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Cereblon inhibits proteasome activity by binding to the 20S core proteasome subunit beta type 4

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ABSTRACT

In humans, mutations in the gene encoding cereblon (CRBN) are associated with mental retardation. Although CRBN has been investigated in several cellular contexts, its function remains unclear. Here, we demonstrate that CRBN plays a role in regulating the ubiquitin–proteasome system (UPS). Heterologous expression of CRBN inhibited proteasome activity in a human neuroblastoma cell line. Furthermore, proteasome subunit beta type 4 (PSMB4), the β7 subunit of the 20S core complex, was identified as a direct binding partner of CRBN. These findings suggest that CRBN may modulate proteasome activity by directly interacting with the β7 subunit.

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1. Introduction

The *cereblon* gene (*CRBN*) was initially identified in association with mild autosomal recessive non-syndromic mental retardation [1]. CRBN is highly conserved from plants to humans and interacts with several cellular proteins. CRBN binds to the large-conductance calcium-activated potassium channel (BK_{Ca}), thereby regulating its surface expression [2]. Another voltage-gated chloride channel (CIC) protein associates with CRBN in the retina [3]. Moreover, CRBN binding to thalidomide mediates its teratogenic effects; CRBN also forms an E3 ligase complex with damaged DNA binding protein 1 [4]. Recently, CRBN was reported to interact with the α1 subunits of AMP-activated protein kinase (AMPK) and prevent the formation of a functional holoenzyme with regulatory subunits β and γ [5]. However, despite the recent research progress with regard to CRBN, its physiological function remains unclear.

The proteasome is a large multi-subunit ATP-dependent protease complex crucial for non-lysosomal protein degradation in eukaryotic cells [6]. The 26S proteasome comprises a 20S catalytic core particle (CP) and two 19S regulatory particles (RPs) surrounding the 20S core [6]. The 20S proteasome comprises the distinct 7 α-subunits and 7 β-subunits transiently associated with others, forming the $\alpha_7\beta_7\beta_7\alpha_7$ structure [7]. Among these 14 subunits, the β1, β2, and β5 subunits have caspase-like, trypsin-like, and chymotrypsin-like activity, respectively [8]. Five of the β-subunits (β1,β2,β5,β6 and β7) are synthesized as precursor proteins bearing N-terminal propeptides [9]. Protein turnover is essential for cellular function, and protein degradation by the 26S proteasome plays a critical role in diverse biological processes [10]. In nerve cells, for example, synaptic plasticity and the formation of specialized zones are highly dependent on protein degradation [11]. Alterations in proteasomal activity are associated with neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's, which are characterized by protein aggregation and misfolding [12]. Here, we show that CRBN interacts with the β7 subunit (PSMB4) of the 20S proteasome and provide evidence of a novel function of CRBN in modulating proteasome activity.

2. Materials and methods

2.1. Cell culture and transfection

SH-SY5Y, N2A, and HEK293FT cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT) and 50 μg/ml gentamycin (GIBCO). Cells were transfected using either Lipofectamine™ (Invitrogen, Carlsbad, CA) or Polyfect™ (Qiagen, Valencia, CA) according to the manufacturer's protocols.

2.2. Construction of proteasome subunit beta type-4 (PSMB4; β7)

The full-length coding sequence of the human proteasome subunit beta type-4 (PSMB4; β7) (NCBI accession no. NP_002787) was subcloned into the pcDNA3.1 and pCS2+MT vectors using a clone (clone ID: hMU011314) purchased from 21C Frontier Human Gene Bank (Daejeon, Korea).

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2.3. Short hairpin RNA (shRNA) CRBN knockdown studies

Short hairpin RNA (shRNA) was used to inhibit CRBN gene expression. The MISSION™ TRC shRNA set, cloned into the pLKO-puro vector (TRCN0000113340-44), was purchased from Sigma. The shRNA plasmids were transfected into HT22 cells and down-regulation of endogenous CRBN was confirmed by Western blot analysis.

