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Functional modulation of AMP-activated protein kinase by cereblon

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ABSTRACT

Mutations in cereblon (CRBN), a substrate binding component of the E3 ubiquitin ligase complex, cause a form of mental retardation in humans. However, the cellular proteins that interact with CRBN remain largely unknown. Here, we report that CRBN directly interacts with the α1 subunit of AMP-activated protein kinase (AMPK α1) and inhibits the activation of AMPK activation. The ectopic expression of CRBN reduces phosphorylation of AMPK α1 and, thus, inhibits the enzyme in a nutrient-independent manner. Moreover, AMPK α1 can be potently activated by suppressing endogenous CRBN using CRBN-specific small hairpin RNAs. Thus, CRBN may act as a negative modulator of the AMPK signaling pathway *in vivo*.

1. Introduction

In humans, the *cereblon* gene has been linked to autosomal recessive nonsyndromic mental retardation (ARNSMR) [1]. CRBN was initially characterized as an RGS-containing novel protein that acted as a K^+ channel-interacting protein in the rat brain [2], and was later shown to interact with a voltage-gated chloride channel (CIC) in the retina [3]. CRBN inhibits the assembly and surface expression of the large-conductance Ca^{2+}-activated K^+ channel α subunit [2]. Recently, CRBN was shown to be the primary target of thalidomide induced teratogenicity and a substrate binding component for the E3-ubiquitin ligase [4]. Despite the association of CRBN to the aforementioned proteins, the molecular characteristics of CRBN and its cellular functions have not been well defined.

To elucidate the cellular functions of CRBN, we searched for CRBN binding partners in rat brain and found a direct interaction between the α1 subunit of AMP-activated protein kinase (AMPK α1) and CRBN. AMPK, a master sensor of energy balance, inhibits ATP-consuming anabolic pathways and increases ATP-producing catabolic pathways [5,6]. AMPK is a serine/threonine protein kinase made up of a heterotrimer of a catalytic α subunit and two regulatory subunits (β and γ) [7]. The α subunit contains three different domains: the catalytic domain, the autoinhibitory domain (AID) and the β-subunit binding domain [8]. The β subunit contains a glycogen-binding domain and the γ subunit contains four cystathionine-β-synthase (CBS) motifs. Of the four CBS motifs present in mammalian γ subunit, two bind AMP/ATP interchangeably (Sites 1 and 3), Site 4 binds AMP non-exchangeably and Site 2 does not bind nucleotide [8,9]. AMPK can be activated by phosphorylation on Thr172 by upstream kinases such as AMPK kinase (AMPKK) [6,10,11]. The β subunit functions as a scaffold for the α and γ subunits, and targets the complex to intracellular sites such as the plasma membrane after myristoylation of its N terminus [12,13] and to intracellular glycogen stores through its glycogen-binding domain [14]. Recently, protein crystallography showed that the C-terminal region of the β subunit of AMPK was important in mediating the interaction between the α and γ subunits [9,15,16]. Although AMPK is thought to play important roles in certain diseases such as diabetes, obesity, and stroke, the protein regulators of this enzyme are poorly understood [17–22]. In this study, we investigated the interaction between CRBN and AMPK, and the effects of CRBN on the functional activity of AMPK.

2. Materials and methods

2.1. Yeast two-hybrid screen

Rat CRBN (rCRBN) was used as bait to screen a rat brain cDNA library (Clontech, Palo Alto, CA). The full-length cDNA encoding rCRBN was subcloned into pGBK-T7, a Gal4 DNA-binding vector (BD Biosciences, San Jose, CA), using standard protocols. Stable expression of the bait protein in yeast strain AH109 was confirmed by immunoblotting using a mouse monoclonal antibody (mAb) specific for the GAL4 DNA-binding domain (Santa Cruz Biotechnology, Santa Cruz, CA). Library scale screening was performed on ~3 million co-transformants according to the manufacturer's instructions. A rat brain cDNA library was cloned into the GAL4 activation-domain vector pACT2 (BD Biosciences) and co-transformed into the yeast AH109 with the bait clone using the Li^+-acetate method. Co-transformants were plated and selected on triple-

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negative synthetic dropout medium (SD/TrpLeuAdenine). Colonies growing on the triple-negative medium were further selected on quadruple-negative medium (SD/TrpLeuHisAdenine, containing 1 mM 3-amino-1,2,4-trizole (3-AT)). In order to confirm initial positives, clones surviving on the quadruple-negative medium were retransformed with the bait vector. Subsequently, the DNA sequences of the confirmed positive clones were determined using the dideoxy chain termination sequencing method.

2.2. Cell culture and transfection

The HEK293FT, SH-SY5Y, HT22, and B103 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% (v/v) fetal bovine serum (FBS, Hyclone). Cells were transfected using either LipofectAMINE™ LTX (GIBCO) or Polyfect™ (Qiagen, Valencia, CA) according to the manufacturer's protocol.

2.3. Construction and generation of CRBN knockdown cell lines

In order to establish CRBN knockdown cells, lentiviral delivery of a short hairpin RNA (shRNA) was used. The MISSION™ TRC shRNA set, cloned into the pLKO-puro vector (TRCN0000113340-44), was purchased from Sigma. Lentiviral particles were generated and concentrated to 10^7 TU/μl (Macrogen, South Korea). HT22 cells infected with virus were selected and maintained using 10 μg/ml puromycin in the medium for 4 weeks, and down-regulation of endogenous CRBN was confirmed by Western blot analysis.

2.4. Site-directed mutagenesis of AMPK α1

A constitutively active mutant (1-548, T172D) and an inactive mutant of AMPK α1 (1-548, D157A) were generated by two sequential polymerase chain reactions using mutagenic primers. Synthetic oligonucleotides were obtained from Cosmo Genetech (Seoul, Korea). To confirm the authenticity of the DNA sequence of each mutant, all constructs were sequenced and/or restriction enzyme digested.

