Proteasome Function Is Regulated by Cyclic AMP-dependent Protein Kinase through Phosphorylation of Rpt6*

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Dysregulation of the proteasome has been documented in a variety of human diseases such as Alzheimer, muscle atrophy, cataracts etc. Proteolytic activity of 26 S proteasome is ATP- and ubiquitin-dependent. O-GlcNAcylation of Rpt2, one of the AAA ATPases in the 19 S regulatory cap, shuts off the proteasome through the inhibition of ATPase activity. Thus, through control of the flux of glucose into O-GlcNAc, the function of the proteasome is coupled to glucose metabolism. In the present study we found another metabolic control of the proteasome via cyclic AMP-dependent protein kinase (PKA). Contrary to O-GlcNAcylation, PKA activated proteasomes both in vitro and in vivo in association with the phosphorylation at Ser120 of another AAA ATPase subunit, Rpt6. Mutation of Ser120 to Ala blocked proteasome function. The stimulatory effect of PKA and the phosphorylation of Rpt6 were reversible by protein phosphatase 1γ. Thus, hormones using the PKA system can also regulate proteasomes in concert with glucose metabolism. This finding might lead to novel strategies for the treatment of proteasome-related diseases.

The proteome is in a dynamic state of synthesis and degradation. Although synthesis plays a role in controlling the concentration of many proteins, many other protein concentrations are controlled by the rate of degradation. Indeed controlling protein half-life by destruction has emerged as a major cellular regulatory mechanism. The destruction process is carried out by diverse proteases in the cell. The two major proteolytic pathways involve either the lysosomes or the ubiquitin-proteasome system. The proteasome is an abundant giant protein substrates in the proteolytic cavity of the core particle. The hexameric ring of the 19 S particle that contacts with the 20 S core particle. By recognizing and unraveling the ubiquitin-conjugated substrates (16) and perhaps by controlling the opening of the core particle (17), the 19 S particle regulates the entry and degradation of the protein substrates in the proteolytic cavity of the core particle. The hexameric ring of the 19 S particle that contacts with the outer α ring of the core particle is composed of six ATPases, which belong to the AAA ATPase family. The six ATPases in the cap have been demonstrated to play important roles in the proteasome function but they are not functionally redundant and have to work coordinately (18) to unfold the protein substrates and transport them into the proteolytic cavity of the 20 S core (16) where proteolysis occurs.

Although the structure and function of the proteasome have been extensively investigated (12), the regulation of its activity remains elusive. The emphasis on regulation of proteolysis by the ubiquitin-proteasome system has been on substrate recognition through polyubiquitination. The proteasome was considered a passive machine that acted uniformly after the substrate proteins were identified. However, hints that the normal proteasome function and substrate specificity depend on the function of Rpt6 has emerged. Although much remains to be learned about the function of Rpt6, this is the first evidence that the proteasome can be regulated in vivo by phosphorylation of a proteasome subunit, which is controlled by a second messenger (cAMP) via PKA.

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CAMP Regulates Proteasomes

...proteolytic activities of the proteasome itself might also be regulated have emerged. The proteasomal activity in muscle cells has been found to be stimulated during starvation (19). The posttranslational modification of the mammalian proteasome by O-GlcNAc2 can inhibit its proteolytic function (20), suggesting that proteasome function is under metabolic control. Phosphorylation, another posttranslational modification, has also been recognized in both core and regulatory subunits of the proteasome (21–23). One function of phosphorylation in the core particle (21) or some subunits in the 19 S regulatory particle (24) might be for the assembly of the intact 26 S proteasome. But the kinases responsible for other phosphorylation and the effect of phosphorylation on other proteasome functions are still unknown. There are indications that proteasome function can be stimulated in a CAMP-dependent manner. Peptidase activity could be elevated by treating osteoblast cells with parathyroid hormone, which functions through a CAMP-dependent mechanism (25). Sp1 proteasome-dependent degradation was stimulated in normal rat kidney (NRK) cells by forskolin treatment (26, 27), which stimulates CAMP formation. Cyclic AMP-dependent protein kinase (PKA), which is the downstream serine/threonine kinase stimulated by CAMP, is involved in many cellular processes (28) especially in energy metabolism (28–30). To more directly determine whether CAMP modulates proteasomal function through phosphorylation, we undertook these studies. We found that PKA can directly stimulate the chymotrypsin-like and trypsin-like activities of proteasomes so that a protein substrate, such as Sp1, can be degraded. Here evidence is presented that another AAA ATPase in the 19 S cap of the mammalian proteasome, Rpt6, is the in vivo target of PKA phosphorylation that results in proteasome stimulation. Mutation of the PKA phosphorylation site in Rpt6, Ser120 to Ala, blocked the proteasome function in vivo. Furthermore the stimulatory effect of PKA on the proteolytic activity of the proteasome and the phosphorylation of Rpt6 were reversible by protein phosphatase 1γ (PP1γ). The linkage of proteasomal function to metabolic regulators like PKA and the O-GlcNAc system underlines the role that proteasomes play in the metabolism in the cell and whole organism.

