Calmodulin Interacts With ATP Binding Cassette Transporter A1 to Protect From Calpain-Mediated Degradation and Upregulates High-Density Lipoprotein Generation

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Objective—To investigate the interaction of ATP-binding cassette transporter A1 (ABCA1) with calmodulin in relation to its calpain-mediated degradation because many calpain substrates bind calmodulin to regulate cellular functions.

Methods and Results—The activity of ABCA1 is regulated through proteolysis by calpain. An immunoprecipitation and glutathione S-transferase pull-down assay revealed that ABCA1 directly binds calmodulin in a Ca\(^{2+}\)-dependent manner. The cytoplasmic loop of ABCA1 contains a typical calmodulin binding sequence of 1-5-8-14 motifs (1245 to 1257 amino acids). The peptide of this region showed binding to calmodulin, and deletion of the 1-5-8-14 motif abolished this interaction. This motif is located near the ABCA1 Pro-Glu-Ser-Thr sequence, and the presence of calmodulin/Ca\(^{2+}\) protected the peptides from proteolysis by calpain. The knockdown of calmodulin by a specific small and interfering RNA increased the degradation of ABCA1 and decreased ABCA1 protein and apolipoprotein A-I–mediated lipid release. Surprisingly, calmodulin inhibitor W7 increased calmodulin binding to ABCA1 and protected it from calpain-mediated degradation, consistent with our previous finding that this compound increased apolipoprotein A-I–mediated cell cholesterol release.

Conclusion—Calmodulin directly binds and stabilizes ABCA1 in the presence of Ca\(^{2+}\) and increases the generation of high-density lipoprotein. (Arterioscler Thromb Vasc Biol. 2010;30:1446-1452.)

Key Words: HDL ■ ABCA1 ■ cholesterol ■ calmodulin ■ calcium ■ calpain

High-density lipoprotein (HDL) plays a central role in the catabolic pathway of cholesterol by transporting it from the peripheral tissues to the liver for conversion to bile acids. Therefore, it is thought to prevent accumulation of cholesterol in extracellular tissues and to act as an antiatherogenic factor. ATP-binding cassette transporter A1 (ABCA1) is a key rate-limiting protein for the generation of HDL in hepatocytes and peripheral cells; helical apolipoproteins, such as apolipoprotein A-I (apoA-I), interact with it to remove cellular phospholipid and cholesterol to form new HDL particles. ABCA1 is one of the target molecules that increases HDL and prevents cardiovascular disease.

The expression of the ABCA1 gene is regulated by the liver X receptor through oxysterol as a signal of cellular cholesterol increase. Interestingly, it is also regulated by sterol-reacting element binding protein 2 in hepatocytes, perhaps to maintain cholesterol homeostasis for the body. The ABCA1 level is also modulated through proteolytic degradation by calpain, and the interaction with helical apolipoproteins to generate HDL stabilizes ABCA1 against this degradation. Several cellular factors also impeded the degradation of ABCA1 by interacting with ABCA1. Pharmacological inhibition of ABCA1 degradation resulted in an increase of HDL biogenesis, an elevation of plasma HDL, and prevention of atherosclerosis.

Many calpain substrates contain the Pro-Glu-Ser-Thr (PEST) sequence. ABCA1 also has a consensus PEST sequence in the cytoplasmic loop and a high PEST score; deletion of this sequence abolished the proteolysis by calpain. The half-life of ABCA1 is less than 2 hours in the absence of helical apolipoproteins, similar to other proteins with a PEST sequence.

