1-wt were incubated with or without 10 μg/ml apoA-I and with or without 10 μM monensin (Mon) for 2 h, and cell surface protein levels were measured by biotinylation.

Fig. 2. Co-localization of ABCA1-wt and ABCA1-dPEST with late endosomal marker LAMP2. HEK293 cells transiently transfected with ABCA1-wt (A) or ABCA1-dPEST (B) (each C-terminally FLAG-tagged) were detected by confocal immunofluorescence microscopy following cell permeabilization and labeling with anti-FLAG antibody (red) and LAMP2 (green) as described under “Experimental Procedures.” Merged signals (yellow) are indicated by the arrow. The images shown represent 1-μm confocal slices. Scale bar, 10 μm.
ABCA1-dPEST with LAMP2 and more prominent plasma membrane localization (Fig. 2B). A direct comparison of cellular distributions was also made using ABCA1-wt and ABCA1-dPEST labeled with different tags. Cells were co-transfected with ABCA1-wt (HA-tagged in the first extracellular loop) and ABCA1-dPEST (FLAG-tagged in the C terminus). ABCA1-wt (HA-tagged) showed both plasma membrane and intracellular localizations (Fig. 3A, green), whereas ABCA1-dPEST (FLAG-tagged) showed more prominent plasma membrane localization in the same cells (Fig. 3, B (red) and C (merge of A and B, yellow)). The different distribution of proteins was not due to the HA or FLAG tagging, as HA-tagged and FLAG-tagged ABCA1-wt constructs showed complete co-localization (Fig. 3, D–F). Incubation of ABCA1-wt or ABCA1-dPEST with apoA-I did not cause any change in their overall cellular distribution (not shown). Together these studies suggested a defect in internalization and endosomal trafficking of ABCA1-dPEST.

To determine the functional consequences of these defects, we developed a method for cholesterol labeling of the late endosomal/lysosomal cholesterol pool. SR-A is well known to bind and internalize modified LDL and deliver it to late endosomes/lysosomes (15). To label late endosomes/lysosomes, HEK293 cells transiently transfected with SR-A and ABCA1 (wt or dPEST) were loaded with cholesterol/acLDL, and then cholesterol efflux to apoA-I was measured (Fig. 4). Cells that were not transfected with SR-A (i.e., they received empty vector) were similarly incubated with cholesterol/acLDL (Fig. 4A). A time course study showed that following the latter procedure (cell surface labeling), ABCA1-dPEST gave rise to higher levels of cholesterol efflux to apoA-I than did ABCA1-wt (Fig. 4A), consistent with previous studies in which cells were labeled with cholesterol/10% fetal bovine serum or cholesterol/cyclo-dextrin (12). However, in cells labeled with cholesterol/acLDL and transfected with SR-A, the opposite result was obtained i.e. there was a defect in cholesterol efflux mediated by ABCA1-dPEST compared with that mediated by ABCA1-wt (Fig. 4B).

To calculate the specific component of efflux attributable to expression of SR-A, the results in Fig. 4B were subtracted from those in Fig. 4A. This showed a major defect for ABCA1-dPEST compared with ABCA1-wt (Fig. 4C). Results from four different experiments in which efflux was measured during a constant time period (4 h) showed the same findings: ABCA1-dPEST showed increased cholesterol efflux from cell surface compared with that shown by ABCA1-wt (2.30 ± 0.18% (mean ± S.E.) versus 1.95 ± 0.18%, t test, p < 0.05, n = 4). However, there was impaired cholesterol efflux from the late endosomal/lysosomal pool in ABCA1-dPEST compared with that in ABCA1-wt (3.05 ± 0.68% versus 5.64 ± 0.94%, t test, p < 0.01, n = 4). ABCA1-dPEST showed only about 40% of SR-A-specific efflux to apoA-I compared with that shown by ABCA1-wt. Taken together the data suggested a major defect in cholesterol efflux from the late endosomal pool from ABCA1-dPEST.

The results in Fig. 4 were not normalized for expression levels of ABCA1 protein. Thus, the experiment was repeated at the 4-h time point, and levels of ABCA1 protein were measured in the cell surface and in total cell lysates. Unexpectedly, SR-A co-transfection resulted in increased levels of both forms of ABCA1, especially in total cell lysates (Fig. 5A). Similar observations were made in five different experiments. The mechanism of this effect is unknown. However, as assessed by confocal microscopy, the cellular distribution patterns of ABCA1-wt and ABCA1-dPEST were not affected by SR-A expression (not shown). As shown in Fig. 5, when the efflux was expressed as cholesterol radioactivity in the medium, cholesterol efflux without SR-A co-transfection was higher for ABCA1-dPEST than for ABCA1-wt, whereas cholesterol efflux with SR-A was higher for ABCA1-wt than for ABCA1-dPEST (Fig. 5B). Normalization of efflux data for cell surface ABCA1 levels (Fig. 5C) or total levels of ABCA1 in cell lysates (not shown) showed that efflux for ABCA1-wt was markedly enhanced by SR-A, whereas efflux for ABCA1-dPEST was not changed by SR-A. This finding indicates that increased efflux by ABCA1-wt with SR-A is not due simply to increased levels of ABCA1 but likely reflects internalization and trafficking of ABCA1-wt to late endosomes/lysosomes. In contrast, ABCA1-dPEST failed to show increased efflux in the presence of SR-A once data were normalized for protein expression levels, suggesting defective internalization and trafficking.