EXPERIMENTAL PROCEDURES

Materials—Purified 26 S proteasome, proteasome substrate Z-GGL-AMC and proteasome inhibitor epoxomycin were purchased from Biomol (Plymouth Meeting, PA). 20 S proteasome was purchased from A. G. Scientific (San Diego, CA). PKA; monoclonal anti-GST antibody; proteasome inhibitors β-lactone, PSI, MGI32, and LLI; and proteasome substrates Suc-LVVY-AMC and Boc-LSTR-AMC were purchased from Sigma. PP1γ was purchased from Roche Applied Science and/or Calbiochem. [γ-32P]ATP was purchased from PerkinElmer Life Sciences. Anti-phosphoserine antibody (clone 4A9) was purchased from Calbiochem.

Recombinant Rpt6 and Mutagenesis—The human Rpt6/Sug1 from human breast cancer cell line MDA 468 was cloned into pGEX and pcDNA 3.1 as described before (31). For point mutations, the pGEX-Rpt6 was modified by site-directed mutagenesis using a four-primer cassette strategy to introduce the substitution (mutated residues are underlined). PCR amplifications were performed with the following oligonucleotides (S’–3’): S120A, CGGGTGGCTCTAAGGAATCGGAGCTACAATCTTGCAAAAGATCCTG; and S215A, GACGTGACTTTATTTCTGTTGTGCTGTGACTGTCAGAGCCCAACCTCC-GAGGCTGCAGG; and downstream, CATTCTCGGAAGATGACGCCTA-

The abbreviations used are: O-GlcNAc, O-linked N-acetylgalcosamin; AMC, amcinomethylcoumarin; FFIU, fluorescence intensity unit; GFP, green fluorescent protein; GST, glutathione S-transferase; NE, nuclear extract; NRK, normal rat kidney; OGT, O-GlcNAc-transferase; PKA, CAMP-dependent protein kinase; PP1, protein phosphatase 1; Boc, t-butoxycarbonyl; Suc, succinyl; Z, benzzyloxy carbonyl; LLI, N-acetyl-l-leucinyl-l-leucinyl-l-norleucinal; DTT, dithiothreitol; LC, liquid chromatography.

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His-tagged proteins (His-Rpt6 and His-Rpt6S120A) were expressed in 293 cells. pcDNA3.1-Rpt6 or pcDNA3.1-Rpt6S120A plasmids were transfected into 80% confluent 293 cells, maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, with Lipofectamine 2000 (Invitrogen). After incubation for 24 h, the transfected cells were harvested. The cell lysate was prepared in lysis buffer containing 20 mM Tris (pH 7.5), 500 mM NaCl, 5 mM imidazole, and 20% glycerol in the presence of 50 mM sodium fluoride and 10 mM sodium orthovanadate. The proteins in the lysate were denatured by adjusting the final concentration to 6 M urea. His-tagged proteins were isolated with His-Mag™ agarose beads (Novagen, Madison, WI) under denaturing conditions. The beads bearing the recombinant proteins were washed with washing buffer containing 20 mM Tris (pH 7.9), 500 mM NaCl, 60 mM imidazole, 50 mM sodium fluoride, and 10 mM sodium orthovanadate. 26 S proteasomes were isolated from 293 cell lysates using proteasome affinity agarose beads bearing ubiquitin-like sequence UbLHRB fused to GST provided in the proteasome isolation kit (Calbiochem) as instructed.