Calmodulin is an acidic Ca\(^{2+}\)-binding protein and orchestrates many regulatory events through the interaction with cellular proteins. Calmodulin-binding proteins are classified into Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent proteins, and calmodulin binds with high affinity to a relatively small α-helical region of many proteins. Many calmodulin-binding proteins are also substrates of calpain because most of the calmodulin-binding proteins contain a PEST sequence. Calmodulin was, indeed, found to regulate calpain-mediated proteolysis by binding to the substrates. Calmodulin binds...
to Ca\(^{2+}\)-ATPase in a Ca\(^{2+}\)-dependent manner at the calmodulin-binding sequence to complete its calpain-mediated cleavage to activate it while EGTA protected it from this cleavage, perhaps by inducing the optimum conformation.\(^{16}\)

Phosphocholine cytidylyltransferase, the enzyme essential for phosphatidylcholine biosynthesis, is degraded by calpain. Both the PEST sequence and a calmodulin binding site are identified in this enzyme, and calmodulin directly binds to and stabilizes phosphocholine cytidylyltransferase against calpain-mediated degradation.\(^{17}\)

Because ABCA1 contains a conserved PEST sequence, calmodulin is potentially involved in its proteolytic degradation. In the present study, we investigated the regulation of ABCA1 degradation in relation to calmodulin. We identified a conserved Ca\(^{2+}\)-dependent calmodulin binding site near the PEST sequence in ABCA1 and demonstrated protection by calmodulin of ABCA1 against calpain-mediated proteolysis.

Methods

Materials

Calmodulin and calmodulin-agarose were obtained from Sigma; rabbit anti–calmodulin monoclonal antibody, Upstate; and control rabbit IgG and protein agarose A/G beads, Santa Cruz Biotechnology. Purified \(\mu\)-calpain was purchased from Calbiochem; and cycloheximide and calmodulin inhibitor W7, Wako. Monoclonal antibody against human ABCA1 (MAB98-7) was generated in rats at MAB Institute against the C-terminus peptide of human ABCA1 (CNFAKDQSDDDHLKDLSLHKN).\(^{18}\) Human apoA-I was prepared from fresh human plasma.\(^{19}\)

Cell Culture and Immunoprecipitation

BALB/3T3 and THP1 cells were incubated in the medium (a 1:1 mixture of Dulbecco’s modified Eagle medium and Ham F12 medium [DF medium] and RPMI1640 medium, respectively) with 10% FBS at 37°C in 5% CO\(_2\).

Cell pellets were harvested with sonication in the 50-mmol/L Tris-hydrochloride buffer (pH 8.0) containing 150-mmol/L NaCl, 5-mmol/L EDTA, and protease inhibitor cocktail (Sigma). Insoluble debris was removed by centrifugation at 12 000 \(\times\)g for 2 minutes at 4°C. The supernatant was collected; and its protein, 500 \(\mu\)g, was rotated with the anti-ABCA1 or anticalmodulin antibody overnight at 4°C and incubated with agarose A/G beads. Proteins bound to the beads were eluted by the SDS-PAGE sample buffer and analyzed by immunoblotting using specific antibodies.

Calmodulin-Agarose Binding Assay

Cell lysate as 250 \(\mu\)g of protein was incubated with 30 \(\mu\)L of 50% calmodulin-agarose bead suspension in Tris-hydrochloride saline for 3 hours at 4°C with 1-mmol/L EGTA or with the various concentrations of calcium chloride. Protein bound to the beads was eluted with the SDS-PAGE sample buffer and analyzed by Western immunoblotting using the anti-ABCA1 antibody.

Glutathione S-Transferase–Fusion Protein and Glutathione S-Transferase Pull-Down Assays

Expression vectors of the peptides corresponding to 1213 to 1349 and to 1264 to 1349 amino acid residue numbers of ABCA1, representing the PEST sequences with and without calmodulin (CaM) recognition sequence 1244 to 1257, respectively (CaM-PST and PST, respectively), were prepared by introducing the EcoRI/BglII fragment from enhanced green fluorescent protein–ABCA1 expression vector into pGEX-5X-1 vector (Amersham Biosciences). The expression of glutathione S-transferase (GST)–CaM–PST and GST-PST fusion proteins was induced in Escherichia coli BL-21 DE3 (Nitron Genie) and purified from cellular lysates by glutathione-Sepharose chromatography, solubilized with 50-mmol/L Tris-hydrochloride containing 10-mmol/L glutathione.

