Apolipoprotein A-I Activates Protein Kinase Ca Signaling to Phosphorylate and Stabilize ATP Binding Cassette Transporter A1 for the High Density Lipoprotein Assembly

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ATP-binding cassette transporter A1 (ABCA1) plays an essential role in the helical apolipoprotein-mediated assembly of high density lipoprotein, and the apolipoproteins stabilize ABCA1 against calpain-mediated degradation during the reaction (2002 J. Biol. Chem. 277, 22426–22429). Protein kinase C (PKC) inhibitors suppressed both ABCA1 stabilization and cellular lipid release mediated by apolipoprotein A-I (apoA-I) but not ABCA1 increase by calpain inhibitors. The increase of ABCA1 and the cellular lipid release by apoA-I were both suppressed by a phosphatidylcholine phospholipase C (PC-PLC) inhibitor but not by the inhibitors of phosphatidylinositol-PLC and phosphatidylinositol 3-kinase. A protein phosphatase inhibitor further enhanced the ABCA1 increase by apoA-I. Biochemical and microscopic evidence indicated that apoA-I activated PKCa, and phosphorylation of ABCA1 was directly demonstrated by apoA-I via PKC. Finally, digestion of sphingomyelin increased ABCA1, and a PC-PLC inhibitor suppressed it. We conclude that apoA-I activates PKCα and phosphorylation of ABCA1 by PC-PLC-mediated generation of diacylglycerol initiated by the removal of cellular sphingomyelin (2002 J. Biol. Chem. 277, 44709–44714), and subsequently phosphorylates and stabilizes ABCA1.

Cholesterol is an essential lipid molecule having many important biological functions, such as regulation of the general physicochemical properties of the biomembrane, formation of its microdomains with sphingomyelin to accommodate various signal-related molecules, direct modification of a key signal protein hedgehog for development, and biosynthesis of steroid hormones and bile acids. On the other hand, cholesterol is synthesized in most somatic cells, whereas its catabolic site is limited to the liver and steroidogenic cells except for partial hydroxylation in the peripheral cells. Therefore, release of cell cholesterol is a major event in cholesterol homeostasis not only for cells but also for whole animal bodies. High density lipoprotein (HDL) plays a central role in this reaction by mediating two distinct pathways: the nonspecific physicochemical exchange of cholesterol and the removal of cholesterol and phospholipid by lipid-free helical apolipoproteins to generate HDL.

Fibroblasts from patients with Tangier disease, a familial HDL deficiency, lack the apolipoprotein-mediated HDL assembly reaction (2, 3), indicating that plasma HDL is generated mainly by this mechanism. Defective mutations have been identified in the gene of ATP-binding cassette transporter A1 (ABCA1) of these patients, indicating the essential role of ABCA1 in the generation of HDL (4–6). Loss of HDL in obca1 knockout mice further confirmed its role (7, 8). The ABCA1 level is shown to be a rate-limiting factor of HDL production by: increasing the apolipoprotein A-I (apoA-I)-mediated release of cellular lipid with the increase of ABCA1 by its transfection (9, 10); treatment with cAMP analogues (11, 12); enhancement of its transcription by liver X receptor and/or retinoid X receptor ligands (13, 14); and the increase of plasma HDL in ABCA1 transgenic mice (15). Thus, the essential role of ABCA1 in the HDL assembly reaction is obvious, although the molecular mechanism for ABCA1 to mediate the cellular lipid release and HDL assembly is still vague.

We and others have discovered that helical apolipoproteins in their free form stabilize ABCA1 against its degradation by thiol protease, most likely calpain, and increase its protein level in cells (16, 17). This is a self-activated positive feedback system for the HDL assembly, so that its molecular mechanism is one of the key factors for regulation of cellular cholesterol homeostasis and plasma HDL level. In this paper, we demonstrate that the protein kinase C (PKC) signaling pathway plays an essential role in the apoA-I-induced stabilization and phosphorylation of ABCA1. This particular signaling is presumably activated by diacylglycerol (DAG) produced by phosphatidylcholine-phospholipase C (PC-PLC) for replenishment of sphingomyelin (SPM) removed by apoA-I (18).