2.4. Western blotting

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto polyvinylidene fluoride (PVDF) or nitrocellulose (NC) membranes. After blocking with 3% BSA in Tris-buffered saline containing 0.2% Tween-20 (TBS-T), the blots were incubated with various primary antibodies including anti-HA (Cell Signaling, 1:1000), anti-Myc (Upstate, 1:2500), anti-green fluorescent protein (GFP) (Abcam, 1:5000), anti-GAPDH (AbFrontier, 1:5000), anti-α-tubulin (Sigma, 1:20000), anti-ubiquitin (Enzo Life Sciences, 1:1000), anti-α2 (Enzo Life Sciences, 1:1000), and anti-β7 (Enzo Life Sciences, 1:5000). The generation of the anti-CRBN antibody is described in a previous report [5]. The blots were then incubated with secondary antibody [anti-rabbit horseradish peroxidase (HRP)-conjugate or anti-mouse HRP-conjugate (Santa Cruz Biotechnology; 1:10000)]. The protein bands were visualized using the enhanced chemiluminescence detection system (ECL; Amersham Pharmacia).

2.5. Co-immunoprecipitation

Cell lysis and protein solubilization were performed in 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, 1 mM PMSF, protease inhibitor cocktail). Clarified cell lysates were incubated with anti-HA, anti-β7, or anti-α2 antibodies for 6 h at 4 °C. Antibody-protein complexes were precipitated with equilibrated Protein G Sepharose™ 4 Fast Flow beads (GE Healthcare) for 1 h at 4 °C.

2.6. Proteasome activity in cell lysates

Cells were lysed by sonication in reaction buffer (30 mM Tris–Cl, pH 7.8, 5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM ATP). Proteasome activity was determined by measuring the fluorescence generated by the release of the fluorogenic AMC (excitation at 380 nm; emission at 460 nm) upon cleavage of the peptidase substrate Suc-LLVY-AMC (Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin).

2.7. Statistical analysis

All values were expressed as the mean ± SEM. An unpaired Student's *t*-test was used to examine the statistical significance of the differences. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. CRBN directly interacts with β7 in vitro

In a previous report, we described the yeast two-hybrid screening of a rat brain cDNA library using the full-length CRBN as bait to identify potential binding partners [5]. To validate the candidate proteins and to narrow down the binding site further, we performed the yeast two-hybrid assay using the candidate proteins and three different regions of CRBN (N-, RGS, and C-domains). The proteasome subunit PSMB4 (β7) was one of the candidate proteins interacting with CRBN through its RGS domain (Fig. 1A). Because the β7 precursor (pro-β7) contains a propeptide within its N-terminus, which is removed during dimerization in the 20S proteasome assembly process [9], we examined whether CRBN interacted directly with pro-β7 or β7 *in vitro*.

GST-fused CRBN was expressed in, and purified from, *Escherichia coli*. Pro-β7 and β7 were radioactively labeled with ^{35}[S]-methionine and binding assays were performed by incubating ^{35}[S]-labeled pro-β7 or β7 with either GST-CRBN or GST alone immobilized on glutathione beads. Both pro-β7 and β7 were pulled down specifically by GST-CRBN (Fig. 1B and C), suggesting that the two proteins interact directly and that the propeptide in pro-β7 does not affect the direct interaction between CRBN and β7.