Tissue Culture and Preparation of NRK Cell Nuclear Extract (NE)—NRK cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, non-essential amino acids, and antibiotics. To test the effect of forskolin and H-89, 100 µM forskolin and/or 1 µM H-89 was added to the cells and incubated for 24 h before harvest. Nuclear extracts were then prepared as described previously (20, 26, 31).

Proteasome Activity Assay in Vivo—293 Cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics. To measure the proteasome activity in vivo, a short degron, CL1 (ACKNWFFSSLHSHFYIHL) (32), was fused to the C terminus of GFP. GFP-CL1 was used as a reporter of proteasome activity in the cell (20, 33). 1.6 µg each of pcDNA3.1-GFP or pcDNA3.1-GFP-CL1 was transfected into 293 cells in 12-well plates using Lipofectamine 2000 (Invitrogen). The cells were incubated at 37 °C for 24–30 h before harvest. To test the effect of forskolin and H-89, 100 µM forskolin and/or 1 µM H-89 was added to the cells and incubated for 24 h before harvest. Nuclear extracts were then prepared as described previously (20, 26, 31).

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Sp1 Degradation Assay—The reconstituted Sp1 degradation was performed as described previously (26, 27). About 10–20 ng of GST–Sp1 was added to the NRK cell NE with a protein concentration of 1 µg/µl and incubated at room temperature for 45 min. The reaction was stopped by adding SDS sample buffer and boiling for 3 min. The samples were separated by SDS-PAGE. GST–Sp1 and GST–SpX fragments were revealed by anti-GST Western blot. To test the effect of PKA or PP1 γ on the degradation of GST–Sp1, NRK cell nuclear extract was pre-treated with 5 units of PKA and/or 10 milliunits of PP1 γ at 30°C for 30 min before the addition of peptide substrates. The incubation was continued at 37°C for 90 min. To test the effect of PP1 γ on the PKA-stimulated peptide activity, the inhibitors were added to the reaction mixture after the PKA treatment and incubated at 37°C for 30 min before the addition of fluoro- genic peptide substrates. The incubation was continued at 37°C for 90 min. To test the effect of PKA and PP1 γ on the degradation of GST–Sp1, NRK cell nuclear extract was pre-treated with 5 units of PKA and/or 10 milliunits of PP1 γ at 30°C for 30 min before the addition of peptide substrates. The FIU was measured as above.

Labeling of Proteins with 32P—To label the proteasome, 5 µg of purified 26 S proteasome or 2 µg of 20 S proteasome was treated with 10 units of PKA together with 5 µCi of [γ-32P]ATP in the proteasome activity assay buffer in a total volume of 30 µl without ATP. After incubation at 30°C for 45 min, the reaction samples were separated by SDS-PAGE. The gel was subjected to Coomassie Blue staining and autoradiography. The labeled bands were excised from the gel. Tandem mass spectrometry was performed on the isolated proteins as described below except that the samples were digested with trypsin.
To determine the modification site of PKA in Rpt6, potential sites of modification, Ser\textsuperscript{120} and Ser\textsuperscript{215}, were mutated from serine to alanine by site-directed mutagenesis. The recombinant proteins were expressed and purified as described above. The glutathione beads bearing GST-Rpt6, GST-Rpt6S120A, GST-Rpt6S215A, or GST-Rpt6S120A/S215A were treated with 5 units of PKA and 5 μCi of [γ\textsuperscript{32}P]ATP at 30 °C for 45 min. The GST beads were then spun down and boiled. The recombinant proteins were resolved with SDS-PAGE and subjected to Coomassie Blue staining and autoradiography.