2.5. Western blot analysis

Proteins were separated by SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes. After blocking with 3% BSA in TBS-T (137 mM NaCl, 20 mM Tris–Cl, pH 7.6, 0.1% Tween 20), the blots were incubated with various primary antibodies: rabbit polyclonal anti-AMPK α1 (Epitomics, Burlingame, CA), rabbit polyclonal anti-phospho-AMPK α1 (Cell Signaling), rabbit polyclonal anti-AMPK β (Cell signaling), rabbit polyclonal anti-AMPK γ1 (C-terminus) (Epitomics), mouse monoclonal anti-Myc (Upstate), rat monoclonal anti-HA (Roche), rabbit polyclonal anti-ACC (Cell Signaling), or rabbit polyclonal anti-phospho-ACC (Cell Signaling). The generation of rabbit polyclonal anti-CRBN antibody against rat CRBN was described in a previous report [2] and its immunoreactivity with human and mouse CRBN was confirmed (Supplemental Fig. 1). The blots were then incubated with horseradish peroxidase (HRP) conjugated anti-rabbit, mouse, or rat secondary antibodies (Santa Cruz), and the protein bands were developed using enhanced chemiluminescence detection (ECL; Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK).

2.6. Co-immunoprecipitation

Cells were solubilized in lysis buffer (RIPA buffer: 20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% NP-40, 1% Sodium deoxycholate, 2 mM Na_3VO_4, 100 mM NaF, PMSF, protease inhibitor cocktail). The supernatant was incubated with various primary antibodies, such as the anti-HA, anti-CRBN, anti-AMPK β, or anti-AMPK α1 antibodies (Invitrogen), overnight at 4 °C. The antibody–protein complexes were precipitated with equilibrated Protein G beads (Amersham Biosciences) at 4 °C for 3 h followed by incubation with lysis buffer at 37 °C for 15 min.

2.7. Immunocytochemistry

Primary hippocampal neurons were prepared as described [23] from embryonic day 18 Sprague–Dawley fetal rats. Cells were fixed in 4% paraformaldehyde (PFA) and 4% sucrose, permeabilized with 0.25% Triton X-100, and stained with mouse anti-AMPKα1 (1:5) and rabbit anti-rCRBN antibody (1:10), overnight at 4 °C. Oregon Green 488-conjugated anti-mouse (Molecular Probes) and Texas Red-conjugated anti-rabbit (Molecular Probes) antibodies were used as secondary antibodies. Cells were visualized using confocal laser scanning microscopy. High resolution digital images were acquired using a 60× objective and were processed in Adobe Photoshop.

2.8. In vitro binding assay

For *in vitro* synthesis of WT AMPKα1, the catalytic domain (1–312), the autoinhibitory plus the β-subunit binding domain (313–548), the constitutively active mutant (α1 T172D), and the inactive mutant (α1 D157A) were subcloned into pcDNA3.1. After confirming the constructs, they were synthesized using the TNT couple® lysate system (Promega). Glutathione *S*-transferase (GST) or GST-fused rCRBN immobilized on Sepharose beads (3–5 μg) was incubated with 3 μL of ^{35}[S]-labeled proteins in TBS-T–TBT binding buffer (137 mM NaCl, 20 mM Tris–Cl, pH 7.6, 0.1% Tween 20, 100 mg/ml BSA and 0.5% Triton X-100), for 2–6 h at 4 °C. Samples were washed three times with the same buffer, followed by an additional three washes with TBS-T. After removal of the washing buffer, protein complexes were eluted by boiling in 20–50 μL of 2× SDS sample buffer (100 mM Tris–Cl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol and 200 mM β-mercaptoethanol), and then separated by SDS-PAGE. Separated protein bands were detected by exposing the gel to BioMax™ X-ray film (Kodak, Rochester, NY).

3. Results

3.1. Identification of CRBN as a novel AMPK α1 binding protein

In order to elucidate the cellular function of CRBN, CRBN binding partners were identified by screening a rat brain cDNA library using a yeast two-hybrid screen. One of the candidate proteins that interacted with CRBN was identified as the α1 subunit of AMPK. Full-length CRBN specifically interacted with AMPK α1 in yeast (Fig. 1A). The interaction between AMPK α1 and CRBN was further characterized using biochemical and cell biological assays. HA-fused CRBN specifically interacted with *myc*-fused AMPK α1 expressed in HEK293FT cells, as detected by co-immunoprecipitation (Fig. 1B). The association between endogenous AMPK α1 and CRBN was also studied in HEK293FT cells. Immunoreactivity against CRBN was detected in protein complexes precipitated by an antibody specific for AMPK α1 (Fig. 1C, 4th blot). The β and γ1 subunits of AMPK were detected in the precipitated complexes (Fig. 1C, 2nd and 3rd blot). We also performed reciprocal co-immunoprecipitation experiments using an antibody specific for CRBN. Immunoreactivity against all three subunits of AMPK, αl, β, and γ1 was detected in the protein complexes precipitated by an antibody specific for CRBN (Fig. 1D), suggesting that CRBN can interact with the α1 subunit of AMPK in the presence of β and γ1. In addition, endogenous CRBN and AMPK α1 co-localized in cultured rat primary hippocampal neurons. Neuronal AMPK α1 was detected in the cell body and dendrites where it displayed a punctate staining pattern (Fig. 2A). Punctate staining of CRBN was also found throughout the cell body and dendrites of hippocampal neurons (Fig. 2B), as previously shown [2]. As illustrated in Fig. 2C, some AMPK α1 co-localized with CRBN in rat hippocampal neurons (*arrowheads*).