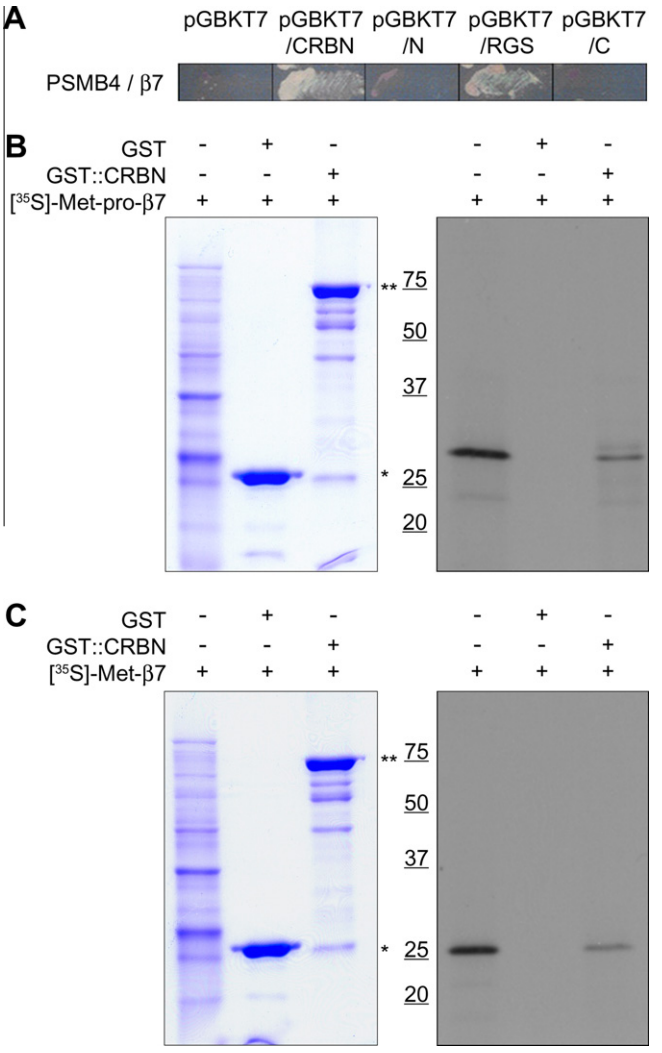

Fig. 1. CRBN as a β7 binding protein. (A) The full-length sequence of CRBN was divided into three domains, N(1–119), RGS(120–255), and C(255–445), and each was cloned into the pGBKT7 vector. PSMB4 (β7) and each of the CRBN-domains were co-transformed into the yeast AH109 strain. N, RGS, and C indicate the N-terminal domain of the *Lon* protease domain, the 'regulators of G protein-signaling'-like domain, and the C-terminal domain, respectively, as previously reported [2]. (B and C) GST and GST-CRBN were detected at approximately ~26 kDa (∗) and 75 kDa (∗∗) (*left* panel, lanes 2 and 3), respectively. Either pcDNA3.1/pro-β7 or pcDNA3.1/ β7 were radiolabeled with [^{35}S]-methionine by *in vitro* transcription and translation using the TNT couple® lysate system (Promega). The plus and minus symbols stand for the presence or absence of the indicated protein.

3.2. CRBN interacts with β7 in SH-SY5Y cells

Next, we examined the interaction between CRBN and pro-β7 or β7 in cultured mammalian cell lines. *Myc*-fused pro-β7 or *myc*-fused β7 with HA-fused CRBN genes were transiently transfected into SH-SY5Y cells and proteins were precipitated with a mouse anti-HA antibody. Western blotting using an anti-*myc* antibody showed that *myc*-fused pro-β7 and *myc*-fused β7 specifically interacted with HA-fused CRBN (Fig. 2A and B). The association between endogenous CRBN and β7 was further characterized in SH-SY5Y cells. Cell extracts were precipitated using either anti-β7 or anti-β2 antibodies. Western blotting using an anti-β7 antibody indicated that immunoprecipitation with the β7 antibody was effective (Fig. 2C, lane 3, second panel) and that β7 was present in protein complexes precipitated by the anti-α2 antibody (Fig. 2C, lane 4, second panel). Moreover, immunoblotting with an anti-α2 antibody showed that α2 (PSMA2) was present in protein complexes precipitated by the β7 antibody (Fig. 2C, lane 3, third panel) and confirmed that immunoprecipitation with the α2 antibody was also effective (Fig. 2C, lane 4, third panel). Immunoreactivity against CRBN was detected in protein complexes precipitated by antibodies specific for β7 and α2. Because α2 is a subunit of the 20S CP, these results suggested that CRBN may associate with the intact proteasome complex *in vivo*. Because β7 and α2 have a molecular weight similar to that of the light chain (LC) of IgG, positive immunoreactivity was detected in IgG immunoprecipitates (Fig. 2C, lane 2, second and third panels). However, immunoreactivity against the heavy chain of IgG (HC) confirmed equal loading of the immunoprecipitate (Fig. 2C, second and third panels).

