E3 ubiquitin ligase RNF2 interacts with the S6' proteasomal ATPase subunit and increases the ATP hydrolysis activity of S6'

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We reported previously that the human RNF2 (RING finger protein 2) protein is an E3 ubiquitin ligase that interacts with the human ubiquitin-conjugating enzyme Hip-2/hE2-25K. In the present study, we show that RNF2 interacts with S6' ATPase, a subunit of the proteasomal 19 S regulatory complex. S6' interacts with RNF2 through its N-terminal RING domain, and RNF2 interacts with S6' through its C-terminal region. Interestingly, the RNF2-S6' interaction increases the ATP hydrolysis activity of the S6' protein. Moreover, S6' ATPase activity is highly increased in the

presence of ubiquitinated proteins. The present study suggests that the E3 ubiquitin ligase RNF2 might have a dual function: facilitating the ubiquitination of its target substrates and recruiting the substrates to the proteasome. Furthermore, ATP hydrolysis in the E3/proteasome complex might act as an important signal for the protein degradation pathway.

Key words: ATP hydrolysis, E3 ubiquitin ligase, proteasomal 19 S regulatory complex, RING motifs, RNF2, S6'.

INTRODUCTION

The ubiquitin–proteasome pathway plays an important role in specific degradation of cellular proteins. Ubiquitin-mediated degradation regulates many cellular processes, including cell-cycle progression, differentiation, signal transduction and disease development [1–4]. The ubiquitin–proteasome pathway generally comprises two steps. The initial phase involves three classes of enzymes, ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Ubiquitin is first activated by the formation of a thioester bond with a cysteine residue of an E1 in an ATP-dependent manner and is then transferred to a cysteine residue of an E2. The E2s, in conjunction with an E3, catalyse the formation of an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ϵ -amino group of lysine residues on target proteins. In the second phase, many polyubiquitinated target proteins are recognized and degraded by the 26 S proteasome complex [5–7].

Even though the enzymatic pathway for ubiquitin conjugation is well established, the mechanisms of recognition of target substrates, interaction of polyubiquitinated proteins and processing of polyubiquitinated proteins by the 26 S proteasome are still unknown. Many studies have suggested that E3 ligases are involved in substrate targeting for degradation by the 26 S proteasome. Although most target proteins are recognized by different E3 ligases, the known E3 proteins are divided into two protein families. The first group of E3 ligases has the HECT (homology to E6AP C-terminus) domain and contains a highly conserved cysteine residue that forms a thioester bond with ubiquitin. The second group is the RING finger domain family, which contains conserved cysteine and histidine residues whose structure is represented through interactions with two zinc ions [8–10].

We previously found that the RNF2/RING1b protein, a RING-HC type of the RING finger family, plays a role as an E3 ligase, and that RNF2 interacts with Hip-2/hE2-25k, a ubiquitin-conjugating enzyme [11]. Our *in vitro* and *in vivo* binding experiments

demonstrate that the RNF2/RING1b protein interacts with S6'/TBP1 (Tat-binding protein-1) ATPase, a subunit of the proteasomal 19 S regulatory complex and a member of the highly conserved AAA (ATPases associated with various cellular activities) protein family [12–14]. We also show that the direct interaction between RNF2 and S6' proteins and the presence of ubiquitinated proteins synergistically increase the ATP hydrolysis activity of the S6' protein. Our results suggest that E3 ligases might recruit substrates to the proteasome in addition to their role as ubiquitin ligase proteins, and that increased ATP hydrolysis activity might be an important degradation signal for various substrates.

MATERIALS AND METHODS

Yeast two-hybrid screening and β -galactosidase assay

The procedure for yeast two-hybrid screening was described previously [11]. In brief, the yeast strain EGY48, which contains a *LEU2* gene under the transcriptional control of multimerized *LexA* operator elements (*LexA* system; Clontech), was transformed with a bait vector expressing a fusion of *LexA* and the human RNF2/RING1b, and with a human foetal brain cDNA library, which expresses cDNAs as fusions to the activation domain. Primary transformants (1×10^7) were screened on medium lacking leucine. For confirming the interaction in yeast, a second *LexA*-dependent reporter bearing a *lacZ* gene was transformed together with the respective expression plasmids, and β -galactosidase activity was measured by standard protocols [11].