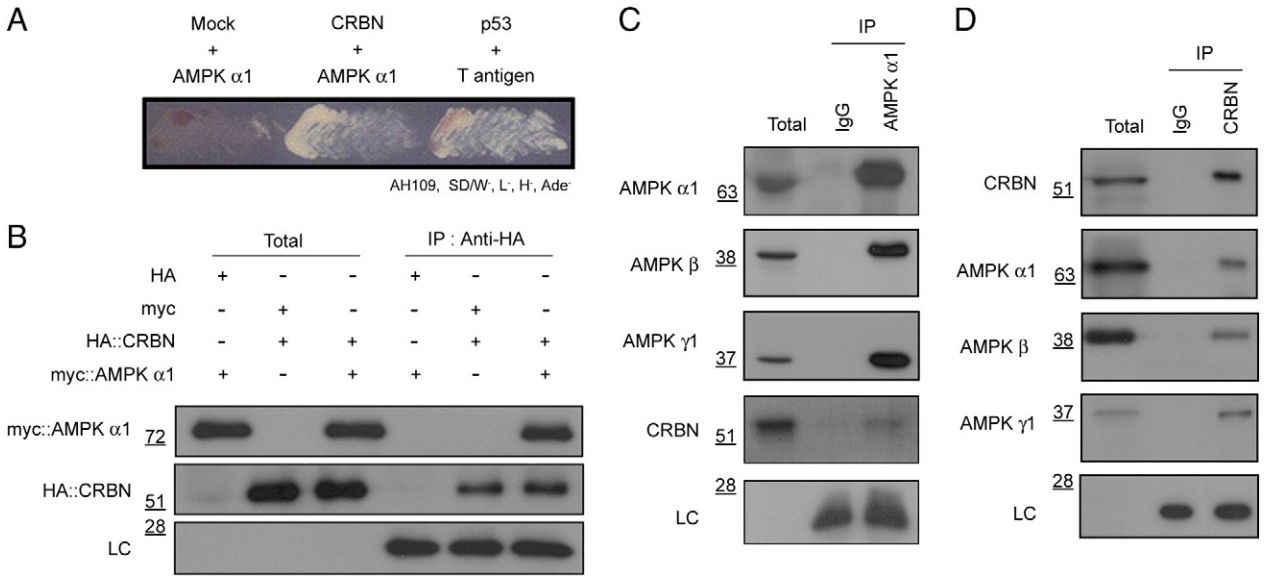

Fig. 1. Interaction of CRBN and AMPK α1. (A) Interaction between CRBN and AMPK α1 in yeast. The full-length CRBN and AMPK α1 were simultaneously co-transformed into the yeast strain AH109 and grown on SD/W$^-$L$^-$ plates. (B) HEK293FT cells were transiently co-transfected with HA::CRBN and Myc::AMPK α1 or empty vector. Cells were harvested after 24 h and CRBN was immunoprecipitated using anti-HA. Western blots of the immunoprecipitates were probed with either anti-Myc or anti-HA. AMPK α1 and CRBN were detected at approximately 72 and 53 kDa, respectively. LC indicates the IgG light chain. The plus and minus symbols indicate the presence or absence of each protein. (C) Co-immunoprecipitation of endogenous CRBN with the endogenous AMPK α1 from HEK293FT cell lysates. HEK293FT cell lysates were solubilized with RIPA buffer and immunoprecipitated with either mouse IgG or mouse anti-AMPK α1. The precipitates were separated by SDS-PAGE and immunoblotted with anti-CRBN, anti-AMPK α1, anti-AMPK β, and AMPK γ1. LC indicates the IgG light chain. (D) Co-immunoprecipitation of endogenous AMPK α1 with endogenous CRBN from HEK293FT cell lysates. HEK293FT cell lysates were solubilized with RIPA buffer and immunoprecipitated with either rabbit IgG or rabbit anti-CRBN. The precipitates were separated by SDS-PAGE and immunoblotted with anti-CRBN, anti-AMPK α1, anti-AMPK β, and AMPK γ1. LC indicates the IgG light chain.

3.2. CRBN competes with AMPK γ1 for AMPK complex formation

Next, we asked whether CRBN interacted directly with AMPK α1 and, if so, whether the binding of these two proteins was influenced by the phosphorylation state of AMPK α1. AMPK α1 can be phosphorylated at Thr172 by two different AMPKK, the tumor suppressor kinase LKB1, and the Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) [24,25]. Phosphorylation of the Thr172 residue is a major determinant of AMPK α activity and we therefore included the constitutively active AMPK α1 mutant (T172D) and an inactive AMPK α1 mutant (D157A) in