EXPERIMENTAL PROCEDURES

Materials—ApoA-I was isolated from fresh human HDL (33). H-7 and HA1004 were purchased from Seikagaku Corporation (Tokyo, Japan). Α-erythro-sphingosine and chelerythrine were from ICN Biomedicals (Costa Mesa, CA) and Research Biochemical International (Natick, MA), respectively. Phorbol 12-myristate 13-acetate (PMA), 4α-

diacylglycerol; PC-PLC, phosphatidylcholine-phospholipase C; SPM, sphingomyelin; apoA-I, apolipoprotein A-I; PMA, phorbol 12-myristate 13-acetate; ALLN, N-acetyl-Leu-Leu-norleucinal; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RT-PCR, reverse transcriptase polymerase chain reaction; CD, cyclodextrin(s); CHX, cycloheximide; PKA, protein kinase A; PI, phosphatidylinositol; MEM, minimum Eagle’s medium; GFP, green fluorescent protein; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase; extra-cellular signal-regulated kinase.
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RESULTS

Cell Culture and Treatments—Human fibroblast cell line cells WI-38 (Biker Cell Bank) were grown to confluence in 100-mm, 60-mm, or 8-well dishes as described (19). In some experiments, ABCA1 expression was enhanced by 1 μg/ml 9-cis retinoic acid (Wako Pure Chemicals) for 16–24 h in fetal calf serum/MEM. HEK293 cells and a clone of those stably expressing human ABCA1-GFP were grown as described (10, 20). Cells were washed twice with phosphate-buffered saline (PBS) and incubated with various compounds and apoA-I in MEM containing 0.1% fatty acid-free bovine serum albumin (BSA/MEM). None of conditions for cell treatment influenced cell viability as determined by LDH release assay (Wako), cellular protein, and lipid level.

Cellular Lipid Release Assay—WI-38 cells were seeded into 6-well trays at a density of 1.0–1.5 × 10⁶ cells/well and grown to a confluent stage. Cellular lipid release was induced by 10 μg/ml apoA-I or 0.5% CD in BSA/MEM. Lipid was extracted from the medium and cells, and total and free cholesterol and choline-phospholipid were measured (12, 19). Cellular protein was determined using a BCA Protein Assay Kit (Pierce).

Western Blotting of ABCA1—Total membrane fraction was prepared, and ABCA1 was analyzed by immunoblotting with rabbit antisera against the C-terminal peptide of human ABCA1 (16, 19, 21). The specificity of the antibody was verified in our previous work, such as detection of specific increase of ABCA1 by CAMP in RAW264 cells (19), identification of the transfected ABCA1-GFP in HEK293 cells by cross-detection of specific increase of ABCA1 by cAMP in RAW264 cells (19), and ABCA1 was analyzed by immunoblotting with rabbit antiserum against the C-terminal peptide of human ABCA1 (16, 19, 21). The density of the visualized protein bands was quantitated using a Fuji BAS 2000 scanner (Fujifilm) was used to visualize and quantitate radiolabeled proteins. The supernatant was analyzed by immunoprecipitation using rabbit anti-human ABCA1 serum and protein A-agarose (Santa Cruz Biotechnology). The supernatant was centrifuged at 100,000 × g before termination of incubation. After the treatment, the cells were lysed with PBS and collected. The cells were lysed with a buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, and 1% (v/v) protease inhibitor mixture) by rotation for 20 min at 4 °C. When the cells were lyzed, the lysate buffer contained phosphatase inhibitor mixture (Sigma) as a final concentration of 1% (v/v). The cell lysate was centrifuged at low speed to remove cellular and nuclear debris. The supernatant was analyzed by immunoprecipitation using rabbit anti-human ABCA1 serum and protein A-agarose (Santa Cruz Biotechnology) after the cell lysate was incubated with protein A-agarose and standard rabbit serum (16). Immunoprecipitated ABCA1 was analyzed by SDS-PAGE and autoradiographed at ~80 °C. A Fuji Radiography Film (FujiFilm) was used to visualize and quantitate radiolabeled proteins.