3.3. Overexpression of CRBN inhibits the ubiquitin–proteasome system

To determine the role of CRBN in the function of the 26S proteasome, the activity of the ubiquitin–proteasome system (UPS) was examined using a GFP reporter vector (GFP-CL1 or GFP^u), which is a widely used florescent reporter for measuring proteasome activity [13]. Fusion of the CL1 degron, a constitutive degradation signal, to the C-terminus of GFP to generate GFP-CL1 (GFP^u) leads to its rapid ubiquitination and proteasomal degradation [13]. Because it is specifically and continuously degraded under a healthy UPS system by cleavage of the 16-residue short degron [14,15], GFP^u degradation is an indicator of 26S proteasome activity.

The HA or HA-fused CRBN gene was transiently co-transfected along with GFP^u into SH-SY5Y cells. GFP^u-positive SH-SY5Y cells were visualized by fluorescence microscopy. Intriguingly, the number of GFP^u-positive CRBN-transfected SH-SY5Y cells increased more than 2.8-fold compared to mock-transfected controls (Fig. 3B and D). Furthermore, treatment of cells with the proteasome inhibitor MG132 as a positive control resulted in a >3.8-fold increase in the number of GFP^u-positive SH-SY5Y cells compared with mock-transfected controls (Fig. 3B and D), indicating the accumulation of undegraded GFP^u. We also confirmed that ectopic expression of CRBN in cells induced the accumulation of unstable GFP^u, but not that of GFP (Fig. 3A and C). In addition, we verified that the ubiquitin-conjugated proteins accumulated in cells overexpressing CRBN when compared with the control cells (Supplementary Fig. 1).

Next, we examined the effects of CRBN expression on the level of GFP^u degradation in three different cell lines. The pcDNA3-HA or pcDNA3-HA/CRBN genes were transiently co-transfected with GFP^u constructs into two neuronal cell lines (human neuroblastoma SH-SY5Y and mouse neuroblastoma N2A) and a non-neuronal cell line (human embryonic kidney (HEK)239FT) to exclude the possibility of cell line specific effects on the UPS system. Western blot analyses with an anti-GFP antibody showed that GFP^u levels were higher in the three cell lines transiently expressing both CRBN and GFP^u than those in the controls (Fig. 3E). These results indicated that ectopic expression of CRBN inhibited GFP^u degradation and thus proteasome activity, and that the inhibitory effects of CRBN may be similar in diverse cell types.