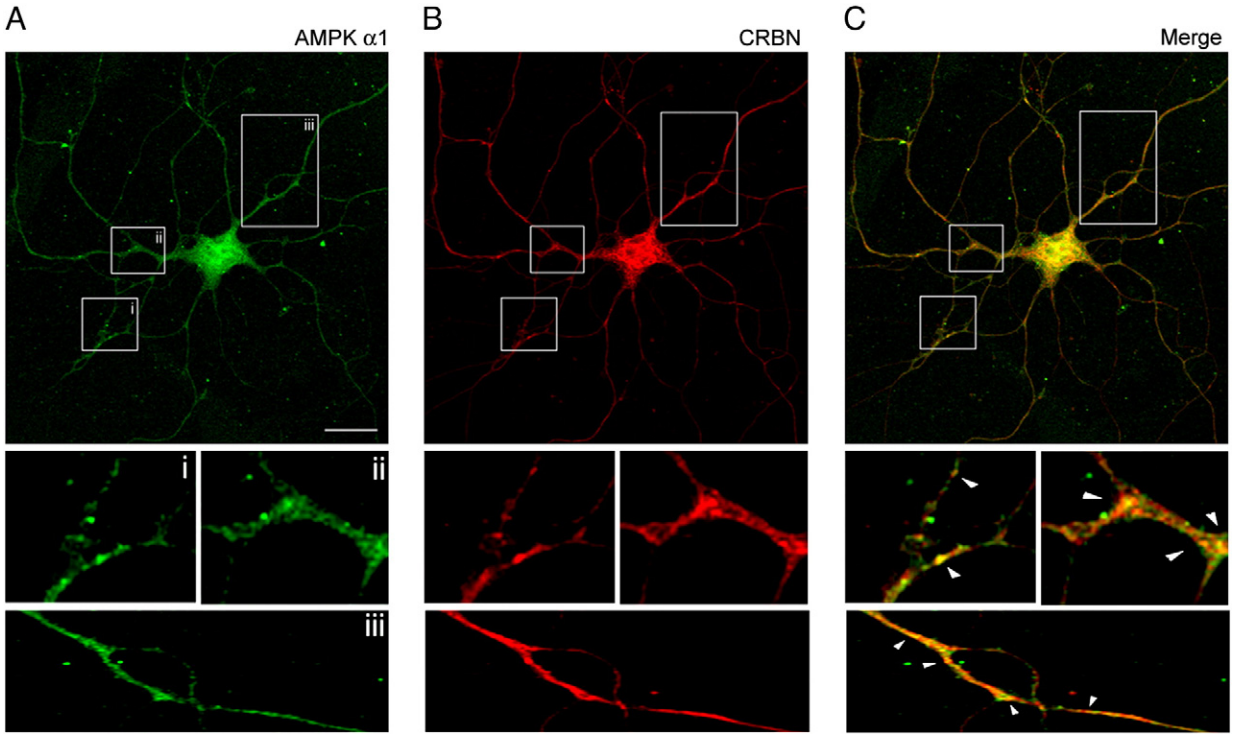

Fig. 2. Colocalization of CRBN and AMPK α1 in hippocampal neurons. Neurons cultured for 14 days *in vitro* were double stained with specific antibodies against CRBN and AMPK α1, and the expression patterns were visualized using a confocal microscope. AMPK α1, CRBN, and merged images are pseudocolored in green, red, and yellow, respectively. Arrowheads indicate co-localized AMPK and CRBN. Scale bar: 20 μm.

our assays [26]. CRBN was expressed as a GST-fusion protein in *E. coli* and purified by affinity chromatography. The wild-type (WT) and mutant AMPK α1 were radio-labeled using ^{35}[S]-Met in a coupled transcription/translation reaction *in vitro*. Binding assays were performed by incubating [^{35}S]-labeled WT, T172D, or D157A AMPK α1 with either GST-CRBN or GST alone immobilized on glutathione beads. All three AMPK α1 (WT, T172D and D157A) were pulled down specifically by GST-CRBN (Fig. 3A), suggesting that the two proteins interact directly and that the phosphorylation of AMPK α1 at T172 is not required for the association between CRBN and AMPK.

In order to identify the AMPK α1 domain that interacts with CRBN, fragments from the N-terminal region (residues 1–312) containing the kinase domain, and a C-terminal region (residues 313–548) harboring the AID and the β-subunit binding domain were generated. While CRBN bound to both fragments, the C-terminal region appeared to interact with CRBN with higher affinity than the N-terminal region (Fig. 3B). We further narrowed down the CRBN-interacting regions of AMPK α1 by constructing eight different deletion mutants (Supplemental Fig. 2A). It was shown by *in vitro* binding assay that two different regions, α1 (1–100) at the N-terminal end and α1(393–473) in the C-terminal region, interacted with CRBN (Supplemental Fig. 2B). By comparing the results of three different constructs, α1(313–422), α1(423–548) and α1(393–473), we were able to show that a small region in the C-terminus covering the amino acid 393–422 would be critical for high-affinity binding to CRBN. It was worth noticing that the N-terminal end of AMPK α1 was predicted to locate closely with the C-terminal region covering the residue 393–473 in a recent structural model [27]. Since the C-terminal β-subunit binding domain of the AMPK α1 subunit is critical for interaction with the β subunit and the C-terminal region of the AMPK α1 contains a regulatory sequence (RS) that is located close to the γ subunit [27], the binding of CRBN to the C-terminal region of the AMPK α1 (residues 313–548) may affect formation of the AMPK complex made up of α, β and γ subunits. Thus, we examined whether an excess of CRBN could influence the formation of the AMPK complex. Since AMPK holoenzymes exist as heterotrimeric complexes [28], the formation of endogenous AMPK complexes was examined by immunoprecipitation in HEK293FT cells (Fig. 3C). In eukaryotic cells, AMPK is predominantly composed of α1βγ1 subunits [7,29]. While the band intensity of the AMPK γ1 subunit was significantly reduced by exogenous expression of HA-tagged CRBN, no such decrease in band intensity was observed for the β subunit. Moreover, HA-tagged CRBN was clearly detected in the AMPK α1 immunoprecipitate. The intensity of the γ1 subunit relative to the α1 subunit was reduced by approximately 60% in response to CRBN expression, but no significant change in the intensity of the β subunit was observed (Fig. 3D). The decreased intensity of the γ subunit in the presence of CRBN was also evident when the AMPK complex was precipitated using the antibody specific for the β subunit (Supplemental Fig. 3). Together with the data showing the binding of CRBN to the C-terminal region of AMPK α1 *in vitro*, these results strongly suggest that CRBN can reduce the affinity of the γ1 subunit for the AMPK complex by directly binding to the α1 subunit.

3.3. Suppression of AMPK activation by ectopic expression of CRBN

The γ subunit of AMPK is a sensor that detects increased levels of AMP evoked by energy imbalance [15,30]. Thus, dissociation of the γ subunit from the AMPK complex by CRBN may influence AMPK activity in energy-restricted conditions. To examine the effect of CRBN expression

