PHOSPHORYLATION OF A PEST SEQUENCE IN ABCA1 PROMOTES CALPAIN DEGRADATION AND IS REVERSED BY APOA-I.

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Running title: PEST Sequence Phosphorylation Regulates ABCA1 Calpain Degradation
Summary

ATP-binding cassette transporter A1 (ABCA1), the defective molecule in Tangier disease, mediates the apoAI-dependent efflux of excess cholesterol from cells. We recently showed that ABCA1 proteolysis by calpain was dependent on a PEST sequence in the cytoplasmic region of ABCA1 and was reversed by apoA-I interaction with ABCA1. We show here that phosphorylation of ABCA1 in HEK293 cells was reduced by 63 ± 2.4% after removal of the PEST sequence (ABCA1delPEST) or by incubation of cells with apoAI (58 ± 3.3%). By contrast, ABCA1delPEST showed no further decrease of phosphorylation upon apoAI treatment. To assess the hypothesis that PEST sequence phosphorylation could regulate ABCA1 calpain proteolysis, we mutagenized S/T residues in the PEST sequence and identified Thr-1286 and -1305 as constitutively phosphorylated residues. The ABCA1-T1286:A/T1305:A mutant was not degraded by calpain and was not further stabilized upon apoA-I treatment. The T1286:A/T1305:A mutant showed a 3.1-fold increase in cell surface expression and a 2.3 fold increase of apoAI-mediated cholesterol efflux compared to wild type ABCA1. In conclusion, we propose a mechanism of regulation of ABCA1 cell surface expression and function in which the interaction with apoA-I results in dephosphorylation of the ABCA1 PEST sequence and thereby inhibits calpain degradation leading to an increase of ABCA1 cell surface expression.
**Introduction**

Atherosclerosis, the major cause of death in industrialized societies (1), is initiated by the deposition of lipoprotein cholesterol in the artery wall and its subsequent uptake by macrophages, giving rise to cholesterol-engorged foam cells (2). Elevated levels of HDL are associated with a decrease in atherosclerosis that is believed to be due in part to the ability of HDL and its apolipoproteins to remove cholesterol from foam cells (3-5). A breakthrough in this area of research has been the identification of mutations in the ATP-binding cassette transporter A1 (ABCA1) (6-8) as the genetic defect in Tangier disease, a disorder characterized by very low plasma HDL levels, defective apolipoprotein-mediated phospholipid and cholesterol efflux from cells (9), macrophage foam cell accumulation in various tissues, including arteries and susceptibility to atherosclerosis (10). The phenotype of Tangier Disease is consistent with the proposed function of ABCA1 as a cell surface transporter that promotes the efflux of cellular phospholipid and cholesterol to lipid-poor apolipoproteins, a process that constitutes the initial step in HDL formation (11,12). Thus, the upregulation of ABCA1 expression may provide a key to HDL formation and promotion of foam cell cholesterol efflux.

The cellular expression of ABCA1 is highly regulated both on transcriptional and post-transcriptional levels (13). The turnover of ABCA1 protein is rapid with a half-life of less than one hour in murine macrophage-like cells and differentiated THP-1 cells (14,15). Recently we showed that ABCA1 protein degradation is regulated by a PEST sequence in the cytoplasmic region of ABCA1 and mediated by calpain protease (16). Interestingly, the interaction of ABCA1 with extracellular apoA-I inhibits calpain protease degradation in a PEST-sequence dependent fashion and thereby increases ABCA1 protein level at the
cell surface (16). Arakawa and Yokoyama independently discovered the stabilization of ABCA1 by apoA-I and suggested its degradation by a thiol protease (15). In the present study, we attempted to elucidate the nature of the signal by which apoA-I can reverse the PEST-dependent ABCA1 degradation by calpain. We first observed that apoA-I promotes PEST sequence dephosphorylation. It has been reported that calpain-mediated degradation can be induced by the phosphorylation of a target protein (17), which led us to the hypothesis that the ABCA1-PEST sequence phosphorylation might regulate ABCA1 calpain proteolysis. We identified Thr-1286 and Thr-1305 as constitutively phosphorylated sites in the ABCA1-PEST sequence and then examined the critical role of the Thr-1286 and Thr-1305 phosphorylation sites in the process of ABCA1 degradation by calpain.
Experimental Procedures

Chemicals and reagents. Human apoA-I (BIODESIGN International, Saco, Maine, USA) was dialyzed against PBS. M2 anti-FLAG antibody was from Sigma (St. Louis, Missouri, USA); The polyclonal ABCA1[159-F11] antibody, directed to the 15 Ct amino acid sequence of mouse and human ABCA1 (CLTSFLQDEKVKESYV), was kindly provided by Dr. R.Barbaras (INSERM U563, Toulouse, France). Protease inhibitor cocktail set was from Roche (Indianapolis, Indiana, USA). Phosphatase inhibitor cocktail set, purified µ-calpain and calpeptin were from Calbiochem-Novabiochem Corp. (San Diego, California, USA). All cell culture reagents were form Invitrogen Life Technologies (USA). All other reagents (analytical grade) were from Sigma (St. Louis, Missouri, USA).