GST-CaM-PST and GST-PST proteins, 2 \(\mu\)g each, prepared as previously described,\(^{20}\) were incubated with 2 \(\mu\)g of purified calmodulin at 30°C for 45 minutes, followed by incubation with 25 \(\mu\)L of immobilized glutathione agarose beads at 4°C for 1 hour. After the incubation and washing of beads 4 times, protein was eluted with the SDS sample buffer and then immunoblotted by the anticalmodulin antibody.

Calpain-Mediated Proteolysis Assay and Calpain Activity In Vivo

The cell lysate was incubated with 0.5 to 3.0 \(\mu\)g of \(\mu\)-calpain at 32°C for 1 hour. Alternatively, GST-CaM-PST or GST-PST protein and cell lysates were incubated with 1.5 \(\mu\)g of purified \(\mu\)-calpain in the same manner. This reaction was discontinued by the SDS sample buffer, and the product was analyzed by electrophoresis in 15% SDS-PAGE. Proteins were stained by Coomassie brilliant blue or transferred to a polyvinylidene difluoride membrane for immunoblotting by anti-GST or anti-ABCA1 antibody.

The cells were pretreated with appropriate compounds for 3 hours and incubated with synthetic calpain substrate t-butyloxycarbonyl-Leu-Met-chloromethylaminocoumarin (BOC-LM-CMAC) (Molecular Probes) for 20 min. After washing with PBS twice, fluorescence of the reaction product was examined in situ with a fluorescent plate reader (model FL600; Bio-Tek Inc) with excitation at 360 nm and emission at 410 nm.

Control of Intracellular Ca\(^{2+}\) Concentration

BALB/3T3 cells were preincubated with Ca\(^{2+}\)-free Hanks’ solution containing EGTA, 1 mmol/L, and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N''-tetraacetoxyethyl ester, 100 \(\mu\)mol/L, for 30 minutes; washed twice; and cultured in the conditions of various extracellular Ca\(^{2+}\) concentrations (0, 10, 50, 100, and 1500 mmol/L calcium chloride) for 60 minutes, as previously reported.\(^{21}\)

Small and Interfering RNA

Mouse calmodulin small and interfering RNA (CUU UCU CCC UAU UCG UAA TT for sense and UUA UCG AAG GAG AAA GCT for antisense) and scramble control were obtained from Ambion and transfected into BALB/3T3 cells using Lipofectamine 2000 transfection reagents (Invitrogen), according to the protocol provided by the manufacturer. The cells were used for the experiments after 48-hour preincubation.

Clearance of ABCA1 Protein and Labeling and Tracing ABCA1 for Endocytosis

Cells were preincubated in DF medium containing 0.02% BSA in the presence of 100-\(\mu\)g/mL cycloheximide for 30 minutes, and cellular ABCA1 protein was analyzed by Western blotting at the indicated times.\(^{6}\)

Cell surface proteins were biotinylated with sulfoconjugated 2-(biotinamid)-rhod-1, 3-dithiopropionate (Pierce) for 1 hour at 4°C, as previously described.\(^{8}\) To trace the labeled surface ABCA1 for internalization, biotinylation of the cell surface protein was cleaved with 50 mmol/L reduced glutathione (Sigma), and the uncleaved biotinylated ABCA1 was determined as the internalized portion.\(^{9}\)

The release of cholesterol and phospholipid from BALB/3T3 cells was measured after incubation with 10-\(\mu\)g/mL apoA-I in DF medium containing 0.1% BSA for 16 hours, as previously described.\(^{22}\) Statistical analysis was performed by Wilcoxon signed rank test.
Results