Plasmids, cell culture and transfections

Truncated S6' constructs, containing deletions of the S6' ORF (open reading frame), were generated by PCR using the pB42AD full-length S6' plasmid as a template. Pfu DNA polymerase (Stratagene) was used for reactions with forward and reverse PCR

Abbreviations used: AAA, ATPases associated with various cellular activities; ALLN, N-acetyl-L-leucyl-L-leucyl-L-norleucinal; DTT, dithiothreitol; GST, glutathione S-transferase; HA, haemagglutinin; HEK, human embryonic kidney; LB, Luria–Bertani; RNAi, RNA interference; TBP1, Tat-binding protein-1; Wt, wild-type.

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primers. Each forward primer contained an EcoRI restriction site, and each reverse primer contained an XhoI site. PCR reactions were performed by standard protocols. The PCR products were purified, digested with EcoRI and XhoI, and cloned into pB42AD, pGEX4T and pET30a expression vectors. For expression of S6' in mammalian cells, the EcoRI- and XhoI-digested PCR products were introduced into pcDNA3-GST tag vector (kindly provided by Dr Y. Seong, Graduate School of Biotechnology, Korea University). The vector was constructed by inserting GST (glutathione S-transferase) sequences into the HindIII/XhoI site of pcDNA3.

HEK-293 (human embryonic kidney) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum and 5% penicillin/streptomycin. Cells were transiently transfected with target vectors by the LipofectamineTM (Invitrogen) method according to the manufacturer's instructions.

Co-precipitation and *in vitro* binding assays

For co-precipitation, HA (haemagglutinin)-tagged RNF2 [11] and GST-tagged S6' were transiently co-transfected into HEK-293 cells in 100-mm-diameter dishes by the LipofectamineTM method. After 48 h, cells were lysed on ice for 15 min with NETN buffer [50 mM Tris/HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40, 10 mM NaF, 1 mM DTT (dithiothreitol), 1 mM PMSF, 2 µg/ml aprotinin and leupeptin], and the extracts were incubated with 100 µl of glutathione-Sepharose beads for 2 h at 4°C. Beads were precipitated by centrifugation at 10000 *g* for 1 min and washed four times with PBST (1% Triton X-100 in PBS), and samples were boiled in loading buffer and separated by SDS/12% PAGE.

For *in vitro* binding assays, GST-tagged S6' proteins were expressed in *Escherichia coli*, and the RNF2 proteins were expressed in HEK-293 cells. From each lysate of HEK-293 cells, 1 mg of proteins were incubated with GST beads carrying GST-tagged S6' proteins in 200 µl of binding buffer (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 0.05% Nonidet P40). The beads were washed four times with binding buffer. The washed beads were boiled in loading buffer, and the supernatants were fractionated by SDS/PAGE and analysed by Western blotting.

Treatment of cells with proteasome inhibitors and actinomycin D

HEK-293 cells were transfected with 2 µg of HA-tagged RNF2, treated with 10 nM actinomycin D (Sigma) for 12 h and incubated further with 20 µM of the proteasome inhibitors MG132 or ALLN (*N*-acetyl-L-leucyl-L-leucyl-L-norleucinal) for 5 h, before cells were harvested and lysed in NETN buffer. From the cell extracts, 30 µg of proteins per sample were separated by SDS/PAGE, transferred on to a membrane and probed with the anti-S6' mouse monoclonal antibody (1:1000 dilution; Affinity) in Western blot analysis. The anti-β-actin monoclonal antibody (1:5000 dilution; Sigma) was used as a control for normalization.

Expression and purification of recombinant proteins

For expression of His₆-tagged S6' and RNF2, we inoculated a single *E. coli* BL21 cell into 250 ml of LB/Kan medium (Luria-Bertani medium containing 50 µg/ml kanamycin) and grew the culture overnight at 37°C. We diluted 150 ml of the overnight culture of cells into 1 litre of LB/Kan medium, and incubated at 37°C for 90 min. Then IPTG (isopropyl β-D-thiogalactoside) was added to final concentration of 1 mM, and the culture was

grown at 37°C for 4 h. After 4 h, the cells were sonicated in lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl and 10 mM imidazole) containing 1 mg/ml lysozyme and His₆-tagged S6 and RNF2 were purified with Ni-NTA (Ni²⁺-nitrilotriacetate) beads. The protein-bound beads were washed five or six times with washing buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl and 20 mM imidazole) and treated with elution buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl and 250 mM imidazole). Protein concentrations were determined by comparing samples with a BSA standard in SDS/PAGE analysis.