Plasmid constructs and cell transfection. WT-mABCA1-FLAG and deleted of the PEST sequence (amino acids 1283-1306) was constructed as described (18). mABCA1-Flag mutation contracts on Thr-1286, Ser-1296, Ser-1302 and The-1305 to Ala were generated by PCR using Pfu polymerase (Stratagene), and confirmed by sequencing. mABCA1-Flag mutation constructs were further called: MutAAAA for mutation to Ala on T-1286/S-1296/S-1302/T-1305, MutTAAT for mutation to Ala on S-1296/S-1302, and MutASSA for mutation to Ala on T-1286/T-1305. HEK293 cells were grown in 6-, 12- or 24-well collagen-coated plates in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum then transiently transfected as previously described (16). 6-, 12-, and 24- wells plates were respectively transfected with 2, 1 and 0.5 µg plasmid DNA per well.
**Mouse peritoneal macrophages.** Peritoneal macrophages were isolated from male mice by peritoneal lavage with PBS 3 days after intraperitoneal injection with 1 ml of 3.85% thioglycollate (Becton Dickinson, Sparks, MD). The isolated cells were plated onto 6-well plates and allowed to adhere by incubation for 4 h at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies). After removal of nonadherent cells by washing with PBS, the cells were further incubated for 2 days in DMEM/10%FBS. To induce ABCA1 protein transcription, cells were incubated overnight with the specific synthetic LXR agonist, TO-901317 (0.5 μM). The day after induction, cells were used for phosphorylation experiments.

**[^32P]-labeling and Immunoprecipitation of ABCA1.** Confluent 6 wells plates transfected HEK293 cells or primary mouse macrophages, were washed with phosphate-free DMEM and incubated in phosphate-free DMEM for 1 h at 37 °C. Cells were then labeled with 500 μCi/well of[^32-P] orthophosphate for 3 h at 37 °C. Following[^32P]-labeling, cells were washed with fresh media, treated as indicated and then lysed at 4°C with RIPA buffer as previously described (16) in the presence of proteases and phosphatases inhibitors. For mouse peritoneal macrophages, the cell lysis proteins were first pre-incubated with protein A/G-agarose (Santa Cruz Biotechnology), then were incubated overnight with 20 μl of rabbit anti-ABCA1[159-F11] immunoserum followed by a 2h incubation with protein A/G-agarose. For HEK293 cells transfected with mABCA1 constructs carrying the FLAG epitopes, the cell lysis proteins were incubated with 20 μl of anti-FLAG affinity gel (Sigma, Saint Louis, USA). For both cells type, the agarose beads were then washed three times with the RIPA buffer and resuspended in 20 μl of 2.5× Laemmli sample buffer. Samples were loaded onto a 7.5% polyacrylamide...
gels, electrophoresed, and transferred to nitrocellulose. Radioactivity associated with the immunoprecipitated ABCA1 was measured with a phosphorimaging screen. Immunoblots were generated by blocking with 5% BSA/PBS for 1 h and incubating with 1:1000 rabbit anti-ABCA1[159-F11] immunoserum or 1:1000 mouse anti-Flag-monoclonal antibody (Sigma) in PBS overnight. Blots were washed with PBS/Tween 0.05% then incubated 1-h with 1:10,000 anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibody in PBS. Following three washes with PBS/Tween 0.05%, protein bands were visualized with ECL reagent. The relative intensities of the bands were determined by densitometry (ImageQuant© 2.2, Molecular Dynamics).

**FLAG cell surface expression assay.** mABCA1 constructs carrying the FLAG epitopes were transfected into HEK293 cells in 6-well plates. The next day, cells were first biotinylated with 0.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce Chemical Co., Rockford, Illinois, USA) at 4°C for 30 minutes. Then cells were lysed with RIPA buffer at 4°C. After centrifugation, the supernatant of cell lysates was incubated with anti-FLAG agarose beads overnight at 4°C. Following centrifugation and washing, the collected agarose beads were resuspended in 20 µl of 2.5× Laemmli sample buffer. Samples were loaded onto a 7.5% polyacrylamide gel, electrophoresed, and transferred to nitrocellulose. Cell surface ABCA1 was revealed by Western analysis with streptavidin–horseradish peroxidase, and then total ABCA1 protein was probed with anti-FLAG antibody.