**Tandem Mass Spectral Analysis**—GST-Rpt6 was pretreated with PKA in the proteasome activity assay buffer as described above. Tandem mass spectral analyses were performed with a Q-Tof 2 mass spectrometer (Micromass, Manchester, UK) using electrospray ionization. Samples had undergone a 16-h chymotryptic digest at 37 °C. The resulting peptides were purified using ZipTips to concentrate and desalt the samples. The samples were then analyzed by LC-tandem mass spectrometry. Liquid chromatography was performed using an LC Packings Ultimate LC system, Switchos microcolumn switching unit, and Famos autosampler (LC Packings, San Francisco, CA). The samples were concentrated on a 300-μm-inner diameter C\textsubscript{18} precolumn at a flow rate of 10 μl/min with 0.1% formic acid and then flushed onto a 75-μm-inner diameter C\textsubscript{18} column at 20 nl/min with a gradient of 5–100% acetonitrile (0.1% formic acid) in 30 min. The nano-LC interface was used to transfer the LC eluent into the mass spectrometer. The Q-Tof 2 mass spectrometer was operated in the automatic switching mode whereby multiply charged ions were subjected to tandem mass spectrometry if their intensities rose above six counts. The tandem mass spectra were processed with the MassLynx MaxEnt 3 software.

**RESULTS**

**Forskolin Stimulation of Proteasome Was Inhibited by H-89**—The activation of PKA by forskolin (34–36), which is produced by Coleus forskohlii and activates adenylate cyclase, in NRK cells is associated with two phenomena. First forskolin treatment dramatically reduces the O-GlcNAc levels on all proteins in these cells (37). Because PKA phosphorylation of glutamine:fructose-6-phosphate transferase 1 inhibits its enzymatic activity (37, 38), halting the conversion of glucose into glucosamine, less UDP-GlcNAc substrate is made available to OGt. The second phenomenon is that forskolin stimulates proteasome-dependent degradation of the transcription factor Sp1 (26, 27) both *in vitro* and *in vivo*. Subsequently it was shown that the proteolytic activity of the 26 S proteasome is inhibited by O-GlcNAc modification and that the stability of Sp1 is related to the O-GlcNAc modification of the 19 S cap rather than to the O-GlcNAc modification of itself (20). The initial thought was that the stimulation of proteasome function by forskolin occurs only through changes in O-GlcNAc modification of the 19 S cap of the 26 S proteasome.

However, it remained possible that forskolin, by activating PKA, could directly activate the proteasome. To test this idea, NRK cells were treated with forskolin and PKA-specific inhibitor H-89. The proteolytic activity of the proteasome was measured with fluorogenic peptide substrates in NRK cell NEs. We have shown the nuclear extract to be a reliable system to measure proteasome function both *in vitro* and *in vivo* (20, 26, 27, 31). In this system, we not only are able to measure the peptidase activity of proteasome but more importantly the degradation of a nuclear protein substrate, Sp1, a proteasome-dependent process. NRK cells treated with forskolin yielded a nuclear extract in which not only the cleavage of both peptide substrates for chymotrypsin-like peptidase, Suc-LLVY-AMC (Fig. 1A) and Z-GGL-AMC (Fig. 1B), was stimulated but also that of the peptide substrate for the trypsin-like peptidase Boc-LSTR-AMC (Fig. 1C). The PKA inhibitor H-89 mostly blocked the ability of forskolin to stimulate these cleavages, suggesting that forskolin acts through PKA. The proteolytic activity stimulated

![Figure 1](image-url)
activity was inhibited by proteasome-specific inhibitors. Untreated NE (mean ± S.D., n = 3) showed that PKA stimulated the degradation of GST-Sp1 in the untreated NRK cell NE (mean ± S.D., n = 3). The PKA-stimulated activity was inhibited by proteasome-specific inhibitors. C, PKA stimulated the degradation of trypsin-like peptide Boc-LSTR-AMC in the untreated NRK cell NE (mean ± S.D., n = 3). The PKA-stimulated activity was inhibited by proteasome-specific inhibitors. Untreated NE (INE) was prepared from NRK cells without forskolin treatment. PKA was added to 10 µl of NE containing 10 µg of cellular proteins. After incubation at 30 °C for 30 min, proteasome inhibitors were added to the indicated reactions and incubated for another 30 min. Proteasome substrates were then added and incubated at 37 °C for 90 min before the FIU was measured. D, PKA stimulated the degradation of Sp1 in the untreated NE. The PKA-stimulated degradation of Sp1 was inhibited by proteasome-specific inhibitors β-lactone (β-lac) and epoxomycin (Epo). After the untreated NRK cell NE was treated with PKA and proteasome inhibitors, GST-Sp1 was added to the reaction and incubated at room temperature for 45 min. The proteins were separated by SDS-PAGE and revealed by anti-GST Western blot. E, Coomassie Blue staining of the purified 26 S proteasome. F, the stimulatory effect of PKA on the degradation of chymotrypsin-like peptide Suc-LLVY-AMC by purified 26 S proteasome was inhibited by H-89 (mean ± S.D., n = 3). G, the stimulatory effect of PKA on the degradation of trypsin-like peptide Boc-LSTR-AMC by purified 26 S proteasome was inhibited by H-89 (mean ± S.D., n = 3). 0.1 µg of highly purified 26 S proteasome was pretreated with PKA and or H-89 at 30 °C for 30 min. Proteasome substrates were then added and incubated for 90 min. Student’s t test was performed (*, p < 0.05; **, p < 0.01). Error bars indicate mean ± S.D.