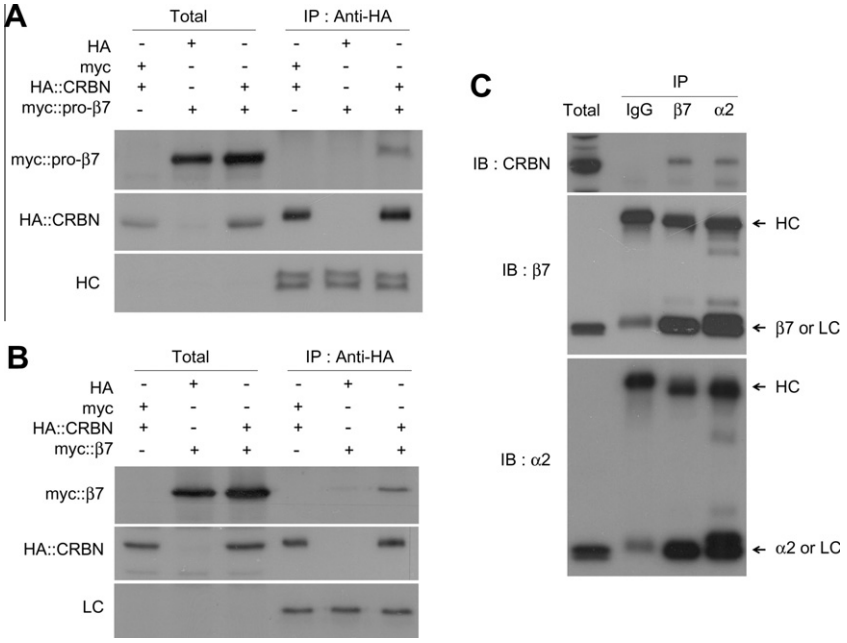

Fig. 2. Interaction between CRBN and β7. (A and B) SH-SY5Y cells were transiently co-transfected with HA::CRBN and Myc::pro-β7 (or Myc::β7) or empty vector. Lysates were immunoprecipitated with an anti-HA antibody and immunoprecipitates were subjected to Western blotting against anti-Myc (*upper* panel) or anti-HA (*middle* panel) antibodies. HC and LC indicate the IgG heavy chain and IgG light chain, respectively. The plus and minus symbols stand for the presence or absence of the indicated protein. (C) SH-SY5Y cell lysates were immunoprecipitated with IgG, anti-β7, or anti-β2 antibodies. The precipitates were separated by SDS–PAGE and immunoblotted against anti-CRBN, anti-β7, and anti-α2 antibodies. HC indicates the IgG heavy chain.