**Cellular cholesterol efflux assays.** The assays were carried out as in ref. (18). Generally, HEK293 cells were labeled by culturing overnight in media containing [³H] cholesterol (0.5 µCi/ml). The next day, cells were washed twice with warm PBS and allowed to incubate in DMEM (1 mg/ml fatty acid free bovine serum albumin) at 37°C for 1h. The
cells were then incubated with fresh DMEM (1 mg/ml fatty acid free bovine serum albumin) with or without free apoA-I (10 µg/ml) at 37°C for 3 hours. The medium was removed and clarified of cellular material, and effluxed cholesterol was quantitated by scintillation counting. The cell layers were lysed in 0.1N NaOH, and the cellular cholesterol was quantitated by scintillation counting. The cholesterol efflux was expressed as the percentage effluxed (media counts / (media counts + cellular counts) ×100).

**Calpain-catalyzed proteolysis of ABCA1.** mABCA1 constructs carrying the FLAG epitopes were transfected into HEK293 cells in 12-well plates. The next day, cells were washed three times with fresh media and placed on ice for 10 minutes. Then cells were permeabilized by addition of 80 µg/ml digitonin in DMEM and incubated on ice for 15 minutes. Next, the cells were washed twice with fresh DMEM, and then purified µ-calpain in DMEM plus 2 mM CaCl₂ was added at the indicated concentration and incubated for 20 minutes at room temperature. Then cells were lysed by addition of 1 ml RIPA buffer with 40 µg/ml calpeptin. Total ABCA1 protein was determined by Western analysis using the anti-FLAG antibody and the relative intensities of the bands were determined by densitometry (ImageQuant© 2.2, Molecular Dynamics).
Results

*ApoA-I promotes dephosphorylation of the ABCA1 PEST sequence.* Transfected HEK293 cells were labeled with $[^{32}P]$ orthophosphate, then incubated with or without apoA-I in medium. ABCA1-FLAG was immunoprecipitated and analyzed by autoradiography and Western blotting. The level of phosphorylation was calculated from the ratio of $[^{32}P]$ / ABCA1 mass in immunoprecipitates, avoiding errors due to differences in recovery. Incubation with apoA-I resulted in a time- and dose-dependent reduction in the phosphorylation of WT-ABCA1, resulting in a maximum decrease of phosphorylation of $58.0 \pm 3.3\%$, $p < 0.01, n = 3$ (Fig 1). The phosphorylation of ABCA1 was also reduced by removal of the PEST sequence ($63.0 \pm 2.4\%, p < 0.01, n = 5$) and in contrast to wild type ABCA1 incubation with apoA-I did not result in any further reduction in phosphorylation (Fig 1A right panel and Fig 1B Time 0). After 1-2h, apoA-I (10 µg/ml) reduced the phosphorylation level of WT-ABCA1 to that seen for ABCA1delPEST (Fig 1B). ApoA-I treatment also appeared to increase ABCA1 protein level for WT-ABCA1 but not for ABCA1delPEST (Fig. 1A), a result consistent with our previous work which showed that stabilization of ABCA1 by apoA-I required the PEST sequence (16). However, differences in recovery of ABCA1 in immunoprecipitates led to variability in this response at individual time-points.

To provide further evidence for the apoA-I effect on ABCA1 phosphorylation *in vivo*, primary mouse macrophages were treated overnight with an LXR activator (TO-901317, 0.5 µM) to induce ABCA1 expression then labeled with $[^{32}P]$ orthophosphate. As evident
from Fig.2, incubation with 10 µg/ml of apoA-I for 2h decreased ABCA1 phosphorylation similar to the response in transfected HEK293 cells.