Reverse Transcriptase (RT)-PCR Analysis—Total RNA was prepared by using a RNeasy Kit (Qiagen) from WI-38 cells at a confluent stage in 100-mm dish or HEK293 cells, and ABCA1 was analyzed. ABCA1 expression was enhanced by 1 μg/ml 9-cis retinoic acid (Wako Pure Chemicals) for 16–24 h in fetal calf serum/MEM. HEK293 cells and a clone of those stably expressing human ABCA1-GFP were grown as described (10, 20). Cells were washed twice with phosphate-buffered saline (PBS) and incubated with various compounds and apoA-I in MEM containing 0.1% fatty acid-free bovine serum albumin (BSA/MEM). None of conditions for cell treatment influenced cell viability as determined by LDH release assay (Wako), cellular protein, and lipid level.

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Reverse Transcriptase (RT)-PCR Analysis—Total RNA was prepared by using a RNeasy Kit (Qiagen) from WI-38 cells at a confluent stage in 100-mm dish after incubation under various conditions. First-strand cDNA synthesized by using total RNA and a SuperScript First-Strand System (Gibco BRL) was amplified by TaqDNA polymerase (Takara) with specific primers for human ABCA1 cDNA and glyceraldehyde-3-phosphate dehydrogenase cDNA for 20, 24, and 28 cycles of PCR (16). The RT-PCR product was analyzed in a 2.5% agarose gel electrophoresis and stained with SYBR Gold Nuclear Acid Gel Stains (Molecular Probes).

PKC Assay—Cells in a confluent stage in 100-mm dishes were incubated in BSA/MEM for 20–24 h prior to stimulation by apoA-I, 10 μg/ml, or PMA, 160 nM, for 20–30 min. The cells were washed three times with ice-cold PBS, scraped, and pelleted by centrifugation at 700 × g for 5 min for membrane preparation. The pellet was resus-
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A PKC activation is initiated by the production of DAG mediated by PC hydrolysis by PC-PLC and/or phosphatidylinositol (PI) turnover by PI-PLC. We therefore undertook experiments to observe the effects of inhibitors of the PLCs. The stabilization of ABCA1 by apoA-I (increase to 180%) was counteracted by D609, a PC-PLC inhibitor (102%), but not by the PI-PLC inhibitor U73122 (206%) and (its inactive analogue, U73343, 190%) (Fig. 5A, panel 1). The ABCA1 stabilization was

and presence of G66976, respectively. Therefore, PKC is involved in the steps initiated by apoA-I and not directly in the proteolysis by calpain.

Fig. 4 shows the effects of PKC inhibitors on the apoA-I-mediated release of cellular lipid. H-7 inhibited the cholesterol release from WI-38 in a dose-dependent manner to reach a maximum of 52% at 50 μM (Fig. 4A). The release of phospholipid also decreased by 20% (Fig. 4B). In contrast, HA1004, a control compound of H-7 and an inhibitor of protein kinase A (PKA), did not influence the apoA-I-mediated lipid release. A potent PKA inhibitor, H-89, however, inhibited the apoA-I-mediated cholesterol and phospholipid release by 56% and 76%, respectively, at 10 μM, consistent with previous observations (27, 28) (data not shown). Nonspecific cholesterol efflux to CD was reduced by neither H-7 nor HA1004 (an apparent increase by H-7 at the highest concentration was because of the apparent decrease of cell protein, for unknown reasons, and the efflux per well and percent efflux were both unchanged) (Fig. 4C). In addition, more specific PKC inhibitors, bisindolylmaleimide I, sphingosine, and G66976, reduced the apoA-I-mediated release of cholesterol and phospholipid by 40–60% and 20–30%, respectively (Fig. 4, D–F). Short-time incubation (4 h) also yielded similar results (data not shown). Other PKC inhibitors such as chelerythrine and long-term treatment with PMA also inhibited apoA-I-mediated cholesterol release (data not shown). The results were thus consistent with the effects of the PKC inhibitors on the apoA-I-induced change of ABCA1.