by forskolin is primarily proteasomal in origin because it can be entirely blocked by multiple proteasome-specific inhibitors (20), demonstrated here with β-lactone. Forskolin treatment is unlikely to have stimulated proteasome activity indirectly only through O-GlcNAc modification because it stimulated all measured peptidases, whereas O-GlcNAc only alters Suc-LLVY-AMC cleavage (20). The results indicate that forskolin stimulates proteasome activity through PKA in a manner that is independent of the effect on O-GlcNAc.

**PKA Stimulated the Peptidase Activity of Proteasomes in the NEs**—To determine whether PKA has a more direct effect on the proteolytic activity, the active catalytic subunit of PKA was added to NE from NRK cells that had not been activated with forskolin. PKA treatment significantly stimulated the cleavage of all three peptides (Fig. 2, A, B, and C). To demonstrate that the stimulated peptidase activity in the NE is proteasomal in origin, multiple proteasome-specific inhibitors, epoxomycin, β-lactone, PSI, and LLnL, were added to the NE prior to measurement of the fluorescence intensity. The stimulated peptidase activity in the NE in this assay was inhibited by all the inhibitors (Fig. 2, A, B, and C). Although the β-lactone inhibition of proteasome degradation of Suc-LLVY-AMC was not as complete as might be expected from the result seen in Fig. 1A, the experimental conditions are not directly comparable because proteasome inhibitors were added to NE following PKA treatment. These experiments demonstrated that the PKA-stimulated peptidase activity in the NE was proteasomal in origin. Because of the complexity of the NE, it remains possible that the effect of PKA on proteasome activity is indirect. Furthermore the cleavage of the peptides by proteasomes may not concur with the degradation of an authentic protein substrate.

**PKA Stimulated Proteasome-dependent Degradation of GST-Sp1 in the NEs**—Forskolin can also stimulate the proteasome-dependent degradation of the transcription factor Sp1. It has been reported that the in vitro degradation of Sp1 in the NE is a two-step process (26). Sp1 is first cleaved between the residues...
Reversed PKA-stimulated Proteasome Activities—To determine whether PKA acts on proteasomes directly, it should be able to stimulate the peptidase activity of the purified 26 S proteasome. As assumed, PKA stimulated both chymotrypsin-like (Fig. 2F) and trypsin-like (Fig. 2G) activities. PKA-specific inhibitor H-89 mostly blocked the stimulatory effect of PKA (Fig. 2, F and G), indicating that PKA activity is required for the stimulation. Although it remains possible that PKA might act through other factors in the NE, these results demonstrate that PKA acts directly on the 26 S proteasome particles.

**Protein Phosphatase 1 γ Inhibited Proteasome Activities**—Protein phosphatase 1 reverses the effect of PKA in glycogen metabolism by dephosphorylating glycogen phosphorylase and glycogen synthase. Three major isoforms of protein phosphatase 1, PP1α, PP1β, and PP1γ, have been characterized (30). Preliminary results indicated that the PP1γ isoform reversed the PKA effect on the proteasome. Therefore, PP1γ was studied further. The results showed that PP1γ treatment inhibited the degradation of both chymotrypsin-like (Fig. 3A) and trypsin-like (Fig. 3B) peptidase activities in the forskolin-activated NE. Furthermore, PP1γ treatment also inhibited Sp1 degradation in the forskolin-activated NE (Fig. 3C). The effect of this phosphatase was also examined on the purified 26 S proteasomes. As expected, PP1γ inhibited both chymotrypsin-like (Fig. 3D) and trypsin-like (Fig. 3E) activities of the purified 26 S proteasomes. Interestingly, the inhibitory effect of PP1γ on 26 S proteasome was just as potent as the proteasome-specific inhibitor epoxomicin (Fig. 3, D and E).