**The ABCA1 PEST sequence is constitutively phosphorylated on threonine residues.**

These results showing reduced phosphorylation of ABCA1 after removal of the PEST sequence led us to identify potential phosphorylation sites within the PEST sequence. Fig.3 shows the conserved PEST sequence among ABCA1 proteins from various species. The PEST sequence contains four potential phosphorylation sites, i.e. Thr-1286, Ser-1296, Ser-1302, and Thr-1305. We generated three mutants in which all four S and T residues, both S residues, or both T residues were mutated to alanine (A) (designated MutAAAA, MutTAAT and MutASSA respectively, Fig.3). Phosphorylation experiments were performed on transfected HEK293 cells expressing WT-ABCA1, ABCA1delPEST, or the S/T mutants. MutAAAA and MutASSA showed a reduction in phosphorylation level comparable to that observed for ABCA1delPEST, while MutTAAT showed similar phosphorylation to wild type ABCA1 (Fig. 4A, mean percentage decrease, for at least 3 different experiments, as compare to the WT-ABCA1 and after recovery protein correction: 57.8 ± 3.1, p<0.01 for MutAAAA; 61.7 ± 4.9, p<0.01 for MutASSA; 63.0 ± 2.4, p<0.01 for ABCA1delPEST and no significant decrease for MutTAAT). Additional phosphorylation experiments using constructs containing point mutants of either Thr-1286 or Thr-1305 showed that both Thr contribute to phosphorylation (data not shown). Thus, these data indicate that Thr-1286 and Thr-1305 are constitutive phosphorylation sites within the ABCA1 PEST sequence.
The PEST phosphorylation sites modulate ABCA1 cell surface expression and function. We next examined the effect of the phosphorylation mutants on the cell surface expression and function of ABCA1. MutTAAT, which has been shown to still contain the PEST phosphorylation sites (Fig. 4), had the same cell surface ABCA1 protein level as WT-ABCA1, whereas mutants on the constitutively phosphorylated sites (MutAAAA and MutASSA), showed an increase of cell surface ABCA1 protein to a level similar to ABCA1delPEST (Fig. 5A; mean fold-increase as compared to WT-ABCA1, n = 3 different experiments: 3.4 ± 0.3, p < 0.001 for MutAAAA; 3.3 ± 0.4, p < 0.001 for MutASSA; 3.1 ± 0.2, p < 0.01 for ABCA1delPEST). In contrast to effects on cell surface expression, there was a smaller and less reproducible increase in recovery of MutAAAA and MutASSA in total cell lysates, suggesting a specific effect on cell surface ABCA1 concentration, as shown previously for the PEST deletion mutant (16).

We have previously shown that deletion of the PEST sequence results in increased cell surface concentration of ABCA1 with a proportionate increase in functional activity (16). We thus investigated whether the stabilization of ABCA1 cell surface protein that resulted from mutation of the PEST sequence T residues was correlated with an increase of ABCA1 protein activity. The increase of cell surface ABCA1 protein obtained with MutAAAA and MutASSA (Fig. 5A) was associated with a more than two-fold increase of apoA-I dependent cellular cholesterol efflux as compared to the wild-type ABCA1 and reached a similar activity level to the ABCA1delPEST (Fig. 5B, mean fold increased as compared to WT-ABCA1, n = 5: 2.3 ± 0.3, p < 0.01 for MutAAAA; 2.3 ± 0.4, p < 0.01 for MutASSA; 2.4 ± 0.3, p < 0.01 for ABCA1delPEST). In contrast, the construct with
conserved PEST phosphorylation sites, MutTAAT, did not increase cellular cholesterol efflux to apoA-I as compared to WT-ABCA1 (1.0 ± 0.2, p < 0.01 for MutTAAT). This is consistent with the lack of increased cell surface expression observed with this mutant (Fig. 5A)

**The PEST phosphorylation sites modulate ABCA1 degradation by calpain and stabilization by apoA-I.**

Our previous work indicated that apoA-I acts via the PEST sequence to counteract a calpain-dependent degradation pathway (16), suggesting that PEST sequence phosphorylation could regulate ABCA1 calpain proteolysis. To address this issue, we performed experiments using calpeptin, a synthetic permeable calpain inhibitor. As shown in Fig. 6A, we confirmed that ABCA1 protein cell surface expression was significantly higher for MutASSA than for the WT-ABCA1. However, calpeptin treatment had no effect on cell surface MutASSA protein level whereas a significant increase was observed for WT-ABCA1 (mean fold increase upon 20 µg/ml calpeptin treatment, n = 3: 1.2 ± 0.3, p < 0.01 for MutASSA; 2.2 ± 0.4, p < 0.01 for WT-ABCA1). Further, to show that PEST sequence phosphorylation modulates ABCA1 degradation by calpain, cells were permeabilized and then treated with purified µ-calpain protease, a ubiquitously expressed subtype of calpain proteases. For WT-ABCA1, this treatment resulted in efficient degradation (Fig 6B, mean percentage decrease upon 0.4 µM calpain treatment: 80.2 ± 4.8%, p < 0.01, n=3), as reported previously (16). However, there was no appreciable degradation of MutASSA, providing direct evidence that ABCA1-mediated calpain proteolysis depends on the phosphorylation state of the PEST sequence.
Because we previously observed that free apoA-I increases ABCA1 protein level in parallel with reduced phosphorylation of the ABCA1 PEST sequence (Fig. 1 and (16)), we next examined the effect of free apoA-I on the ABCA1 mutant for the PEST sequence phosphorylation sites (MutASSA). We observed that the ability of free apoA-I to increase WT-ABCA1 cell surface protein level in HEK293 transfected cells was abolished for MutASSA (Fig. 5A; mean fold increased upon 10 µg/ml apoA-I incubation, n = 3: 2.31 ± 0.17, p < 0.01 for WT-ABCA1; 0.82 ± 0.21, p < 0.01 for MutASSA). This last result confirms that free apoA-I increases ABCA1 protein level in a process strictly dependent on the PEST sequence phosphorylation sites.