PKC-related signal transduction is involved in the apoA-I-induced cellular events (22–25), the effect of PKC modulators on this reaction was examined in WI-38 human fibroblasts (Fig. 3). ApoA-I-induced increase of ABCA1 (284%) was suppressed by H-7, bisindolylmaleimide I, and sphingosine to 132, 142, and 140%, respectively (Fig. 3A), whereas PKC activation by PMA increased ABCA1 (170–180% by PMA and 103% by 4αPMA) (Fig. 3B). G66976, a specific inhibitor of Ca²⁺-dependent PKCs and -βI (26), almost canceled the apoA-I-mediated 2–3-fold increase of ABCA1 at either 4 or 24 h, and it prevented retardation of the ABCA1 decay by apoA-I when translation was blocked by CHX to the same extent based on density (Fig. 3C). On the other hand, the inhibitors of PKC did not influence the mRNA level of ABCA1 (Fig. 3D). In the absence of apoA-I, ABCA1 level was not significantly influenced by PKC inhibitors, and the increase of ABCA1 by the calpain inhibitors ALLN and calpeptin was unaffected by G66976 (Fig. 3E). The increase was to 160 and 180% by ALLN and 192 and 170% by calpeptin in the absence and presence of G66976, respectively. Therefore, PKC is involved in the steps initiated by apoA-I and not directly in the proteolysis by calpain.

Fig. 2. Calpain inhibitors suppress ABCA1 degradation. A, WI-38 cells were incubated with ALLN for 1 h, and the membrane fraction was analyzed for ABCA1 by Western blotting. B, WI-38 cells were incubated with 50 μM ALLN in the presence of CHX (20 μg/ml) for 2 h. The membrane fraction was analyzed for ABCA1. C, cells were incubated with calpain inhibitors (100 μM ALLN, 100 μM calpeptin, and 100 μM PD150606) for 3 h, and ABCA1 protein was analyzed. D, WI-38 cells pretreated with 9-cis retinoic acid to enhance ABCA1 expression were pulse-labeled with 0.2 μCi/ml [35S]methionine/cysteine mixture for 2 h followed by chase with or without 100 μM ALLN for 2 h. ABCA1 was immunoprecipitated from 500 μg of cell lysate protein. E, HEK293 cells and those expressing ABCA1-GFP were pulse-labeled and treated as described in D, and the cell lysate, 500 μg of protein, was used for immunoprecipitation analysis of ABCA1.

192% at 24 h). On the other hand, equivalent cholesterol removal by CD (19) resulted in decrease of ABCA1 (64%), suggesting that cholesterol removal is not a trigger of the ABCA1 stabilization. The slow catabolic rate of ABCA1 by apoA-I was demonstrated in the presence of cycloheximide (CHX) (Fig. 1B) and by a pulse labeling experiment (Fig. 1C). In contrast, ABCA1 mRNA was not influenced by apoA-I for 4 h; rather, it decreased after 24 h, moderately by apoA-I, and markedly by CD, presumably because of the decrease of cell cholesterol (Fig. 1D). Retardation of ABCA1 catabolism by apoA-I was also shown for the transfected ABCA1-GFP in HEK293 (20) (Fig. 1E).

Consistent with previous work (16, 17), a thiol protease inhibitor, ALLN, retarded ABCA1 degradation in WI-38 and HEK293/ABCA1-GFP. ABCA1 was apparently increased up to 170% by ALLN (Fig. 2A) and up to 150% by calpain inhibitors calpeptin and PD150606 (Fig. 2C), respectively, in WI-38. The retarded degradation of ABCA1 by ALLN was demonstrated by inhibiting translation by CHX and by pulse-labeling in WI-38 (Fig. 2, B and D) and in HEK293 (Fig. 2E). Thus, the calpain-mediated degradation of ABCA1 was shown to be a common pathway to regulate cellular ABCA1.
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not influenced by the PI 3-kinase inhibitor LY294002 either (180%), further supporting the hypothesis that PI turnover is not involved in this reaction (Fig. 5A, panel 1). Our recent finding indicated that acute reduction of SPM in cells by digestion or by apoA-I-mediated removal induces SPM replenishment by the PC-PLC-mediated PC hydrolysis that generate phosphorylcholine and DAG (18). Therefore, the effect of SPM digestion on ABCA1 was examined. ABCA1 was increased by sphingomyelinase treatment to 200–250% (Fig. 5A, panel 2, SPMase), and both Go6976 and D609 partially inhibited this effect by reversing the 140% increase to 101 and 99%, respectively (Fig. 5A, panel 3). The effect of the PLC inhibitors on apoA-I-mediated lipid release was consistent with the effects of these inhibitors on the increase of ABCA1 by apoA-I; only D609 suppressed the release of cholesterol and phospholipid by apoA-I (Fig. 5B, panels 1–3).