ABCA1-Calmodulin Interaction in a Ca\(^{2+}\)-Dependent Manner

The interaction of ABCA1 with calmodulin was examined in the lysate of BALB/3T3 cells. ABCA1 was degraded by \(\mu\text{-calpain}\) in a dose-dependent manner (Figure 1A). The interaction of ABCA1 with calmodulin was demonstrated by coimmunoprecipitation with the anti-ABCA1 antibody and with the anti-calmodulin antibody, whereas knockdown of each protein abolished these apparent cross interactions (Figure 1B). Binding of the membrane ABCA1 to calmodulin-agarose beads required Ca\(^{2+}\) showing the interaction to be Ca\(^{2+}\)-dependent (Figure 1C).

The interaction of ABCA1 with calmodulin was further examined in cultured cells in the medium containing various Ca\(^{2+}\) concentrations or in the presence of calcium-chelating reagents. Chelating of calcium ion increases ABCA1 protein (Figure 1D, left, and E) as the result of a decrease of calpain activity (Figure 1F). On the other hand, precipitation of calmodulin with ABCA1 increased with calcium (Figure 1D, right). The chelating treatment markedly decreased this association and increased ABCA1 as much as other reagents to block ABCA1 degradation (Figure 1E, bottom).

Calmodulin Recognition Motif in the Cytoplasmic Loop of ABCA1

Database analysis identified the PEST sequence in the cytoplasmic loop of ABCA1 (Figure 2A). The Ca\(^{2+}\)-dependent calmodulin-binding motifs 1-5-8-14 were found in the cytoplasmic loop of ABCA1 as 1244 to 1257 (Figure 2B), located near the PEST sequence (1283 to 1306). To analyze whether this motif binds calmodulin in a Ca\(^{2+}\)-dependent manner, GST-fusion peptides of the ABCA1 cytoplasmic loop were generated with and without this motif (GST-CaM-PST and GST-PST, respectively) (Figure 2A and C). The GST pull-down assay indicated that the GST-CaM-PST protein interacts with calmodulin in the presence of Ca\(^{2+}\), but the
GST-PST protein does not (Figure 2D). The 1-5-8-14 motif in the cytoplasmic loop of ABCA1 was shown to bind to calmodulin in the presence of Ca\textsuperscript{2+}/H\textsubscript{11001}.

Calmodulin Binding Protects ABCA1 Against Calpain-Mediated Degradation

The reactivity of GST-CaM-PST protein to calpain was examined in vitro (Figure 3A). GST-CaM-PST protein (44 kDa) was completely degraded by calpain in the absence of calmodulin. In the presence of calmodulin and Ca\textsuperscript{2+}/H\textsubscript{11001}, GST-CaM-PST protein was partially degraded; 34- and 28-kDa peptides were identified as limited cleavage products. These results were confirmed by immunoblotting with an anti-GST antibody (Figure 3B). For GST-CaM-PST alone, a 26-kDa band was detected as the GST protein copurified in the sample. Preincubation with calmodulin resulted in limited proteolytic digestion of GST-CaM-PST by calpain to produce 34- and 28-kDa peptides; GST-CaM-PST was almost completely degraded without the pretreatment. The results demonstrated that calmodulin binds to ABCA1 in the presence of Ca\textsuperscript{2+} by the 1-5-8-14 motif, and this reaction leads to limited calpain-mediated digestion of ABCA1 in vitro.