RNF2 RNA knockdown

For RNF2 RNAi (RNA interference) DNA vector-based knockdown, 53 bp oligonucleotide duplexes that contain sequences specific to a portion of the RNF2 coding sequences were cloned into the pSilencerTM 1.0-U6 siRNA expression vector (Ambion) following the manufacturer's instructions. The cloned sequence was 5'-TGGCAATTGATCCAGTAATTTCAAGAGAA-TTACTGGATCAATTGCCATTTTTT-3'. The vectors expressing the hairpin RNAs were transfected into HeLa cells using the polyethyleneimine (Sigma) method. After 48 h, the cells were analysed for RNF2 expression by Western blotting.

ATPase activity assays

In vitro ATPase activity was assayed by a modified Malachite Green method [15,16]. Purified His₆-tagged S6' (20 ng), BSA (20 ng) and/or RNF2 (40 ng) proteins were mixed with 100 µl of reaction buffer (20 mM Hepes, pH 7.2, 1 mM DTT and 5 mM MgCl₂) containing 200 µM ATP and were incubated for 40 min at 37°C. After incubation, the reactions were quenched with 900 µl of Malachite Green reagent (0.034% Malachite Green oxalate, 1.1% ammonium molybdate, 1 M HCl and 0.04% Tween 20). The quenched reaction mixtures were incubated for 5 min at room temperature (25°C), and samples were measured at 655 nm with a Benchmark microplate reader.

For *in vivo* ATPase activity, HA-tagged RNF2 [11] and GST-tagged S6' were transiently co-transfected into HEK-293 cells. After cells were lysed, the extracts were incubated with 100 µl of glutathione-Sepharose beads for 2 h at 4°C. Protein-bound beads were precipitated by centrifugation at 10000 *g* for 1 min and washed four times with 1 ml of reaction buffer without ATP. Washed beads were incubated for 40 min at 37°C with 100 µl of reaction buffer containing 200 µM ATP. After incubation, the reactions were quenched with 900 µl of Malachite Green reagent for 5 min at room temperature. The resulting reactions were measured at 655 nm.

In vitro ubiquitination assays

Ubiquitination experiments were carried out according to a previously published method [11]. Beads carrying GST-tagged RNF2-Wt (wild-type) or RNF2 H69Y (50 pmol) were combined with 20 ng of mammalian E1 (Calbiochem), 20 ng of E2 (hE2-24K; purified in our laboratory) and 10 µg of ubiquitin (Sigma) in ubiquitination buffer (50 mM Tris/HCl, pH 7.4, 2 mM ATP, 5 mM MgCl₂, 2 mM DTT, 1 mM creatine phosphate and 15 units of creatine phosphokinase and 30 µl of *E. coli* BL-21 cell lysate), and the mixture was incubated for 1.5 h at 30°C. The beads were washed three times with ATPase reaction buffer lacking ATP. For ATPase assays, 1/10 of the beads was mixed with 100 µl of reaction buffer containing 200 µM ATP and S6' (40 ng) and incubated for 40 min at 37°C. The reactions were terminated with Malachite Green reagent, and the resulting reaction mixtures were measured at 655 nm.

Figure 1 S6' protein interacts with RNF2

The specificity of the interaction between RNF2 and S6' was identified by co-transforming the full-length LexA-RNF2 protein with the indicated proteins, and the strength of the interactions was determined by liquid β -galactosidase assays. Results are means $\pm$ S.D. for three independent experiments. AD, activating domain.

For ubiquitin conjugates binding assay, the ubiquitin reaction mixtures containing purified GST-RNF2-Wt or RNF2 H69Y (500 pmol), 500 ng of mammalian E1, 1 μ g of E2, and 30 μ g of ubiquitin (Sigma) were incubated for 1.5 h at 30°C. The resulting mixture was added to the His₆-agarose bead-immobilized S6' protein and rotated at 30°C overnight. Ubiquitin conjugates were detected by Western blotting.