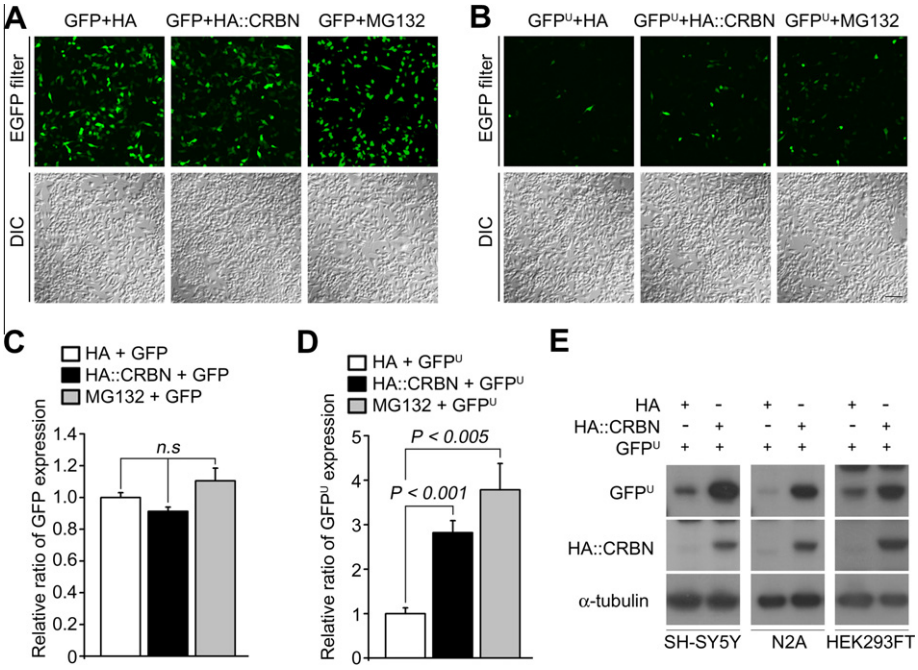

Fig. 3. CRBN overexpression increased the accumulation of GFP^u. (A and B) SH-SY5Y cells were co-transfected with HA or HA::CRBN and pEGFP (or pGFP^u) (7:1 ratio), and then cultured in the presence or absence of 1 μM MG132 1 h prior to analysis. For a precise comparison of fluorescence intensities, all images were obtained using the same laser source. Scale bar: 100 μm. (C) GFP-positive cells were scored using a fluorescence microscope as described in (A), and relative expression is presented as the mean ± SEM. (D) GFP^u-positive cells were scored using a fluorescence microscope as described in (B), and relative expression is presented as the mean ± SEM. (E) HA or HA::CRBN were co-transfected with pGFP^u (7:1 ratio) into human neuroblastoma SH-SY5Y, mouse neuroblastoma N2A, and human embryonic kidney (HEK293FT) cells. Cell lysates were subjected to Western blotting against anti-GFP, anti-HA, and anti-α-tubulin antibodies to detect GFP^u, HA::CRBN, and α-tubulin, respectively. The plus and minus symbols stand for the presence or absence of the indicated protein. The results shown are representative of four independent experiments.

3.4. CRBN expression modulates the chymotrypsin-like activity of the proteasome

Next, the effects of CRBN expression on the activity of the proteasome were tested in mammalian cell lines. The pcDNA3-HA or pcDNA3-HA/CRBN genes were transiently transfected into SH-SY5Y, and chymotrypsin-like activity was measured using Suc-LLVY-AMC, a widely used fluorogenic proteasome substrate. The free AMC released by Suc-LLVY-AMC hydrolysis is excitable at 380 nm; fluorescence intensity is therefore proportional to the chymotrypsin-like activity of the proteasome.

Proteasome activity was approximately 20% lower in CRBN-transfected cells than in mock-transfected cells (Fig. 4A, upper). The assay conditions were validated by pretreatment of cells with 1 μM MG132 for 1 h prior to harvest, which dramatically decreased proteasome activity by 66% (Fig. 4A, upper), indicating that the assay was reliable. The expression of HA-fused CRBN and the use of equal amounts of total protein for the assay were confirmed by Western blotting (Fig. 4A, bottom). Taken together, these results suggest that ectopic expression of CRBN down-regulates proteasome activity.

We further examined the effect of endogenous CRBN on proteasome activity in shCRBN-transfected cells. Interestingly, knockdown of endogenous CRBN expression enhanced proteasome activity by about 44% compared with that in scramble control cells (Fig. 4B, upper). The reliability of the assay conditions was demonstrated by the 46% reduction in proteasome activity observed in MG132-treated cells. Down-regulation of endogenous CRBN by shRNA and the loading of equal amounts of total protein for the assay were confirmed by Western blot analysis (Fig. 4B, bottom). These results further supported the inhibition of proteasome activity by CRBN.