Calmodulin Modulates Stability of ABCA1 and Its Activity In Vivo

The intracellular roles of calmodulin in regulation of ABCA1 expression were investigated. Knockdown of calmodulin resulted in decrease of ABCA1 as the total (Figure 4A, left) and cell surface (Figure 4A, right), without changing intracellular calpain activity (data not shown). The degradation of ABCA1 was accelerated when calmodulin was knocked down (Figure 4B and C) because it decreased to 20% of the original level in the knockdown cells after 120 minutes while it remained 50% in the control. It was recently reported that ABCA1 is degraded by calpain after its endocytosis,\textsuperscript{8} so that the rate of endocytosis of ABCA1 was examined and found unchanged by calmodulin knockdown (Figure 4C). Finally, the activity of ABCA1 was evaluated by measuring the release of cellular cholesterol and phospholipid by apoA-I. Figure 4D shows that the lipid releases were both decreased by knockdown of calmodulin (cholesterol, 2.45 ± 0.31 versus 1.70 ± 0.32; phospholipid, 4.92 ± 0.48 versus 3.99 ± 0.18, mean ± SD). The results indicated that ABCA1 turnover was...
significantly accelerated in the calmodulin-knockdown cells; this resulted in decreased HDL biogenesis.

**Binding to ABCA1, but Not “Activity” of Calmodulin, Modulates Stability of ABCA1**

We reported that calmodulin inhibitor W7 increased the apoA-I–mediated release of cellular phospholipid and cholesterol.23 W7 increased ABCA1 protein in total cell (Figure 5A and B) and cell surface (Figure 5B). When the cell lysate was incubated with \( \text{Ca}^2+ \)-calpain, ABCA1 decreased along with the increase of the 120-kDa band that reacts to anti-ABCA1 C-terminus peptide antibody (Figure 5C, left), consistent with the view that ABCA1 is cleaved at the region near the PEST sequence and calmodulin binding site. When the cells were preincubated with 10-\( \mu \)-mol/L W7, ABCA1 increased and became resistant to the calpain-mediated degradation (Figure 5C, right, and Figure 5D). Under the condition that W7 increased ABCA1 by inhibiting calpain-mediated degradation, the apoA-I–mediated cellular lipid release increased in a dose-dependent manner, consistent with our previous work.23

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Figure 4. Effects of calmodulin knockdown on stability and activity of ABCA1. A, ABCA1 protein. In BALB/3T3 cells, calmodulin was knocked down by small interfering RNA (siRNA) (C). Total cell lysate (left) and biotinylated surface proteins (right) were analyzed for ABCA1 and CaM. B, ABCA1 degradation. Cells treated with siRNA were preincubated with 100-\( \mu \)-g/mL cycloheximide for 30 minutes and chased, and ABCA1 was analyzed. The graph shows ABCA1 standardized for \( \beta \)-actin. C, Endocytosis of ABCA1. Cell surface protein was pulse labeled by biotinylation. After incubation at 37°C, the internalized ABCA1 was analyzed as described in the Methods section, “Tracing ABCA1 for Endocytosis.” The graph shows internalized ABCA1 standardized for calnexin. D, HDL biogenesis. Release of free cholesterol (FC) and phospholipid (PL) from cells by apoA-I, 10 \( \mu \)-g/mL, was measured. Data are given as mean±SD for 4 samples, with *P<0.05 from control.

Figure 5. Effects of calmodulin inhibitor W7. A and B, W7 increases ABCA1 protein. BALB/3T3 cells were incubated with W7 for 5 hours, and total cell lysate and cell surface–biotinylated protein were analyzed for ABCA1. C and D, W7 inhibited calpain-mediated ABCA1 degradation. W7 was added to culture medium for 3 hours at 37°C, and cell lysate was incubated with \( \mu \)-calpain for 1 hour at 32°C. Alternatively, after incubation with W7 for 5 hours, cells were washed and made permeable by 80-\( \mu \)-g/mL digitonin for 15 minutes on ice, washed, and treated with 0.2-\( \mu \)-mol/L \( \mu \)-calpain. The reaction products were analyzed for ABCA1. E, W7 increased apoA-I–mediated lipid release. Cells were incubated with the indicated concentration of W7 for 16 hours and 10-\( \mu \)-g/mL apoA-I. Release of cholesterol and phospholipid was measured. Data are given as mean±SD for triplicate assays, with *P<0.05. F, Cells were cultured with W7 for 5 hours at 37°C and harvested. Cell lysates were immunoprecipitated with antibodies against calmodulin (CaM) or ABCA1 and analyzed for ABCA1 and CaM.
proteins.12 The binding of calmodulin induces conformational change in some calmodulin binding proteins and protects their calpain cleavage sites,24 consistent with the current finding with ABCA1.