RESULTS AND DISCUSSION

The RING finger domain of RNF2 interacts with the S6' protein

To identify RNF2-mediated target proteins, we used the human RNF2 protein as bait in yeast two-hybrid screening. Yeast cells expressing LexA-RNF2 were transformed with a human foetal brain cDNA library, and nine independent positive clones were identified. Five of the clones were derived from a single unique gene, which was revealed to be human S6'/TBP1, a subunit of the 19 S regulatory complex (proteasome). Yeast two-hybrid assays with various proteins (Figure 1) showed that RNF2 interacted with only the S6' protein, but not with other proteins, confirming a specific interaction between RNF2 and S6'. It is interesting that RNF2 does not interact with the HN₃ protein, a subunit of the 20 S proteasome core.

To define the RNF2 protein region that is responsible for the interaction with S6', we constructed deleted versions of RNF2 for expression as LexA-BD (binding domain) fusion proteins in yeast (Figure 2A). The interaction strength between S6' and RNF2-deleted versions was analysed by the β -galactosidase assay. As shown in Figure 2(A), S6' interacted with RNF2-Wt and RNF2- Δ C (C-terminal deletion), but not with RNF2- Δ N (N-terminal deletion), suggesting that the N-terminal region of RNF2, including the RING finger domain, is essential for the interaction. To verify the role of the RING finger domain as the S6'-interacting region, we investigated RING domain mutation plasmids: RNF2 C51W/C54S and RNF2 H69Y. Yeast two-hybrid experiments showed that S6' interacted with neither the double-mutation RNF2 C51W/C54S protein nor the single-mutation RNF2 H69Y protein, indicating that the RING domain in RNF2 has an important role in the interaction with the S6' protein (Figure 2A).

To confirm the interaction domain further, we performed an *in vivo* binding assay in mammalian cells (Figure 2B). HEK-293 cells were transiently co-transfected with pcDNA3-GST-S6' and HA-tagged RNF2 or deletion constructs. Transfected cells were harvested, and extracts were incubated with glutathione-Sepharose beads. The beads were precipitated by centrifugation at

Figure 2 RING finger domain of RNF2 interacts with S6'

(A) Schematic representation of wild-type RNF2 and various mutation constructs. The interaction activities were determined by liquid β -galactosidase assays (right). The arrows indicate positions of point mutations in RNF2. (B) Co-precipitation assay. HEK-293 cells were co-transfected with pcDNA3-GST-S6' and HA-tagged RNF2 mutation constructs. After 48 h, the cells were harvested, and GST precipitates were resolved by SDS/PAGE. Bands were detected by using an anti-HA antibody (top panel), and the blot was stripped and re-probed with an anti-GST antibody (middle panel) to illustrate that relatively equivalent amounts of RNF2 were expressed. For each crude extract, 10% was used for immunoblotting with the anti-HA antibody (bottom panel). IP, immunoprecipitation; IB, immunoblot. Molecular-mass sizes are given in kDa.

10000 g for 1 min, and the samples were separated by SDS/12% PAGE, and probed with an anti-HA antibody. As shown in Figure 2(B), S6' interacted with RNF2-Wt and RNF2- Δ C, but not with RNF2- Δ N, RNF2 C51W/C54S or RNF2 H69Y, confirming the interactions detected through the yeast two-hybrid assays.

The C-terminus of S6' interacts with RNF2

The AAA motif of the S6' protein has been reported to consist of four conserved domains [17]: putative ATP-binding motif GPP-GXGKT, ATP hydrolysis motif DEID, and two RNA/DNA helicase motifs XAT and H/QRXGRXXR (Figure 3A). The H/QRXGRXXR domain was also reported to play a role in ATP hydrolysis [18]. To define the S6' protein region that is responsible for the interaction with RNF2, we constructed several truncation mutations of S6' and then determined the regions binding to RNF2 by yeast two-hybrid assays. As shown in Figure 3(A), RIPI 19, which contains the 138-residue C-terminus of S6', appears to be the minimal region required for RNF2 binding.