**PKA-stimulated Proteasome Activity of Purified 26 S Proteasome**—To determine whether PKA acts on proteasomes directly or through other factors in the NE, we used highly purified 26 S proteasomes (>95% purity) (Fig. 2E). If PKA acts on 26 S proteasome directly, it should be able to stimulate the peptidase activity of the purified 26 S proteasome. As assumed, PKA stimulated both chymotrypsin-like (Fig. 2F) and trypsin-like (Fig. 2G) activities. PKA-specific inhibitor H-89 mostly blocked the stimulatory effect of PKA (Fig. 2, F and G), indicating that PKA activity is required for the stimulation. Although it remains possible that PKA might act through other factors in the NE, these results demonstrate that PKA acts directly on the 26 S proteasome particles.

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subunits in 20 S proteasome were also labeled with $^{32}$P (Fig. 4A). However, these subunits were not labeled in intact 26 S proteasomes. The failure to label any proteins in the core particle of the intact 26 S proteasome is not likely a consequence of quantity. There is likely a stoichiometrically equivalent amount of the 45-kDa substrate in the 26 S proteasome to proteins in the 20 S core. The failure to label these subunits in the 26 S proteasome could be a result of steric hindrance imposed by the 19 S cap on the 20 S subunits. Alternatively the core particle in intact 26 S proteasomes could be different (e.g. already modified) from the 20 S core particle. For example, assembly of the 20 S particle into intact 26 S proteasomes may require phosphorylation (21). Because it is thought that the degradation of protein substrates in the cell is mainly the function of the intact 26 S proteasome, the relevant phosphorylation might be the one seen in the 26 S particle. The phosphorylation of the 20 S core particle by PKA, however, deserves further investigation. It might play an important role in the assembly of the 26 S proteasome (21) or in the regulation of the peptidase and $\alpha$-secretase activities of the 20 S proteasome (22).

**Rpt6 Was Identified as the Subunit Modified by PKA**—The 45-kDa band, phosphorylated in the 26 S proteasome by PKA, was excised from the gel and identified by tandem mass spectrometry. Two fragments of Rpt6 (Sug1), TMLEQLDGFEATK and IAELMPGASGAEVK, were identified, suggesting that the 45-kDa band was mammalian Rpt6, an AAA ATPase (39) that resides in the 19 S cap of the proteasome. Interestingly, Rpt6 is not modified by OGT (20). To assess whether Rpt6 could be modified by PKA, recombinant Rpt6 fused to GST was expressed and purified with glutathione-Sepharose beads. The beads bearing the recombinant GST-Rpt6 protein were then treated with PKA and $\gamma$-$^{32}$P]ATP. GST-Rpt6, but not GST alone (37), was labeled by PKA treatment (Fig. 4B). The radioactive band was excised from the gel, and the radioactivity was counted. About 9.5% of 2 $\mu$g of the recombinant protein was labeled with phosphate by PKA after 45 min in vitro. The phosphorylation of GST-Rpt6 was greatly reduced by adding PP1 to the reaction (Fig. 4B), indicating that PP1 reverses PKA-induced phosphorylation of Rpt6. These data make it likely that Rpt6 is the major subunit being modified by PKA and that the phosphatase PP1 is sufficient to remove the modification. However, the modification might also be removed by other protein phosphatases.