To affirm the observations made by using PKC inhibitors, we measured PKC activation by apoA-I. ApoA-I induced translocation of PKC to the membrane fraction indicating PKC activation by apoA-I (Fig. 6A). Western blotting analysis showed that WI-38 cells predominantly express PKCa, and PKCβ and PKCγ at very low levels (data not shown); Go6976 selectively inhibits Ca2+-dependent PKCa and PKCβI (26). We therefore assumed that PKCa is likely to be a PKC isotype involved in apoA-I-induced ABCA1 stabilization. To test this idea, PKCa tagged with GFP was transiently expressed in WI-38, and the transfected cells were stimulated by apoA-I. PMA induced translocation of the PKCa-GFP to plasma membrane as well as to cytoplasmic vesicular-like compartments (Fig. 6B, top), and a similar translocation profile was demonstrated by apoA-I at 10–20 min after stimulation (Fig. 6B, middle), consistent with the profile of PKCa-GFP activation by other stimuli (29–32). The time course of the PKCa-GFP translocation by apoA-I was consistent with the PKC activation shown in Fig. 6A. PKCa-GFP translocation by apoA-I was completely blocked by Go6976 (Fig. 6B, bottom). D609 (50 μg/ml) also inhibited the apoA-I-induced translocation of PKCa-GFP (data not shown).

PKC catalyzes phosphorylation of serine and/or threonine residues in specific amino acid residue sequences, and ABCA1 contains several serine and threonine residues that are potentially phosphorylated by PKC. We therefore investigated phosphorylation of ABCA1 by apoA-I. ABCA1 was phosphorylated with PKA activation by dibutyryl cAMP in both WI-38 and HEK293 cells expressing ABCA1-GFP, consistent with previous work by others (27, 28) (Fig. 7, A and B). A PKC activator, PMA, also phosphorylated ABCA1 in both cells as much as PKA did, first showing that ABCA1 can be phosphorylated by PKC. ApoA-I also induced phosphorylation of ABCA1 in both cells (Fig. 7, A and B). After standardization for a slight increase in ABCA1 protein mass by these treatments, the increase in phosphorus incorporation was estimated as 335, 380, and 350% by cAMP, PMA, and apoA-I, respectively. The apoA-I-induced phosphorylation of ABCA1 was suppressed by Go6976 (Fig. 7C). D609 apparently induced background phosphorylation of ABCA1 for unknown reasons but canceled the change in phosphorylation by apoA-I. Finally, the effect of okadaic acid, an inhibitor of protein phosphatases 1 and 2A, was examined. It increased phosphorylation of ABCA1 in the apoA-I-treated cells, indicating that phosphatase 1 and/or phosphatase 2A play a role in the dephosphorylation of ABCA1 (Fig. 7D). Okadaic acid increased ABCA1, especially in the presence of apoA-I (Fig. 7E), again strongly suggesting that ABCA1 phosphorylation is associated with its stabilization. Thus, apoA-I-induced ABCA1 stabilization is linked with ABCA1 phosphorylation by PKC, activated by the apoA-I-cell interaction.