**CK2 and PKA inhibitors do not affect phosphorylation of the PEST sequence of ABCA1.**

Upon examination of the PEST sequence of ABCA1, we found that Thr-1286 and -1305 are respectively potential CK2 and PKA phosphorylation sites (19). To determine if CK2 and PKA kinases could be involved in the PEST sequence phosphorylation, we examined the effect of the CK2 inhibitor, apigenin (17) and a PKA inhibitor, H-89 (20) on ABCA1 phosphorylation. As shown in Fig. 7A, treatment of HEK293 transfected cells with 20 µM apigenin for 3 hours does not alter phosphorylation of either WT-ABCA1 or MutASSA. Increasing apigenin concentration (30, 40 µM) or longer incubation time (8h) also failed to change ABCA1 phosphorylation (data not shown). These data suggest that CK2 does not mediate either phosphorylation of the PEST sequence of ABCA1, or of the whole ABCA1 protein. Treatment of HEK293 transfected cells with 20 µM H-89 PKA
inhibitor for 3 hours induces a significant decrease of the WT-ABCA1 phosphorylation (Fig. 7B, mean percentage decrease as compared to the control without H-89: 35.4 ± 3.4%, p < 0.01, n=3). Furthermore, we observed that H-89 PKA inhibitor does not affect cell surface expression of ABCA1 (data not shown). This result is consistent with two recent independent studies (20,21) showing that PKA promotes ABCA1 phosphorylation and subsequent apoA-I-dependent phospholipid efflux in an independent fashion to ABCA1 protein stability. However, we observed a similar decrease in the phosphorylation of MutASSA upon H-89 treatment (Fig. 1B, mean percentage decrease as compare to the control without H-89: 29.6 ± 4.1%, p < 0.01, n=3), which suggests that PKA is not involved in the phosphorylation of the PEST sequence of ABCA1 but act on other ABCA1 phosphorylation sites as suggested by Haidar et al. (20).
**Discussion**

By mutating serine and threonine residues to alanine, we provide evidence for the basal phosphorylation of threonine residues (1286 and 1305) within the PEST sequence of ABCA1. Interestingly, phosphorylation of these residues is abolished by incubation with extracellular apoA-I, in a process that parallels the PEST-dependent stabilization of ABCA1 by apoA-I (16). These findings indicate that apoA-I stabilization of ABCA1 is mediated by reduced PEST sequence phosphorylation, which in turn leads to decreased calpain proteolysis of ABCA1. Mutation of the PEST phosphorylation sites leads to increased cell surface expression and functional activity of ABCA1, suggesting that modulation of kinase or phosphatase activity at the PEST sequence could represent a new therapeutic approach to up-regulation of cellular ABCA1 activity.

Our findings indicate that phosphorylation of the PEST sequence has a key role in the stabilization of ABCA1 by apoA-I. The time course and dose response of apoA-I induced dephosphorylation of the PEST sequence paralleled the effects of apoA-I on stabilization of ABCA1 (16) and mutants of the threonine residues that prevent phosphorylation produced similar effects to apoA-I on cell surface concentration of ABCA1 (Fig. 5) and were not additive to the effects of apoA-I (Fig. 6C), strongly suggesting that the effects of apoA-I on ABCA1 stabilization were mediated by decreased PEST phosphorylation. The phosphorylation defective mutant also was resistant to exogenous calpain (Fig. 6B) and showed no additional increase in level in the presence of calpain inhibitor, calpeptin,
indicating that apoA-I reduces phosphorylation of PEST threonine residues and thereby abolishes calpain proteolysis.

The mechanism linking the binding of extracellular apoA-I to changes in ABCA1 PEST phosphorylation is unknown. Our earlier work on the apoA-I mediated stabilization of ABCA1 suggested that the mechanism involves either a conformational change in ABCA1 brought about by binding of apoA-I to ABCA1, or a local change in the membrane secondary to apoA-I mediated phospholipid efflux. A mutant (ABCA1-W590S) showed normal levels of binding of apoA-I but decreased phospholipid efflux (22,23). This mutant was not stabilized by apoA-I implying that phospholipids efflux is required for apoA-I-mediated ABCA1 stabilization (16). However, we could not exclude the possibility that apoA-I binds to this mutant in an incorrect orientation, preventing an appropriate conformational change. Thus, either a conformational change of ABCA1 brought about by binding of apoA-I, or a membrane alteration secondary to phospholipid efflux could lead to altered access of a kinase or phosphatase acting at the PEST sequence (i.e. kinase/phosphatase substrate availability). Alternatively, we cannot exclude the possibility that apoA-I binding or phospholipid efflux induces a signaling cascade that leads to altered kinase or phosphatase activity.

