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The COP9 signalosome controls ubiquitinylation of ABCA1

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ABSTRACT

ATP-binding cassette protein A1 (ABCA1) mediates the transfer of cellular free cholesterol and phospholipids to apolipoprotein A-I (apoA-I), an extracellular acceptor in plasma, to form high-density lipoprotein (HDL). ABCA1 has been suggested to be degraded by proteasome in cholesterol-loaded macrophages, however, the mechanism and regulation of proteasomal degradation of ABCA1 remain unclear. In this study, we analyzed the putative interaction between ABCA1 and COP9 signalosome (CSN), a key molecule in controlling protein ubiquitination and deubiquitination. CSN2 and CSN5, subunits of COP9 CSN complex, were coprecipitated with ABCA1 when coexpressed in HEK293 cells and proteasomal degradation was inhibited by MG132. Overexpression of CSN2 increased endogenous CSN7 and CSN8, and decreased ubiquitinylated forms of ABCA1. These results suggest that CSN is a key molecule which controls the ubiquitinylation and deubiquitinylation of ABCA1, and is thus an important target for developing potential drugs to prevent atherosclerosis.

Introduction

The maintenance of cellular cholesterol homeostasis is important for normal human physiology; its disruption can lead to a variety of pathological conditions, including cardiovascular disease [1]. ATP-binding cassette protein A1 (ABCA1), a key factor in cholesterol homeostasis, mediates the secretion of cellular free cholesterol and phospholipids to an extracellular acceptor, apolipoprotein A-I (apoA-I), to form high-density lipoprotein (HDL) [2,3]. HDL formation is the only known pathway for the elimination of excess cholesterol from peripheral cells. Defects in ABCA1 cause Tangier disease [4–6], in which patients have a near absence of circulating HDL, prominent cholesterol–ester accumulation in tissue macrophages, and premature atherosclerotic vascular disease [1,7].

Because cholesterol is an essential component of cells, however, excessive elimination of cholesterol could result in cell death, and ABCA1-mediated cholesterol efflux is highly regulated at both the transcriptional and post-transcriptional levels. The degradation of ABCA1 is regulated [8–10] and is carried out via several pathways; (i) cell-surface ABCA1 is endocytosed and recycled back to the plasma membrane or delivered to the lysosomes through early and late endosomes for degradation [11,12], (ii) calpain protease degrades ABCA1 on the plasma membrane [13,14] and intracellularly, especially when apoA-I does not bind to ABCA1 [15]. ABCA1 is also degraded through the ubiquitin–proteasome pathway [16], however, the mechanism and regulation of ABCA1 through the ubiquitin–proteasome pathway is still unclear, although it is supposed to be important in free cholesterol-loaded macrophages.

Recently, it was reported that COP9 signalosome (CSN) complex plays an important role in the ubiquitinylation and deubiquitinylation of various proteins, such as IκBα [17]. The CSN complex consists of eight subunits (CSN1–CSN8), and CSN5 and CSN6 subunits are active centers of dissociation of the ubiquitin like protein Nedd8 from cullin [18]. Because the attachment of Nedd8 (neddylation) to cullin is essential to stimulate recruitment of an ubiquitin-loaded E2 enzyme to cullin-based ubiquitin E3 ligase, this deneddylation activity of CSN is necessary for normal regulation of cullin-based ubiquitin ligase activity. Other subunits, such as CSN1, CSN2, and CSN4, were reported to enhance CSN assembly and stabilize the CSN complex [19–21]. In this study, we analyzed a putative interaction between ABCA1 and CSN subunits. Our results suggest that the CSN complex associates with ABCA1 and controls ubiquitinylation and deubiquitinylation of ABCA1.

Materials and methods

Materials. We generated a mouse monoclonal anti-ABCA1 antibody, KM3110, which recognizes the C-terminal 20 amino acids of human ABCA1 [9]. Monoclonal mouse anti-Flag antibody (M-2) and monoclonal mouse anti-ubiquitin antibody (P4D1) were purchased from Santa Cruz Biotechnology (CA, USA). Polyclonal rabbit anti-CSN7 and anti-CSN8 were purchased from Affiniti/Biomol (PA,

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USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and anti-rabbit IgG were purchased from Bio-Rad (CA, USA). Protein G-sepharose was purchased from Sigma–Aldrich (Tokyo, Japan). MG132 was purchased from Calbiochem (WI, USA). TO901317 was purchased from Cayman Chemical (MI, USA). Acetyl-LDL was purchased from Biomedical Technologies (MA, USA). Other chemicals were purchased from Sigma (MO, USA), GE Healthcare Biosciences (NJ, USA), Wako Pure Chemical Industries (Osaka, Japan), or Nacalai Tesque (Kyoto, Japan).

DNA construction. We generated expression constructs for human CSN2 and CSN5 by cloning PCR-amplified full-length cDNA into the expression vector pcDNA3.1. In addition, a sequence encoding the Flag tag was fused to the 5′ terminal of the CSN sequence. pcDNA3.1-ABCA1 [9] was used for the expression of ABCA1.