**DISCUSSION**

We have demonstrated that: 1) ApoA-I stabilizes ABCA1 against calpain-mediated degradation in human fibroblast WI-38; 2) PKC activity is involved in the ABCA1 stabilization by...
apoA-I in an upstream step(s) of the calpain-mediated proteolysis; 3) DAG produced by PC-PLC triggered by apoA-I-mediated SPM removal is likely to activate PKC/H9251 for the initiation of a signal cascade; and 4) ABCA1 is phosphorylated by PKC/H9251 and thus activated, and this is associated with its stabilization. Thus, PC-PLC/PKC/H9251 signaling activated by apoA-I is involved in the molecular mechanism for apolipoprotein to stabilize ABCA1. The signaling pathway can be initiated by SPM replenishment reaction for its removal by apoA-I to produce diacylglyceride by PC-PLC (18) (Fig. 8).

Apolipoprotein-mediated release of cholesterol and phospholipid to assemble HDL is one of the essential reactions for cellular cholesterol homeostasis and production of plasma HDL (1). ABCA1 plays a key role in this reaction, shown by defect of apolipoprotein-mediated cellular lipid release and the disappearance of plasma HDL in familial HDL deficiencies caused by its mutations (2–6), in ABCA1 knockout mice (7, 8), WHAM chicken (33), and probucol treatment (34, 35). The increase of ABCA1 expression results in enhancement of the apolipoprotein-mediated HDL production by cells in vitro and an increase of plasma HDL in vivo (9–15). Cellular ABCA1 level is thus one of the rate-limiting factors for cellular lipid release and HDL production. Transcriptional regulation of ABCA1 is known to involve liver X receptor and retinoid X receptor (13, 14). Degradation of ABCA1 is also involved in the regulation of its cellular level. Unsaturated fatty acids and excess free cholesterol loading enhance degradation of this protein (36, 37). Apolipoproteins in their free form but not HDL-bound form stabilize ABCA1 by protecting it from calpain-mediated degradation (16, 17).

On the other hand, many reports indicate that HDL or HDL-apoproteins modulate various cellular signaling pathways. HDL stimulates PC-PLC, PC-PLD, and PKC in relation to cellular cholesterol release (22, 23, 38), activates PI-PLC, Ras, Raf-1, Mek-1, and MAP kinase (39), and interrupts the sphingosine kinase pathway (40). HDL-mediated Ras/MEK/MAP kinase activation requires scavenger receptor B1, whereas lipid-free apoA-I does not activate this signaling cascade (41). HDL promotes generation of DAG by both PI turnover pathway and PC hydrolysis (42, 43). PI-PLC activation is mediated by HDL-associated lysosphingolipid, which is related to cell proliferation but not to cholesterol efflux (44). It is not clear whether these HDL-mediated signal activations are lipid-free apoli-
poprotein-related events, although a portion of the HDL apoproteins may dissociate to interact with cells (1).

We have shown that PKC is involved in apolipoprotein-mediated cholesterol release by modulating its incorporation to the HDL (24, 25). More recently, we demonstrated that the apoA-I-mediated removal of cellular SPM causes its replenishment by transfer of phosphorylcholine from PC to ceramide (18). This reaction is mediated by PC-PLC and generates DAG. The results seem consistent with previous reports on the involvement of PC derivatives in apoA-I-mediated cholesterol release (45), impairment of PC-PLC activation by HDL in cells from Tangier disease (46), and PI-PLC activation by HDL but not by apoA-I (44). Thus, PKC may be activated by both HDL and lipid-free apolipoprotein, whereas stabilization of ABCA1 is specific to lipid-free apolipoprotein (16, 17); therefore the apolipoprotein-mediated PC-PLC signaling pathway is more likely associated with this reaction through PKCα activation.

ABC transporters play critical roles in the transport of a wide range of molecules across biological membranes, not only eukaryotes but also prokaryotes. The activities of some ABC proteins are regulated by their phosphorylation. Cystic fibrosis transmembrane regulator is phosphorylated by both PKA and PKC to regulate its function (47, 48). The phosphorylation and regulation of the activity of multidrug resistance P-glycoprotein by PKC and PKA have also been reported (49, 50). Furthermore, apoA-I-mediated cholesterol release and ABCA1 phosphorylation are both enhanced by activation of PKA by cAMP analogue (27, 28). The same report (27) shows that mutation of its phosphorylation site (Ser-2054) results in impairment of the apoA-I-mediated lipid release, but PKA-dependent ABCA1 phosphorylation does not seem to be involved in its stability. PKA is also known to induce ABCA1 transcription in some cells (12, 51), so that it might play dual roles in regulation of ABCA1 activity.