It has been previously shown that phosphorylation by protein kinase CK2 within the IκBα-PEST sequence promotes calpain-mediated degradation of IκBα, by enhancing the binding of calpain to the PEST sequence (17). However, in contrast to the relatively well defined mechanism by which PEST sequences can enhance ubiquitination of substrates
(24), there is no general agreement on the mechanism by which PEST sequences enhance calpain activity on target proteins. Although the PEST sequence contained a potential CKII consensus sites, experiments using inhibitors excluded a role of CKII in PEST sequence phosphorylation.

Two different groups have recently reported that ABCA1 was constitutively phosphorylated in vivo on RAW macrophages (21) and human skin fibroblasts (20) and also in transfected HEK293 cells (21). In both studies, protein kinase A (PKA) has been showed to be involved in the ABCA1 phosphorylation and See et al. have suggested by in vitro kinase assay that Ser-1042 and Ser-2054, located in the nucleotide binding domain of ABCA1, are major phosphorylation sites for PKA. Furthermore the authors reported that ABCA1 phosphorylation by PKA on Ser-2054 regulate apoA-I-dependent cholesterol and phospholipid efflux but do not affect apoA-I binding or ABCA1 protein stability. Here we confirmed that ABCA1 is constitutively phosphorylated in primary mouse macrophages and transfected HEK293 cells. We observed that phosphorylation of ABCA1 was decreased by about 60% after mutation of Thr-1286 and -1305 residues in the PEST sequence domain of ABCA1. Therefore, the 40% of phosphorylation level maintained after mutation of Thr-1286 and -1305 is consistent with other potential sites of phosphorylation outside the PEST sequence, such as Ser-1042 and Ser-2054. In contrast with the phosphorylation site on Ser-2054 that does not affect ABCA1 protein stability (21), we showed by mutagenesis that the phosphorylation on Thr-1286 and -1305 residues in the PEST sequence domain of ABCA1, decreases ABCA1 protein stability and thereby ABCA1 cell surface levels and activity. Therefore, these data
confirmed that PKA is involved in the phosphorylation of ABCA1 protein, but act on a different domain than the PEST sequence, possibly on Ser-1042 and Ser-2054 as previously suggested (21).

As in earlier studies of the PEST deletion mutant, the predominant effect of mutation of the threonine phosphorylation sites was to increase cell surface ABCA1 concentration, with a smaller increase in ABCA1 in total cell lysates. This suggests that PEST sequence dependent degradation primarily involves ABCA1 in the plasma membrane. This could reflect targeting of calpain to plasma membrane ABCA1. An alternative possibility is that calpain cleavage of ABCA1 leads to exposure of an internalization motif that is normally involved in endocytic removal of ABCA1 from the plasma membrane. It is interesting to note the parallel effects of the different mutants on cell surface concentration and cholesterol efflux activity (Fig. 5). This suggests that lipid efflux is primarily mediated by cell surface ABCA1. However, other evidence suggests intracellular trafficking of both apoA-I and ABCA1 (25-27), and further studies will be needed to settle the issue of whether cellular cholesterol efflux involves internalization and recycling of ABCA1.

Studies in ABCA1 transgenic and knock-out animals, as well as in humans with Tangier Disease, suggest that macrophage ABCA1-mediated lipid efflux is anti-atherogenic (10,28,29). Up-regulation of ABCA1 expression mediated by LXR activators is also associated with anti-atherogenic effects (30) but is complicated by fatty liver and hypertriglyceridemia (31,32). Thus, alternative ways to increase ABCA1 activity are being sought. The stabilization of ABCA1 by apoA-I occurs in vivo (16) and appears
relevant to the anti-atherogenic effects of apoA-I infusion or overexpression (33-36). This stabilization mechanism might provide a way to specifically target ABCA1. Several approaches could be envisaged, such as peptides or antibodies that mimic the effect of apoA-I binding to ABCA1. The present results suggest a novel approach involving small molecules that would decrease phosphorylation of the threonine residues within the PEST sequence of ABCA1. There are a large number of kinases and phosphatases in the mammalian genome, and recent clinical development of kinase inhibitors in the treatment of chronic myelogenous leukemia (37) suggests the future potential for this approach in drug development.
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Footnotes

This work was supported by grant HL22682 and LM was a recipient of the fellowship “Lavoisier” from the “Ministère Français des Affaires Etrangères”.