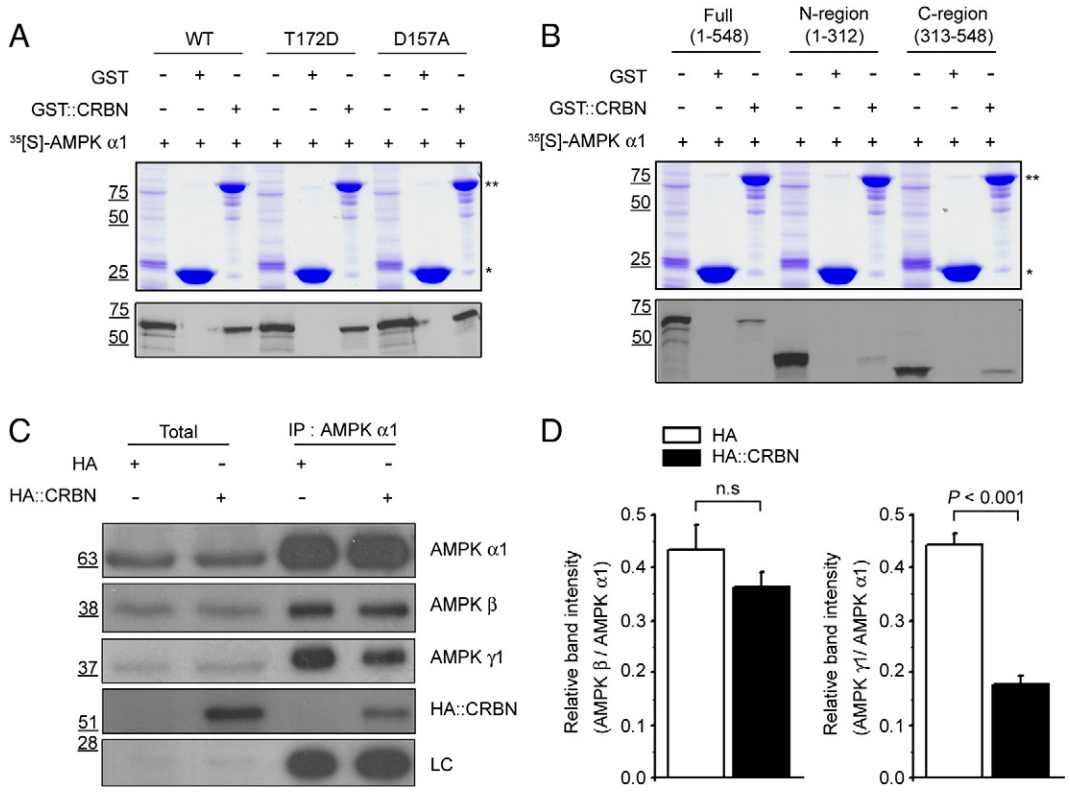

Fig. 3. Disruption of the interaction between the α and γ subunit of AMPK by CRBN. (A) GST and GST-CRBN were detected at approximately ~26 kDa (*) and 79 kDa (**) (upper panel, lanes 2, 5, 8 and 3, 6, 9), respectively. The wild-type (WT), constitutively active mutant (T172D), and inactive mutant (D157A) AMPK α1 were subcloned into a modified pcDNA3 vector, transcribed to mRNA, and then translated *in vitro* using the TNT couple® lysate system (Promega) with [^{35}S]-Met. The plus and minus symbols indicate the presence or absence of each protein. (B) Full-length protein (1–548), N-terminal fragment [residues 1–312, kinase domain], and C-terminal fragment [residues 313–548; autoinhibitory domain (AID) and β-subunit binding domain of AMPK α1] were obtained as described in (A). (C) Cell lysates were prepared from HEK293FT cells transfected with pcDNA3-HA (HA) or pcDNA3-HA/CRBN. Proteins were precipitated with anti-AMPK α1 and probed with anti-AMPK α1, anti-AMPK β, anti-AMPK γ1, and anti-HA. Endogenous AMPK α1, β, γ1, and expressed HA-CRBN were detected at approximately ~68, 38, 37, and 53 kDa, respectively. LC indicates the IgG light chain. (D) Relative band intensity was measured by densitometric analysis of the blot in (C). The results shown are the means ± SD of four independent experiments.

on AMPK activation, HEK293FT cells were transiently transfected with pcDNA3-HA or pcDNA3-HA/CRBN, using CRBN sequences from human, mouse or rat. The intensity of the phosphorylated AMPK α1 (phosphor-AMPK α1) band, which indicates the level of activation of the enzyme, was significantly decreased by exogenous expression of each of the CRBN genes, compared to mock (pcDNA3-HA) transfected cells (Fig. 4A). The level of AMPK α1 activation was compared by quantifying the ratio of phosphorylated AMPK α1 to total AMPK α1 (P-AMPK α1/AMPK α1) (Fig. 4B). Expression of human, mouse, and rat CRBN decreased the AMPK activation by 46%, 42%, and 42%, respectively (Fig. 4A and B). We also examined the effects of rat CRBN expression in three different neuronal cell lines. The pcDNA3-HA or pcDNA3-HA/CRBN constructs were transiently transfected into human neuroblastoma SH-SY5Y, mouse hippocampal HT22, and rat neuroblastoma B103 cells. Western blot analyses revealed that the band intensity of phosphor-AMPK α1 was significantly reduced upon ectopic expression of HA-CRBN in all three cell lines tested (Fig. 4C). The relative band intensity of P-AMPK α1/AMPK α1 was decreased by 44% in SH-SY5Y, 56% in HT22, and 41% in B103 cells (Fig. 4D). These results indicate that ectopic expression of CRBN can inhibit the activation of AMPK in diverse cell types and that the inhibitory effects of CRBN from three different species are comparable.

The effects of CRBN expression under conditions known to activate endogenous AMPK were examined. AMPK can be activated by chemical activators, such as AICAR, or by serum starvation [31,32]. Activation of AMPK, as indicated by the phosphorylation of AMPK α1, was detected in HEK293FT cells in response to serum starvation alone or in combination with AICAR treatment (Fig. 5A). To evaluate the effects of CRBN on AMPK activation in response to serum starvation, HEK293FT cells that had been transiently transfected with pcDNA3-HA or pcDNA3-HA/CRBN were incubated under serum-deprived conditions for 24 h. In control cells transfected with pcDNA3-HA, a significant increase in the phosphorylation of AMPK α1 was observed in response to serum starvation (Fig. 5B, lanes 1 and 3, 2nd panel). However, the expression of CRBN potently inhibited phosphorylation of the α1 subunit, both in the presence and the absence of serum (Fig. 5B, lanes 2 and 4, 2nd panel). The effects of CRBN expression on AMPK activation in the absence or presence of serum are summarized in Fig. 5C. The ectopic expression of CRBN decreased the relative intensity of phosphor-AMPK α1 by 43% under normal culture conditions and by 34% under serum-deprived conditions. We also examined the effects of CRBN on the downstream AMPK signaling pathway. The reduction in AMPK α1 activation resulted in the suppression of phosphorylation of the acetyl coenzyme A carboxylase (ACC), a well characterized AMPK substrate (Fig. 5B, lanes 2 and 4, 4th panel). The level of ACC phosphorylation was compared in the presence or absence of CRBN expression by quantifying the ratio of phosphorylated ACC to total ACC (P-ACC/ACC). Ectopic expression of CRBN decreased the relative intensity of P-ACC by 48% under normal culture conditions and by 36% under serum-deprived conditions (Fig. 5D). These results indicate that overexpression of CRBN decreases the phosphorylation of AMPK α1 and, thus, suppresses AMPK activity in a serum-independent manner.