Cell culture and transfection. Human embryonic kidney (HEK)-293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in 5% CO_2. They were continuously kept in a subconfluent state. Transient transfection was performed using LipofectAMINE Plus Reagent (Invitrogen). To obtain mouse peritoneal macrophage cells, C57Black6/J female mice (10 weeks old) were injected with 2.0 ml PBS containing 4.0% thioglycollate intraperitoneally, and the macrophages were harvested 3 days later by peritoneal lavage. The harvested cells were plated in cell culture plates in RPMI1640 supplemented with 10% fetal bovine serum. Twenty-four hours after plating, the adherent macrophages were incubated with 50 μg/ml acetyl-LDL and 3 μM TO901317 for 24 h to induce the expression of ABCA1. Animal experiments were conducted in accordance with institutional policies following approval from the Animal Experimentation Committee of the Graduate School of Agriculture, Kyoto University.

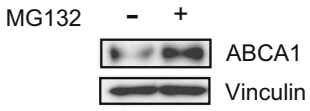

Fig. 1. Proteasomal degradation of ABCA1 in macrophages. Peritoneal macrophages from C57 mice were incubated with 50 μg/ml acetyl-LDL and 3 μM TO901317 for 24 h to induce ABCA1 expression. The macrophages were incubated with or without 20 μM MG132 for 4 h. Cell lysates were subjected to immunoblotting using antibodies against ABCA1 and vinculin. Vinculin was used as a loading control.

Immunoprecipitation. Thirty–fifty percent confluent HEK293 cells were transiently cotransfected with pcDNA3.1-ABCA1 and pcDNA3.1-CSN. After 24 h, cells were washed with serum-free growth medium and incubated with serum-free growth medium containing 0.02% bovine serum albumin (BSA) in the presence or absence of 50 μM MG132 for 4 h. Cells were washed with PBS and lysed in ice-cold PBS containing 1% Nonidet P-40 and protease inhibitors, 100 μg/ml (*p*-amidinophenyl)methanesulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Cell lysates were incubated with 5 μg anti-Flag antibody M2 for 1 h at 4 °C. The immunocomplexes were incubated with protein G-sepharose for 1 h and washed four times with lysis buffer. The bound proteins were separated by SDS–PAGE (7–15%) and analyzed by immunoblotting using the antibodies. Western blotting bands were quantified using a Fujifilm LAS-3000 imaging system.

Results

Proteasomal degradation of ABCA1 in cholesterol-loaded macrophages

When mice peritoneal macrophages, loaded with cholesterol with acetyl-LDL, were treated with 20 μM MG132, a proteasome inhibitor, for 4 h, the amount of ABCA1 increased 1.8-fold compared to without treatment (Fig. 1). This was consistent with previous reports which showed increased ABCA1 in cholesterol-loaded macrophages by lactacystin treatment [14]. These results suggested that ABCA1 is degraded by proteasome in cholesterol-loaded macrophages.

CSN complex associates with ABCA1

As the first step, to reveal the mechanism and regulation of proteasomal degradation of ABCA1, we analyzed the putative interaction between ABCA1 and CSN, a key molecule in controlling protein ubiquitination and deubiquitination. HEK293 cells were transiently transfected with expression vectors for ABCA1 and Flag-tagged CSN5, a subunit of the CSN complex, and ABCA1 was immunoprecipitated with anti-ABCA1 antibody. As shown in Fig. 2A, Flag-tagged CSN5 was coimmunoprecipitated with ABCA1 in the presence of MG132, but not in its absence. The association of ABCA1 and Flag-tagged CSN5 in the presence of MG132 was confirmed by their coprecipitation with anti-Flag antibody (Fig. 2B).

It was reported that the CSN5 subunit, not as a subunit of the CSN complex, independently interacts with the target protein,

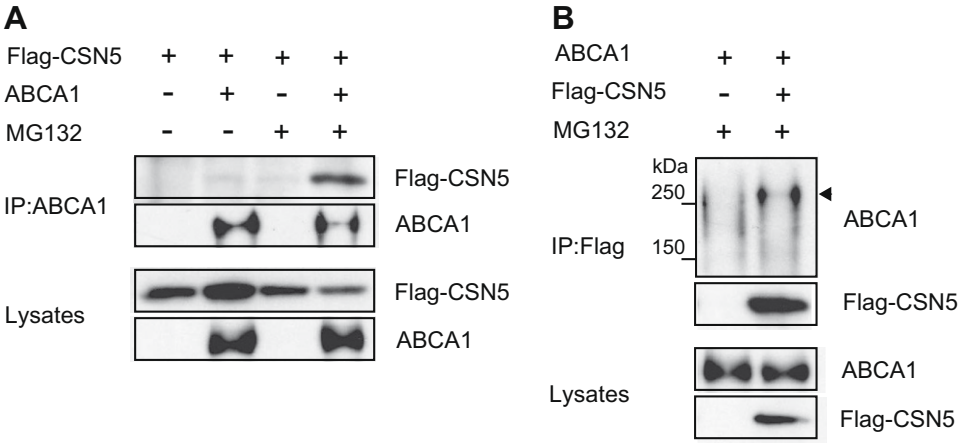

Fig. 2. CSN5 associates with ABCA1 in the presence of proteasome inhibitor. (A) HEK293 cells were transiently cotransfected with ABCA1 and Flag-CSN5. Twenty-four hours after transfection, cells were incubated with or without 50 μM MG132 for 4 h. Cell lysates were immunoprecipitated (IP) with anti-ABCA1 antibody. Immunocomplex and cell lysates were subjected to immunoblotting using antibodies against Flag and ABCA1. (B) Cell lysates from cells treated with MG132 were immunoprecipitated with anti-Flag antibody. Immunocomplex and cell lysates were subjected to immunoblotting using antibodies against ABCA1 and Flag.