Abbreviations: ABCA1, ATP-binding cassette transporter A1; PEST, proline-glutamic acid-serine-threonine; apo, apolipoprotein; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; CK2, protein kinase CK2 (formerly casein kinase II); PKA, protein kinase A.
Figure legends

Fig 1. **ApoA-I decreases PEST-ABCA1 phosphorylation.** ABCA1-FLAG or ABCA1delPEST-FLAG were transiently transfected in HEK 293 cells, then labelled with 500 µCi/well of [$^{32}$P] orthophosphate as described under “Experimental Procedures”. Cells were washed then incubated with DMEM media containing apo A-I (10 µg/ml) for indicated times (A-B) or with increasing concentration of apo A-I for 2 hours (C). Cells were lysed, and ABCA1 protein was immunoprecipitated with FLAG antibody, resolved on SDS-polyacrylamide gel, and transferred to nitrocellulose. Phosphorylated ABCA1 (32P-ABCA1) was revealed by autoradiography then total ABCA1 protein was probed with FLAG antibody. In (B) and (C) the level of ABCA1 phosphorylation was determined from phosphorylation signal normalized for total ABCA1 protein level. The results are representative of two or more independent experiments. The *error bars* represent the standard deviations.

Fig 2. **ApoA-I decreases endogenous ABCA1 phosphorylation in primary mouse macrophages.** Primary mouse macrophages in 6 wells were labelled with 500 µCi/well of [$^{32}$P] orthophosphate as described under “Experimental Procedures”. Cells were washed then incubated with DMEM media with or without apo A-I (10 µg/ml) for 2 hours. Cells were lysed, and ABCA1 protein was immunoprecipitated with rabbit anti-ABCA1[159-F11] affinity-purified polyclonal antibody, resolved on SDS-polyacrylamide gel, and transferred to nitrocellulose. Phosphorylated ABCA1 (32P-
ABCA1) was revealed by autoradiography then total ABCA1 protein was probed with rabbit anti-ABCA1[159-F11] antibody.

Fig 3. **Schematic representation of ABCA1 and the conserved phosphorylation sites in the PEST sequence.** The PEST sequences in ABCA1 proteins identified in human (h), mouse (m) and chicken (c) are shown to indicate their conservation. The conserved serine and threonine residues are highlighted. Mutants were constructed involving both T and S residues (AAAA), only S residues (TAAT) or only T residues (ASSA).

Fig 4. **Mutation of Thr residues (1286 and 1305) decreases ABCA1 Phosphorylation**

WT-mABCA1-FLAG, mABCA1delPEST-FLAG, mABCA1-FLAG mutated to Ala on T-1286/S-1296/S-1302/T-1305 (MutAAAA), mutated to Ala on S-1296/S-1302 (MutTAAT), or mutated to Ala on T-1286/T-1305 (MutASSA), were transiently transfected in 6 wells HEK-293 cells (2 µg plasmid DNA per well). Cells were labelled with 500 µCi/well of $[^{32}\text{P}]$ orthophosphate as described under “Experimental Procedures”, then lysed. ABCA1 protein was immunoprecipitated with FLAG antibody, resolved on SDS-polyacrylamide gel, and transferred to nitrocellulose. Phosphorylated ABCA1 (32P-ABCA1) was revealed by autoradiography then total ABCA1 protein was probed with FLAG antibody.
Fig 5. **Increased ABCA1 cell surface protein level and efflux activity with Thr-1286 and Thr-1305 mutations.**

HEK293 cells were transfected with either empty vector (mock), WT-ABCA1, ABCA1delPEST or ABCA1 construct carrying the indicated mutations (MutTAAT, MutAAAA, MutASSA). (A) Cells were biotinylated at the surface as described under “Experimental Procedures”, then lysed. ABCA1 protein was immunoprecipitated with FLAG antibody, resolved on SDS-polyacrylamide gel, and transferred to nitrocellulose. Cell surface ABCA1 was revealed by Western analysis with avidin-horseradish peroxidase, and then total ABCA1 protein was probed with FLAG antibody. The results are representative of three independent experiments. (B) Cell were labeled overnight with 1mCi/ml [3H] cholesterol for a next day cholesterol efflux experiment which was carried out with or without apoA-I (10 µg/ml) for 3h as described under “Experimental Procedures”. Counts of [3H] cholesterol were determined for the medium and cells and expressed as apoA-I-dependent efflux (apoA-I-dependant efflux minus medium only dependant efflux). The results are representative of five or more independent experiments with measurements performed in triplicate. The *error bars* represent the standard deviations and, for each experiment, *p* < 0.01.
Fig 6. **ABCA1 mutated on Thr-1286 and Thr-1305 is not degraded by calpain and is not stabilized by apoA-I.**