It has been reported that apoA-I internalization and recycling is involved in ABCA1-mediated cholesterol efflux (16). We measured cell-associated and residual [125I]apoA-I in cells after a 0.5-h incubation of [125I]apoA-I and a 1-h chase at 37 °C (Fig. 5D–F). As shown in Fig. 5, when the efflux was expressed as cholesterol radioactivity in the medium, cholesterol efflux without SR-A co-transfection was higher for ABCA1-dPEST than for ABCA1-wt, whereas cholesterol efflux with SR-A was higher for ABCA1-wt than for ABCA1-dPEST (Fig. 5B). Normalization of efflux data for cell surface ABCA1 levels (Fig. 5C) or total levels of ABCA1 in cell lysates (not shown) showed that efflux for ABCA1-wt was markedly enhanced by SR-A, whereas efflux for ABCA1-dPEST was not changed by SR-A. This finding indicates that increased efflux by ABCA1-wt with SR-A is not due simply to increased levels of ABCA1 but likely reflects internalization and trafficking of ABCA1-wt to late endosomes/lysosomes. In contrast, ABCA1-dPEST failed to show increased efflux in the presence of SR-A once data were normalized for protein expression levels, suggesting defective internalization and trafficking.

It has been reported that apoA-I internalization and recycling is involved in ABCA1-mediated cholesterol efflux (16). We measured cell-associated and residual [125I]apoA-I in cells after a 0.5-h incubation of [125I]apoA-I and a 1-h chase at 37 °C (Fig.
ABCA1 Promotes Cholesterol Efflux from Late Endosomes

6). Consistent with increased cell surface expression of ABCA1-dPEST, there was increased cell association of [125I]apoA-I for cells expressing ABCA1-dPEST compared with those expressing ABCA1-wt; however, internalized [125I]apoA-I at the end of 1 h (residual) was less for cells expressing ABCA1-dPEST, consistent with decreased ABCA1-dependent internalization of [125I]apoA-I. The ratio of internalized ligand/cell-associated ABCA1, as described under “Experimental Procedures.” One day after transfection, cells were incubated for 4 h with [3H]cholesterol/acroLDL (total cpm in cells after labeling: 7356 ± 149 (mean ± S.E.) in ABCA1-wt and 6859 ± 314 in ABCA1-dPEST without SR-A co-transfection; 13,862 ± 420 in ABCA1-wt and 12,462 ± 483 in ABCA1-dPEST with SR-A co-transfection). Efflux was then performed with or without 10 μg/ml apoA-I in 0.1% BSA/DMEM and expressed as the percentage of cpm in medium/total cpm (medium + cell lysate). At time points shown, aliquots of medium were removed for liquid scintillation counting. Results are shown as the net efflux to apoA-I in C, at each time point, SR-A dependent efflux was calculated by subtracting results in A from those in B. Representative data are shown for one of two independent experiments.

The present studies provide strong new evidence to support ABCA1 promotes cholesterol efflux from late endosomes and that trafficking of ABCA1 to this site may be involved in this process. In macrophages expressing similar amounts of ABCA1, there were 2–3-fold higher levels of cholesterol efflux to apoA-I than to other cell-surface proteins, compared with techniques that initially label predominantly the plasma membrane (cholesterol/fetal bovine serum or cholesterol/cyclodextrin) (10). Also, Niemann-Pick C1-deficient cells in which there is defective release of cholesterol from late endosomes showed a severe defect in ABCA1-mediated cholesterol efflux (10). Neufeld et al. (11) showed localization of ABCA1 to late endosomes and trafficking of ABCA1 between late endosomes and the cell surface. Moreover, Tangier disease fibroblasts showed an accumulation of cholesterol and Niemann-Pick C1 protein in late endosomes, leading to the suggestion that the trafficking of ABCA1 to late endosomes might be involved in cholesterol efflux from this location (19). However, such co-localization studies do not prove that ABCA1 in late endosomes has a functional role.

The present studies provide strong new evidence to support
a functional role of ABCA1 in late endosomes by showing that a mutant ABCA1 with a defect in internalization from the cell surface also has a defect in cholesterol efflux after cholesterol labeling of late endosomes via SR-A. In contrast, the mutant shows higher cholesterol efflux after cell surface labeling, indicating specificity for the defect in late endosomal cholesterol efflux. The magnitude of the defect in late endosome cholesterol efflux (Figs. 4C and 5) represents a minimal estimate because movement of [3H]cholesterol between late endosomes and cell surface pools undoubtedly occurs during the SR-A-mediated [3H]cholesterol/acLDL uptake and labeling procedures.

In studies carried out before the discovery of the role of ABCA1 in lipid efflux, Takahashi and Smith (16) showed that a mutant ABCA1 with a defect in internalization from the cell surface probably degrades ABCA1 at the plasma membrane. Thus, the primary defect causing increased cell surface concentration of ABCA1-dPEST is likely decreased calpain proteolysis. The defect in internalization could arise because of alteration of an internalization motif or saturation of the internalization machinery secondary to increased cell surface concentration. However, the second explanation seems unlikely as increased cell surface concentration of ABCA1-wt secondary to apoA-I treatment (12) did not result in a defect in internalization (Fig. 1C). The PEST sequence does not contain any classical internalization motifs (18). Although it disrupts a putative dileucine-based motif, this was shown to be functionally important. Thus, it seems likely that the effects of deleting the PEST sequence on the internalization of ABCA1 either involve a nonclassical signal within the PEST sequence or are mediated indirectly, e.g. secondary to a conformational change involving a distant internalization motif.

The model suggested by our data is that in late endosomes cholesterol is released from degraded lipoproteins and deposited in the surrounding membrane. The activity of ABCA1 may serve to maintain a cholesterol gradient across the membrane. Although apoA-I can accept cholesterol from ABCA1 at the cell surface, it may also be internalized in association with ABCA1, picking up cholesterol in late endosomes followed by re-secretion from the cells. The latter could be a major pathway for ABCA1-mediated cholesterol efflux in macrophage foam cells.

REFERENCES