3.4. Up-regulation of AMPK activity by knockdown of endogenous CRBN

Although AMPK activity was clearly suppressed by the ectopic expression of CRBN, it was unclear whether endogenous CRBN regulated AMPK activity. We therefore examined the regulation of AMPK signaling in the HT22 cell line, in which endogenous CRBN was suppressed by stable expression of an shRNA specific for CRBN. Western blotting was used to confirm the suppression of endogenous CRBN at the protein level (Fig. 6A). Moreover, we confirmed that the expression level of endogenous LKB1 and CaMKKβ in the CRBN knockdown (KD) cell line was not significantly different from that in

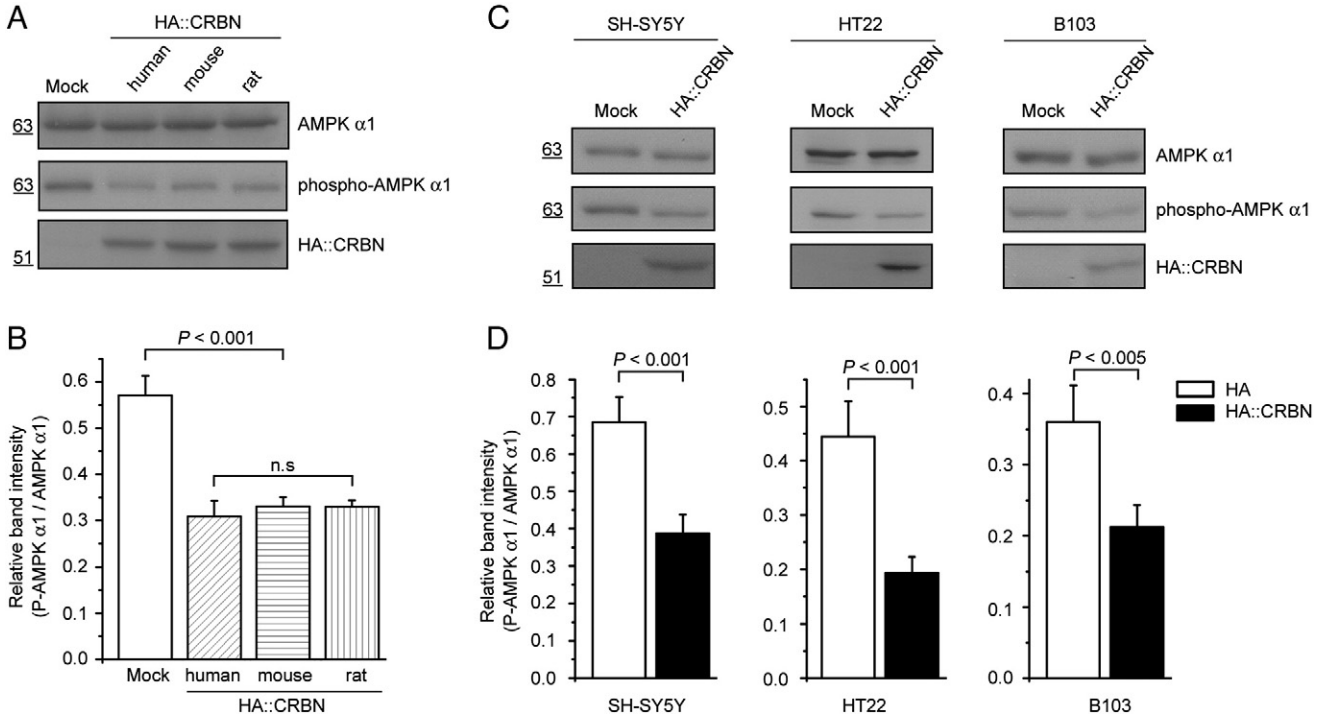

Fig. 4. Decreased AMPK activation in cells expressing CRBN. (A) HEK293FT cells were transiently transfected with pcDNA3-HA (Mock) or pcDNA3-HA/CRBN (HA::CRBN) of human, mouse, or rat origin, respectively. Proteins were separated on SDS-PAGE and immunoblotted with anti-phosphorylated-AMPK α1, and anti-HA. Anti-AMPK α1 was used to probe for equal protein loading. (B) To determine the effects of CRBN expression on AMPK activation in total and phosphorylated AMPK, the relative band intensity was measured by densitometric analysis of the blot in (A). The results shown are the means ± SD of four independent experiments. (C) Human neuroblastoma SH-SY5Y cells, mouse hippocampal HT22 cells, and rat neuroblastoma B103 cells were transfected with pcDNA3-HA (Mock) or pcDNA3-HA/CRBN (HA::CRBN), respectively. Proteins were subjected to immunoblotting using anti-phosphorylated-AMPK α1 and anti-HA. Anti-AMPK α1 was used to probe for equal protein loading. (D) The relative band intensity was measured by densitometric analysis of the blot in (C) to determine the effects of CRBN expression on AMPK activation in total and phosphorylated AMPK. The results shown are the means ± SD of four independent experiments.