WT-mABCA1-FLAG or mABCA1-FLAG mutated to Ala on T-1286/T-1305 (MutTSSA) were transiently transfected into HEK293 cells. Cells were then treated with or without calpeptin (3h, 20 µg/ml, Panel A), µ-calpain (as indicated under “Experimental Procedures”, Panel B) or apoA-I (2h, 10 µg/ml, Panel C). Panel A and C, cell surface proteins were biotinylated as described under “Experimental Procedures”. After cell lysis and immunoprecipitation with anti-FLAG antibody, cell surface ABCA1 was revealed by Western-blot analysis with avidin-horseradish peroxidase, and then total ABCA1 protein was probed with FLAG antibody. The results are representative of three independent experiments. The relative intensities values of the bands are as follows: WT-ABCA1, 1.1 ± 0.2 versus 2.3 ± 0.3 upon calpeptin treatment and 1.1 ± 0.2 versus 3.6 ± 0.2 upon apoA-I treatment; MutASSA, 2.5 ± 0.3 versus 2.6 ± 0.2 upon calpeptin treatment and 2.9 ± 0.3 versus 2.7 ± 0.2 upon apoA-I treatment. Panel B, Cells were lysed then total cell lysis proteins were immunoblotted with FLAG antibody. The results are representative of three independent experiments. The relative intensities values of the bands are as follows: WT-ABCA1, 1.1 ± 0.2; 0.8 ± 0.1; 0.2 ± 0.1 upon µ-calpain 0, 0.1 and 0.4 µM respectively, and MutASSA, 1.9 ± 0.2; 1.7 ± 0.1; 1.8 ± 0.2 upon µ-calpain 0, 0.1 and 0.4 µM respectively.
Fig 7. **Effect of apigenin (CK2 inhibitor) and H-89 (PKA inhibitor) on ABCA1 phosphorylation.**

WT-mABCA1-FLAG or mABCA1-FLAG mutated to Ala on T-1286/T-1305 (MutASSA), were transiently transfected in 6 wells HEK-293 cells (2 µg plasmid DNA per well). Cells were labelled with 500 µCi/well of [³²-P] orthophosphate as described under “Experimental Procedures”. Cells were washed then incubated with either 0 (carrier DMSO), 20 µM apigenin (panel A), or 0 (carrier methanol), 20 µM H-89 (panel B) for 3h. Cells were lysed and ABCA1 protein was immunoprecipitated with FLAG antibody, resolved on SDS-polyacrylamide gel, and transferred to nitrocellulose. Phosphorylated ABCA1 (32P-ABCA1) was revealed by autoradiography then total ABCA1 protein was probed with FLAG antibody.
Fig 1.

**A**

| Time (h) | 0 | 0.5 | 1 | 2 |
|----------|---|-----|---|---|
| ApoA-I   | - | +   | - | + |
| 32P-ABCA1 |   |     |   |   |
| ABCA1    |   |     |   |   |

**B**

| Ratio 32P / protein level (arbitrary unit) |
|------------------------------------------|
| 1.5                                      |
| 1.0                                      |
| 0.5                                      |
| 0.0                                      |

**C**

| Ratio 32P / protein level (arbitrary unit) |
|------------------------------------------|
| 1.5                                      |
| 1.0                                      |
| 0.5                                      |
| 0.0                                      |

**Time Course**

**Dose Response (2H)**
Fig 2. 

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Fig 3.

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Fig 3.
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Fig.4
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Fig. 5

A

Cell Surface ABCA1

Total ABCA1 protein

B

% ApoA-I dependent efflux (% of total cellular cholesterol)

Mock

WT-ABCA1

Mut AAAA

Mut TAAT

Mut ASSA

PestLess-ABCA1
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A

| Calpeptin (20 µg/ml) | Vector | WT-ABCA1 | Mut ASSA |
|----------------------|--------|----------|----------|
| -                    | -      | +        | -        |
| Cell Surface ABCA1   |        |          |          |

B

| µ-calpain (µM)       | Vector | WT-ABCA1 | Mut ASSA |
|----------------------|--------|----------|----------|
| 0                    | 0      | 0.1      | 0.4      |
| ABCA1 protein        |        |          |          |
| β-actin              |        |          |          |

C

| ApoA-I (10 µg/ml)   | Vector | WT-ABCA1 | Mut ASSA |
|---------------------|--------|----------|----------|
| -                   | -      | +        | -        |
| Cell Surface ABCA1  |        |          |          |
| Total ABCA1 protein |        |          |          |

Fig. 6
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Fig. 7

**A**

|           | WT-ABCA1 | Mut ASSA |
|-----------|----------|----------|
| Apigenin (20 µM) | -       | +        |
| 32P-ABCA1   | -       | +        |
| ABCA1 protein | -       | +        |

**B**

|           | WT-ABCA1 | Mut ASSA |
|-----------|----------|----------|
| H-89 (20 µM) | -       | +        |
| 32P-ABCA1   | -       | +        |
| ABCA1 protein | -       | +        |
Phosphorylation of a pest sequence in ABCA1 promotes calpain degradation and is reversed by APOA-I
Laurent Martinez, Birgit Agerholm-Larsen, Nan Wang, Wengen Chen and Alan R. Tall
J. Biol. Chem. published online July 17, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307161200

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