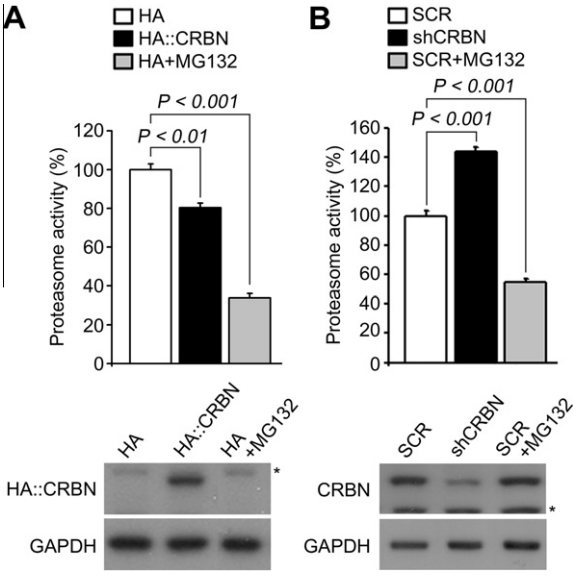

Fig. 4. Regulation of proteasome activity by CRBN expression. (A) SH-SY5Y cells were transfected with either HA or HA::CRBN, and then incubated in the presence or absence of 1 μM MG132 1 h prior to the preparation of cell extracts. Proteasome activity in the cell extracts was measured using a Suc-LLVY-AMC fluorogenic substrate (upper). The expression of HA::CRBN and the use of equal amounts of protein in the assay were analyzed by Western blotting with anti-HA and anti-GAPDH antibodies, respectively (bottom panels). (B) Cells were transfected with either scrambled shRNA as a control (SCR) or shCRBN, and then incubated in the absence or presence of 1 μM MG132 one hour prior to the preparation of cell extracts. Proteasome activity was measured as described in (A). Endogenous CRBN expression and the equal amount of total proteins used for the proteasome activity were analyzed by Western blotting with anti-CRBN and anti-GAPDH antibodies, respectively. The asterisk indicates the position of cross-reactive bands (bottom). The results shown are representative of four independent experiments. Error bars represent the SEM.

4. Discussion

The present study shows that CRBN interacts with the proteasome by binding to its β7 subunit, thereby inhibiting its enzymatic activity. Overexpression of CRBN resulted in the accumulation of GFP^u (Fig. 3B, D, and E) and decreased the chymotrypsin-like peptidase activity of the proteasome (Fig. 4A) in different cell lines. Furthermore, the increased proteasome activity induced by knockdown of endogenous CRBN (Fig. 4B) further supported the notion that CRBN can negatively regulate proteasomal activity *in vivo*.

Although the mechanism underlying the modulation of proteasome activity by CRBN was not identified, one possibility is that CRBN interferes with the assembly of the 20S proteasome. We demonstrated that CRBN directly interacted with β7, one of the subunits of the 20S CP, *in vitro*. Assembly of the 20S proteasome is a stepwise process during which incorporation of the β7 subunit occurs late, and drives the dimerization of the two half-proteasome precursor complexes. Incorporation of the β7 subunit is a rate-limiting step in precursor complex dimerization [16]. The N-terminal propeptide of pro-β7 is automatically cleaved off during dimerization [9] and the C-terminal extension of the β7 subunit intercalates into the groove between opposing β1 and β2 subunits, stabilizing the 20S proteasome complex [17]. We showed that the interaction between CRBN and β7 was not affected by the pro-β7 propeptide, suggesting that CRBN may bind to β7 directly and could affect the intercalation into the groove. Furthermore, the complex formation of CRBN with endogenous β7 and α2 (Fig. 2C) further supports the role of CRBN as an interacting protein for the β7 subunit of the 20S proteasome.

Although the effects of CRBN on the association of other proteasomal subunits, or the stability of the 26S proteasome, remain to be elucidated, several reports support our hypothesis that proteasome activity can be regulated via interactions with proteasomal subunits. For example, α-synuclein, a well-known protein associated with familial Parkinson's disease (PD), binds to S6 and induces proteasome dysfunction in neurons [18–20]. Recently, another familial PD-associated protein, parkin, was found to interact with Rpn1, Rpn10, Rpt5, and Rpt6 within the 19S regulatory subunit of the proteasome [21]. The interaction of parkin with the 19S regulatory particle promotes proteasome activity by enhancing the assembly, or levels, of the 26S proteasome [21]. The interaction between CRBN and β7 may, therefore, play a role in the regulation of proteasome activity, especially considering that β7 incorporation is the rate-limiting step in the dimerization of proteasome half-mers.

The strong expression of CRBN in the brain suggests that it may play an important role in neural cells [2]. Therefore, our findings showing the modulation of proteasome activity by CRBN may provide a valuable link between the cellular function of CRBN and the molecular etiology of mental retardation in relation to mutations in the CRBN gene. In conclusion, CRBN interacts with the 26S proteasome via its β7 subunit and may negatively regulate proteasomal activity *in vivo*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.108.

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