We confirmed these results by modified *in vitro* binding assays with GST-fusion proteins. GST-tagged S6' deletion constructs were expressed in *E. coli*, and HA-tagged RNF2 was expressed in HEK-293 cells. The lysed HEK-293 cell extracts were incubated with glutathione-Sepharose beads pre-loaded with GST-tagged S6' deletion proteins. The beads were precipitated by

Figure 3 Identifying the RNF2-interacting domain in S6' proteins

(A) Schematic representation of wild-type and various deletion-mutation S6' constructs. The strength of interaction between RNF2 and each deletion-mutation S6' construct was measured using the β -galactosidase assay. RIP1 (RNF2-interacting protein) 17 and 19 were obtained from our yeast two-hybrid results. (B) The binding region was determined by modified GST pull-down assays. HA-tagged RNF2 was expressed in HEK-293 cells and was assessed for its ability to form a stable complex with various GST-fused S6' deletion-mutation constructs. SDS/PAGE analysis of the GST-fused S6' deletion-mutation constructs. GST-tagged S6'1-175 and GST-tagged S6'175-439 (a gift from Dr I. T. Satoh) have been described previously [29].

centrifugation at 10000 *g* for 1 min, and the precipitants were separated by SDS/12% PAGE and then probed with an anti-HA antibody. Figure 3(B) shows that the 138-residue C-terminal region of S6' interacts with RNF2, confirming the interactions detected through the yeast two-hybrid assays. Taken together, these results indicate that the C-terminal region of S6' is essential for maintaining the stability of the protein-protein interaction.

S6' is not the target substrate of RNF2 E3 ligase

Generally, in the proteasome-mediated degradation system, RING finger E3 ligases recognize and interact with target substrates. To investigate whether S6' could be degraded by RNF2 E3 ligase, we transfected HEK-293T cells with HA-RNF2 and treated then with proteasome inhibitors ALLN or MG132 in either the absence or the presence of actinomycin D, which inhibits mRNA synthesis. The endogenous levels of S6' were detected by immunoblot with a mouse monoclonal antibody against S6'. As shown in Figure 4(A), the levels of S6' were not much changed in the presence of proteasome inhibitor, suggesting that S6' is not among the target substrates of RNF2 E3 ligase-mediated degradation. The levels of endogenous α -synuclein were investigated as a positive control. Actinomycin D abolished the increase in α -synuclein induced by MG132, indicating that actinomycin D inhibited the transcription of α -synuclein.

We tested the effect of knocking down RNF2 expression on the level of S6' using short interfering hairpin RNAs. As shown in Figure 4(B), the RNAi led to an approx. 80% reduction in RNF2 protein levels. However, we did not observe alterations in protein levels of the S6' protein, suggesting that S6' is not a target of RNF2-mediated degradation.

Figure 4 S6' protein is not a target substrate for RNF2-mediated proteasomal degradation

(A) HEK-293T cells were transfected with HA-RNF2 and treated with the proteasome inhibitors ALLN or MG132 in the absence or presence of actinomycin D (Act D) were harvested and lysed, and cell lysates were used for immunoblotting with the anti-S6' mouse monoclonal antibody. The anti- β -actin monoclonal antibody was used for normalization of protein amounts and the α -synuclein proteins (Syn) were used as the positive control for actinomycin D treatment. (B) Short hairpin RNA-mediated knockdown of RNF2 expression in HeLa cells. Cells expressing hairpin RNAs were analysed for RNF2 expression by Western blotting. Molecular-mass sizes are given in kDa in both panels.