During the reviewing period of this paper, Martinez et al. (52) reported that apoA-I dephosphorylated mouse ABCA1 transiently expressed in HEK293 cells in its PEST sequences in relation to its stabilization (52). They demonstrated that Thr-1286 and Thr-1305, potential phosphorylation sites by casein kinase II and PKA, respectively, are phosphorylated, but the inhibitors of either kinase did not alter the PEST sequence phosphorylation or ABCA1 stabilization.

It is not fully clear how the phosphorylation of ABCA1 by PKC is involved in its stabilization against calpain-mediated proteolysis. Phosphorylation of ABCA1 by PKC may alter its conformation to make it resistant to calpain-mediated degradation. Alternatively, some other molecule(s) may be involved in the mechanism. Several proteins have been shown to interact with ABCA1 to regulate its functions, including Cdc42 (53) and Fas-associated death domain protein (54). It is noteworthy...
that Cdc42 and Fas-associated death domain protein are phosphorylated and regulated by PKC (55–58). In addition, a PDZ protein, β2-syntrophin, also interacts with ABC family proteins ABCA1 (59) and MRP2 (60) or another membrane protein, ICA512 (61). Phosphorylation of either β2-syntrophin or the membrane protein may regulate the interaction (60), and dissociation of ICA512 from β2-syntrophin accelerates its calpain-mediated degradation (61). Thus, PKC could regulate the stabilization of ABCA1 through its interaction with the “third party” protein(s). Identification of the phosphorylation site of ABCA1 by PKC is important for the further understanding of this reaction. Also, phosphorylation and dephosphorylation of PEST sequence in ABCA1 may control its stability via unidentified kinases and phosphatases.

In conclusion, we have demonstrated the signaling cascade for stabilization of ABCA1 by apolipoproteins. ApoA-I generates HDL by removing cellular lipids including SPM, induces PC-PLC-dependent DAG production as a product of SPM replenishment, and activates PKCs. Consequently, ABCA1 is phosphorylated, and this process results in the protection of ABCA1 against calpain-mediated proteolytic degradation to increase its levels in the cells.

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**Fig. 7. Phosphorylation of ABCA1 by apoA-I.** A, HEK293 and 293/ABCA1-GFP cells were incubated with 0.25 mCi/ml [32P]orthophosphate for 3 h. ApoA-I (10 μg/ml) was added into the labeling period. The phosphorylated ABCA1 by apoA-I was analyzed by Western blotting. B, WI-38 cells were pulse-labeled with 0.5 mCi/ml [32P]orthophosphate for 3 h and stimulated by apoA-I for the final hour of the labeling period. Cell lysate, 150 μg of protein, was used for the ABCA1 immunoprecipitation. D, WI-38 cells were pretreated with 9-cis retinoic acid for 18 h, pulse-labeled with 0.5 mCi/ml [32P]orthophosphate for 3 h, and stimulated by apoA-I for the final hour of the labeling period. Cells were washed twice with PBS and chased in the presence of okadaic acid (50 nM) for 4 h. ABCA1 was analyzed by immunoprecipitation. E, WI-38 cells were incubated with apoA-I (10 μg/ml) for 1 h, washed, and then incubated with okadaic acid (5 nM) for 4 h. ABCA1 was analyzed by Western blotting.

**Fig. 8. A putative model for the signaling cascade for apoA-I-induced ABCA1 stabilization.** ApoA-I removes cellular free cholesterol (FC), PC, and SPM in the presence of ABCA1 activity, and thereby SPM in plasma membrane decreases. This triggers PC-PLC-mediated PC hydrolysis to generate phosphorylcholine for the replenishment of SPM, and this reaction also generates DAG. DAG activates PKC, and the activated PKCo phosphorylates ABCA1. These reactions eventually lead to the protection of ABCA1 from its degradation by calpain.