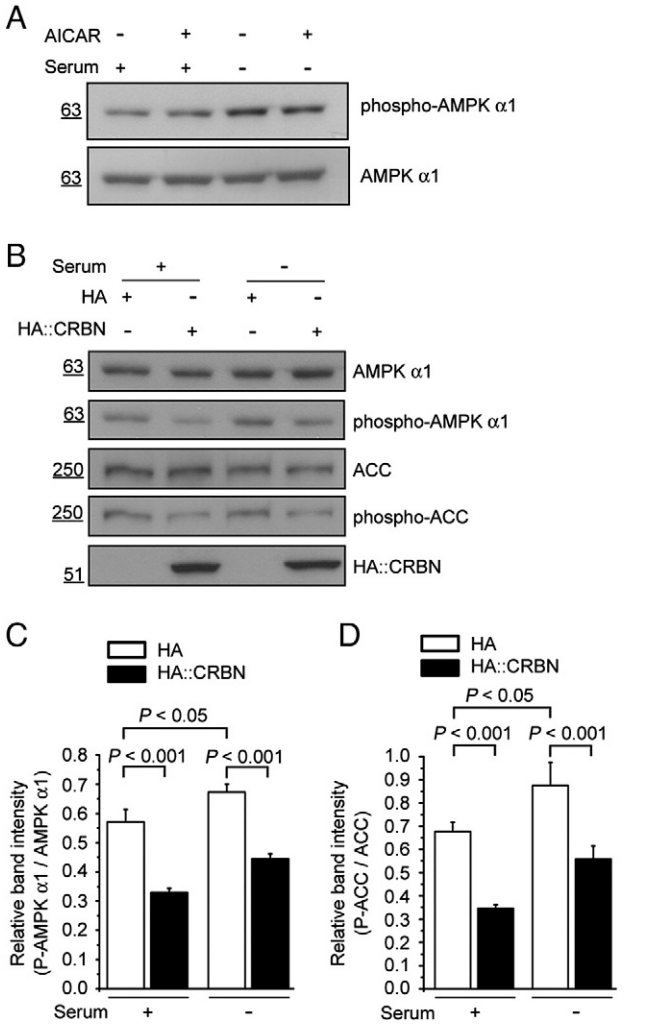

Fig. 5. Effects of exogenous CRBN on AMPK activation by serum deprivation. (A) HEK293FT cells grown in the presence or absence of serum were incubated in the presence or absence of 1 mM AICAR for 24 h. Western blot analysis was performed with anti-AMPK α1 (or anti-phospho-AMPK α1). Anti-AMPK α1 was used to probe for equal protein loading. (B) Cell lysates were prepared from HEK293FT cells transfected with pcDNA3-HA (HA) or pcDNA3-HA/CRBN (HA::CRBN). Proteins were subjected to immunoblotting using anti-phospho-AMPK α1 and anti-ACC. Anti-AMPK α1 and anti-ACC were used to probe for equal protein loading. Endogenous AMPK α1 (or phospho-AMPK α1), ACC (or phospho-ACC), and expressed HA-CRBN were detected at approximately ~63, 260, and 53 kDa, respectively. The plus and minus symbols indicate the presence or absence of each protein. (C) Relative band intensity of was determined by densitometric analysis of (B). The results shown are the means ± SD of four independent experiments.

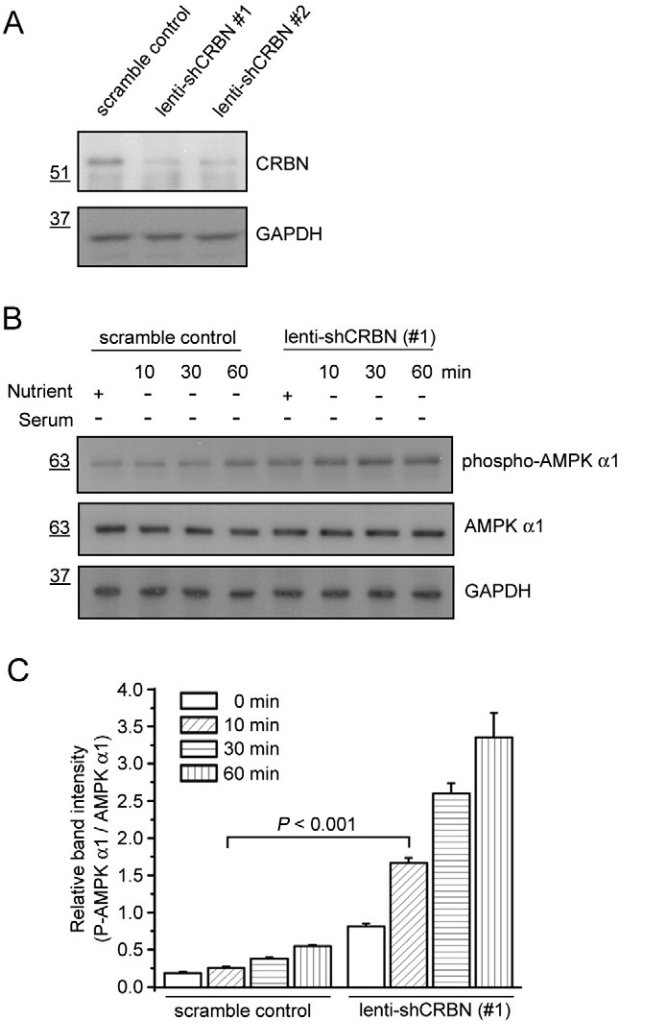

Fig. 6. Increase in AMPK activation by suppression of endogenous CRBN. (A) CRBN knockdown cell lines were generated by infecting mouse hippocampal HT22 cells with a lentivirus carrying a short hairpin RNA (shRNA). Lysates were immunoblotted with rabbit anti-CRBN. Anti-GAPDH was used to probe for equal protein loading. (B) CRBN knockdown cells were grown in the presence of serum for 24 h and, subsequently, in the absence of serum for 3 h. Cells were then washed with HBSS three times and incubated for the indicated times (10, 30, and 60 min) in HBSS. (C) Relative band intensity of was determined by densitometric analysis of the blot in (B). The results shown are the means ± SD of four independent experiments.

the control cell line (Supplemental Fig. 4). When cells were placed in a nutrient-free medium (Hank's buffered salt solution, HBSS), as described previously [33], phosphorylation of AMPK α1 slightly increased, in a time-dependent manner, in control cells expressing a scrambled shRNA. In contrast, AMPK activation was dramatically increased, by up to seven-fold, in the CRBN knockdown (KD) cell line within 60 min (Fig. 6C). These results further support the hypothesis that CRBN can function as an endogenous repressor of AMPK activity.