S6'-RNF2 interaction increases ATP hydrolysis activity of S6'

The S6' protein belongs to the AAA family, which is conserved in the subunits of the 19S regulatory proteasome complex. We investigated whether the binding of RNF2 and S6' is functionally linked to the ATP hydrolysis activity of the S6' protein. For *in vitro* ATPase activity assays, we used a modified Malachite Green method. Purified His₆-tagged S6' and RNF2 proteins were mixed in reaction buffer containing 200 μ M ATP and were incubated for 40 min at 37°C. The hydrolysed phosphate from ATP was quenched with Malachite Green reagent, and absorbance was measured at 655 nm with a microplate reader. As shown in Figure 5(A), the S6' protein hydrolysed ATP, but BSA or RNF2 alone showed background levels, as expected; the ATPase activity level of S6' was arbitrarily set to 100%. Interestingly, three independent experiments showed that the binding of RNF2 to the S6' protein increased the hydrolysis of ATP (Figures 5A and 5B). The specificity of increased hydrolysis was verified by ATPase assays with several proteins. Figure 5(C) shows that the ATPase activity of S6' is increased only in the presence of the RNF2 protein. To

Figure 5 Binding of RNF2 and S6' proteins stimulates ATP hydrolysis

(A) For ATP hydrolysis assays, we pre-incubated S6' (20 ng), BSA (40 ng) and/or RNF2 (40 ng) proteins for 30 min to form a complex, and then incubated further for 40 min in the presence of 200 μ M ATP. The amount of hydrolysed phosphate was quantified by the Malachite Green assay. The ATPase activity level of S6' was arbitrarily set to 100% as a positive control, and results are means $\pm$ S.D. for three independent experiments (top panel). Lower panels: Coomassie Blue staining of the amounts of proteins used in each reaction. To detect the amount of protein, the volume was loaded up to ten times (lower panels). (B) Plot of ATPase activity of the same proteins and complexes as in (A) against time. (C) In the presence of S6', ATP hydrolysis assays were performed with 40 ng of various proteins. (D) ATP hydrolysis assays were carried out with S6' (20 ng) and RNF2-Wt, RNF2-ΔN and RNF2 H69Y proteins (40 ng). Results are means $\pm$ S.D. for three independent experiments. (E) ATPase activity of the S6' protein was examined by the Malachite Green assay in the presence of GST alone or pcDNA3-GST-S6' co-precipitates in HEK-293 cells. Emp, empty vector. Results are means for three independent experiments.

confirm further the ATP hydrolysis activity of S6', we performed *in vitro* ATPase assays with S6'-binding mutation proteins, RNF2 H69Y and RNF2-ΔN. As we expected, RNF2 H69Y and RNF2-ΔN did not increase the ATPase activity of S6' (Figure 5D).

We tested further the increased ATPase activity of S6' by the binding of RNF2 by *in vivo* binding experiments in HEK-293 cells, which were followed by *in vitro* ATPase assays. pcDNA3-

GST-S6' and wild-type RNF2 or RNF2 mutation proteins were transiently co-transfected into HEK-293 cells and harvested, and the transfected cell extracts were incubated with glutathione-Sepharose beads. The beads were precipitated by centrifugation at 10000 *g* for 1 min and assayed for ATPase activity. ATPase activity was observed in the presence of RNF2, but not in the presence of RNF2 proteins with single or double point mutation,

indicating that mutated RNF2 proteins do not interact with S6' in HEK-293 cells (Figure 5E). As expected, GST-S6' alone showed a background level of ATPase activity. These studies suggest that the interaction of RNF2 with S6' is important for the increased ATPase activity of S6'.

S6' ATPase activity increases in the presence of ubiquitinated proteins

The S6' protein was reported previously to recognize polyubiquitin signals [19]. Therefore we tested whether ubiquitinated proteins synergistically increase S6' ATPase activity through the binding of RNF2. We used RNF2 or the RING-domain-mutation plasmid RNF2 H69Y for *in vitro* ubiquitination assays; RNF2-H69Y was described previously as being not ubiquitinated [11]. The ATPase assays shown in Figure 6(A) indicated that, in the presence of the ubiquitinated proteins, the ATPase activity of the RNF2-S6' complex was approx. 3-fold higher than that of S6' in the presence of RNF2 H69Y (top panel). To confirm the ubiquitin conjugates presence in the RNF2-S6' complex, we carried out co-precipitate assay used with equal amounts of ubiquitination mixture and His₆-agarose beads immobilized S6' protein. Since the polyubiquitin conjugate has a high specificity, up to ten times as much of RNF2, RNF2 H29Y and S6' proteins were used. In Figure 6(A), RNF2-Wt interacted with ubiquitinated proteins (upper middle panel) and with S6' (lower middle panel). In our previous study, the RNF2 protein was not auto-ubiquitinated [11]. These data demonstrate that ubiquitinated proteins synergistically increase S6' ATPase activity. Taken together, our results suggest that ATP hydrolysis, when it depends on the co-operation of S6' with E3 ligases and ubiquitinated substrates, might act as an important signal.