Previously, it was found that calmodulin inhibitor W7 increased the apoA-I–dependent cell cholesterol release,23 apparently being contradictory to the current finding that calmodulin binding protects ABCA1 and upregulates its activity. Surprisingly, W7 increased calmodulin binding to ABCA1 and inhibited its calpain-mediated degradation. Therefore, the physical interaction of calmodulin with ABCA1 may interfere with its proteolysis; however, it is unrelated to other regular activities of calmodulin. Thus, previous data with W723 are consistent with the current observation. This view was supported by the findings that calpeptin does not further increase ABCA1 in the presence of W7 and that the effects of W7 were abolished by knockdown of calmodulin. It remains unknown why W7 increases calmodulin binding to ABCA1 despite its calmodulin inhibition.

We reported that ABCA1 in the plasma membrane is internalized by endocytosis and hydrolyzed by calpain, presumably in the endosome.8 The calpain inhibitor, calpeptin, increased ABCA1 and apoA-I–mediated lipid release,13,25 but did not influence ABCA1 endocytosis.8 When surface ABCA1 interacts with a helical apolipoprotein, such as apoA-I, it becomes resistant to calpain and is recycled to increase surface ABCA1. Consequently, inhibition of ABCA1 endocytosis decreases its degradation and increases it in the surface to enhance HDL biogenesis, by such compounds as cytochalasin D8 or spiroquinone and diphenoquinone, oxidized products of probucol.11 The current findings (Figure 4) showed that knockdown of calmodulin did not influence ABCA1 endocytosis, so that inhibition of ABCA1 degradation by calmodulin is likely the result of ABCA1 resistant to calpain.

However, a role of the PEST sequence in ABCA1 degradation is unclear. Deletion of the sequence prevented ABCA1 internalization, increased cell surface ABCA1,13 and decreased cholesterol efflux from late endosomes,26 suggesting that the PEST sequence associates with ABCA1 internalization despite the fact that it is unrelated to any internalization motif. These findings may agree with the observation that ABCA1 removes cholesterol from the late endosome27; however, they are inconsistent with many other reports8,28,29 that ABCA1 generates HDL particles mainly at the plasma membrane.

Phosphorylation of the PEST sequence reportedly promoted calpain-mediated degradation of ABCA1.30 Phosphorylation of PEST sequences generates a strong negative charge at the region that may bind calcium; this may locally activate calpains.31 The dephosphorylation-mimicking form of ABCA1 by deleting the PEST sequence became insensitive to the apoA-I treatment against the calpain-mediated proteolysis,30 supporting this hypothesis. On the other hand, previous reports showed that ABCA1 can be phosphorylated by protein kinases A,20 C,25 and CK233 in relation to regulation of ABCA1 functions. CK2 phosphorylation sites are the Thr1242, Thr1243, and Ser1255 residues in the cytoplasmic loop domain of ABCA1; dephosphorylation of these residues increased the functional activity of ABCA1 but did not significantly affect ABCA1 turnover.33 The calmodulin binding site of ABCA1 is located at 1244 to 1257, at a close or overlapping position to a CK2 phosphorylation site, so that the phosphorylation by CK2 may have interference through calmodulin binding.
In conclusion, ABCA1 is regulated by its calcium-dependent interaction with calmodulin. The mechanism is apparently by direct interaction between the 2 molecules but not through the regular mechanism for the activity of calmodulin.

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Disclosures
Dr Yukomaya is involved in the establishment of a venture company (HYKES Laboratories).

References
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