4. Discussion

AMPK is a multifunctional metabolic sensor that maintains cell survival in human diseases associated with metabolically abnormal conditions [34]. However, the mechanisms that regulate AMPK *in vivo* are still poorly understood. By searching for proteins that bind CRBN, we demonstrated that CRBN directly interacts with the α subunit of AMPK and reduces the enzymatic activity of AMPK by suppressing phosphorylation of its catalytic subunit. The interaction between AMPK α1 and CRBN was confirmed *in vivo* as well as *in vitro* and the binding of CRBN reduced the level of the γ subunit in the AMPK complex (Fig. 3C), suggesting its potential role as an endogenous negative modulator.

We observed that the activation of endogenous AMPK is greatly enhanced in CRBN KD cells (Fig. 6C) further implies that at least some CRBN may be present in the endogenous AMPK complex as a binding partner and limit the activation of AMPK. Under nutrient-reduced conditions, CRBN KD cells exhibited a faster and more substantial activation of AMPK, with a 2-fold increase at 10 min and a 7-fold increase at 60 min, compared to the control cells, which exhibited a 2-fold increase at 30 min and a 3-fold increase at 60 min (Fig. 6C). Negative regulation by CRBN may block the hyper-activation of AMPK in both presence and absence of sufficient nutrients. One possible interpretation is that CRBN may interfere with the flexibility of the AMPK complex, potentially affecting the interaction between the RS (regulatory sequence) with a neighboring sequence of α subunit and γ subunit. Although the X-ray structures of AMPK complex were revealed recently by three independent groups [9,15,16], the structures

did not include the kinase domain of the α subunit or the N-terminal region of the β subunit. One of structural studies showed that an interaction was possible between the RS of the α subunit and the γ subunit [16]. It was confirmed in a separate study that the RS of the α subunit is indeed near the AMP-binding site (Site 1) of the γ subunit [27]. Based on these recent structural studies, it is possible that Thr172 is better protected from dephosphorylation in the absence of CRBN than in its presence, because CRBN interacts with the AMPK α1 subunit via separate binding sites in the N-terminal and C-terminal regions (Fig. 3B). This idea is supported by the fact that CRBN interacts strongly with AMPK α1 via a C-terminal region covering amino acid 394–422 (Supplemental Fig. 2B) and that this CRBN-interacting region is co-localized [27] or very close to the RS [8]. Alternatively, CRBN could interfere with the stability of the AMPK complex and cause the dissociation of the γ subunit, especially when CRBN is overexpressed. Dissociation of the γ subunit may have implications for the suppression of AMPK activity, since the AMP-binding γ subunit could induce a conformational change in the kinase domain of the α subunit that protects AMPK from Thr172 dephosphorylation [27]. Thus, the direct binding of CRBN to the α subunit and the liberation of the γ subunit from the AMPK complex may result in suppression of enzymatic activity and lack of phosphorylation of downstream targets such as ACC (Fig. 5B).

Initially, the *CRBN* gene was identified in a group of ATNSMR patients as having a single C to T point-mutation at position 1274 [1]. This nonsense mutation is likely to cause premature termination and a deletion of the last 24 amino acids of human CRBN. However, the molecular pathology of the CRBN mutation remains elusive. To our knowledge, there are no reports clearly showing the expression of a truncated CRBN in affected individuals. It will be of interest to find out whether the inhibitory effects of CRBN on AMPK are modified by the mutation in ATNSMR patients. Since CRBN interacts with the α1 subunit of AMPK via its N-terminal *Lon* domain (Supplemental Fig. 5), it remains to be seen whether the truncated CRBN missing the C-terminal 24 amino acids exhibits any differences in binding to the AMPK α1 subunit and suppression of AMPK activity.

In a recent report, CRBN was characterized as a substrate binding component of the E3-ubiquitin ligase and a primary target of thalidomide teratogenicity [4]. Since AMPK α1 interacts directly with CRBN, the AMPK α1 subunit may be a previously unidentified substrate for the CRBN E3 ubiquitin ligase. However, the subcellular location of AMPK α1 differs from the CRBN E3 ubiquitin ligase complex. While AMPK α1 is located throughout the cytoplasm and the nucleus [35,36], the majority of the E3 complex containing DDB1 (damaged DNA-binding protein 1) and Cul4 (Cullin 4) is found in the nucleus [37]. Notably, the nuclear localization of CRBN as a component of the E3 ligase complex was conducted only with exogenous CRBN [4]. In addition, according to previous reports [2,3,38], most of the immunoreactivity against endogenous CRBN is found in the cytoplasm, including the soluble and light membrane fractions, in three . Thus, whether CRBN plays a role in many different cellular functions, as do many other substrate binding components of the E3 ubiquitin ligase [39–42], remains to be determined.

As a key sensor of metabolic stress, AMPK has been associated with many human diseases. While many researchers have suggested that the activation of AMPK protects cells from hypoxic/ischemic damage [32], several reports revealed opposing roles for AMPK in the induction of oxidative stress and neuronal death in stroke [43,44]. The AMPK signaling cascade may differ depending on the upstream activating kinase, such as LKB1 or CaMKKβ [25,45]. Thus, the protective or suppressive effects of CRBN in human metabolic diseases remain an intriguing possibility. The suppression of AMPK activity by CRBN may provide a valuable link between the cellular function of this novel protein and the pathology of the human mutation. In conclusion, CRBN may negatively regulate the functional activity of AMPK *in vivo* by binding directly to its α1 subunit.

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