Several laboratory groups have reported that E3 ligases interact with 26 S proteasome subunits [20–25]. Studies show that Ubr1p interacts with the proteasomal proteins Rpn2p, Rpt1p, and Rpt6p, and UFD4p interacts with Rpt4 [20,21]. Recently, the E3 ligase pVHL was reported to bind to the S6' 19 S proteasome subunit, leading to degradation of the Hif1 α substrate [26]. In the present study, we show that RNF2, an E3 ubiquitin ligase, interacts with S6' and, furthermore, that the RNF2-S6' interaction increases the ATPase activity of S6'. The RING finger domain of RNF2 and the C-terminal region of S6' are required for their direct interaction. Our studies suggest that the RNF2-S6' interaction might act as a signal of ATP hydrolysis.

On the basis of our studies, we propose a model of substrate degradation in the ubiquitin proteasome system. In normal circumstances, or in a low level of ubiquitination, substrates are not degraded by the 26 S proteasome complex, since the substrate is not sufficiently ubiquitinated to initiate ATP hydrolysis. However, in the presence of ubiquitinated substrates, substrate-linked E3 might bind to the proteasome and initiate ATP hydrolysis, leading to degradation of substrates. In the present study (Figure 5), however, in the absence of ubiquitinated substrates, ATPase activity was observed from the S6' protein, although the activity was much weaker than in the presence of ubiquitinated substrates. Indeed, yeast two-hybrid screening identified S6' as a binding partner of pVHL E3 ligase, and other E3 ligases, such as Ubr1p and UFD4, have also been reported to bind the specific proteasomal subunits without multi-ubiquitin chains. Hence, we believe that S6' not only recognizes a multi-ubiquitin chain, but also interacts with E3 ligases. Consequently, in the ubiquitin-proteasome pathway, ATPase activity might be increased by several signals, such as multi-ubiquitin chains, substrates or E3 ligases. Indeed, we have consistently identified several chaperones interacting with RNF2 in our yeast two-hybrid screening experiments (S.-J. Lee and

Figure 6 ATPase activity of S6' increases in the presence of ubiquitinated proteins

(A) ATP hydrolysis assays with purified S6' and either RNF2-Wt (Wt-RNF2) protein, which has ubiquitin ligase activity, or mutant RNF2 H69Y protein (Mut-RNF2), which does not have ubiquitin ligase activity [11]. GST-RNF2-Wt and GST-RNF2 H69Y immobilized to the glutathione-Sepharose beads were combined with E1, E2 and ubiquitin in ubiquitination buffer. The beads were washed and incubated with the S6' protein for 40 min. A one-tenth volume of the reaction mixture was used for ATPase assays. To detect ubiquitin conjugates, the His₆-agarose bead-immobilized S6' protein was incubated with ubiquitination mixture containing RNF2-Wt or RNF2 H69Y. The beads were precipitated, washed and subjected to SDS/PAGE. Anti-ubiquitin antibodies were used to detect ubiquitin conjugates (top panel). The same blot was reprobbed with anti-GST antibodies (middle). The bottom panel shows that the equal S6' levels were used. Results are means $\pm$ S.D. for three independent experiments. IP, immunoprecipitate; IB, immunoblot. (B) The plot of absorbance against time for the RNF2-Wt or RNF2 H69Y proteins.

S. Kang, unpublished work). The precise roles of ATPase activity in the ubiquitin-proteasome pathway are unclear, but many studies have suggested that the ATPase activity of chaperones and 19 S regulatory complex proteins might contribute to the refolding of substrates or channel-gating into the proteolytic chamber of the 20 S complex [27,28]. We think that RNF2-S6' complexes or RNF2 chaperones might control the refolding and degradation of target substrates through the level of ATP hydrolysis. Nevertheless, further studies are needed to elucidate the relationship

between the proteasome-mediated degradation pathway and chaperone-mediated refolding pathway.

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