**Rpt6 Was Phosphorylated by PKA at Ser$^{120}$**—To identify the site that is modified by PKA, we searched for phosphorylation sites with NetPhos 2.0 Server (Center for Biological Sequence Analysis, Technical University of Denmark). Two potential sites, Ser$^{120}$ and Ser$^{215}$, were predicted to be modified. We mutated these sites to Ala (Fig. 5A). The wild type GST-tagged Rpt6 and the indicated mutants were treated with PKA and $\gamma$-$^{32}$P]ATP. Although wild type GST-Rpt6 and mutant GST-Rpt6S215A could still be labeled by $^{32}$P, mutant GST-Rpt6S120A could not (Fig. 5B). The point mutation of Ser$^{120}$ to Ala totally prevented labeling of Rpt6 with $\gamma$-$^{32}$P]ATP by PKA, indicating that Ser$^{120}$, but not Ser$^{215}$, is the PKA phosphorylation site in Rpt6.

**FIGURE 4. Rpt6 was the subunit modified by PKA.** A, autoradiography showed proteasome subunits labeled with $^{32}$P by PKA. Arrows indicate the bands labeled with $^{32}$P. B, recombinant GST-Rpt6 was labeled with $^{32}$P by PKA. After the proteins were resolved with SDS-PAGE, the gel was subjected to Coomassie Blue staining (bottom panel) and autoradiography (top panel). Con, control.

**FIGURE 5. Rpt6 was modified by PKA at Ser$^{120}$.** A, diagram showing the site-directed mutagenesis of Rpt6. Ser$^{120}$ was the modification site of PKA. GST-Rpt6, GST-Rpt6S120A, GST-Rpt6S215A, or GST-Rpt6S120A/S215A was treated with 5 $\mu$Ci of $\gamma$-$^{32}$P]ATP and PKA. The proteins were resolved with SDS-PAGE followed by Coomassie Blue staining (bottom panel) and autoradiography (top panel). C, diagram showing that the phosphorylation site of PKA is conserved through evolution. PS, phosphoserine.
A search for the phosphorylation sites in Rpt6 for organisms ranging from yeast to human showed that this site is well conserved except that the predicted site is Ser119 in yeast and *Drosophila* Rpt6. However, they all have the same characteristic PKA phosphorylation motif (RXX(S/T)) (Fig. 5C).

Ultimately to further confirm that Rpt6 is phosphorylated at Ser\textsuperscript{120}, tandem mass spectrometry was performed. As expected, the results confirmed that Ser\textsuperscript{120} was phosphorylated after PKA treatment. The observed phosphorylated fragment was RNdpSYTLH. The average mass of this fragment with phosphate was 1085.0353 Da. The monoisotopic mass was 1084.4339 Da.

**FIGURE 6.** Tandem mass spectrometry showed that Ser\textsuperscript{120} was phosphorylated by PKA. GST-Rpt6 was pretreated with PKA in proteasome activity assay buffer and resolved with SDS-PAGE. The GST-Rpt6 band was excised from the gel after Coomassie Blue staining. Tandem mass spectrometry was performed. The observed fragment was RNdpSYTLH. The average mass of the fragment with phosphate was 1085.0353 Da. The monoisotopic mass was 1084.4339 Da.

**In Vivo Phosphorylation of Rpt6 Was in a PKA-dependent Manner—**To determine whether endogenous Rpt6 is phosphorylated at antibody-detectable serines, the proteins in highly purified 26 S proteasome were resolved with SDS-PAGE and blotted with anti-phosphoserine antibody. Several bands were detected in the blot (Fig. 7A). When the blot was stripped and reprobed with anti-Rpt6 antibody, one of the phosphoserine antibody-detected bands was identified as Rpt6 (Fig. 7A). This indicates that endogenous Rpt6 is phosphorylated at a serine residue. Furthermore, 293 cells were transfected with plasmids encoding wild type His-Xpress-Rpt6 or mutant His-Xpress-Rpt6\textsuperscript{S120A}. His-tagged recombinant proteins were isolated from the cell lysates and resolved with anti-phosphoserine Western blot. Only the wild type Rpt6 was detected with anti-phosphoserine antibody; the PKA phosphorylation site mutant Rpt6\textsuperscript{S120A} was not (Fig. 7B). Therefore, endogenous Rpt6 is phosphorylated at Ser\textsuperscript{120}, the target of PKA. Because the 26 S proteasomes were purified from unstimulated cells (Fig. 7A), the phosphorylation must occur in basal conditions without external PKA stimulation.

To determine whether the phosphorylation of Rpt6 results from the activation of PKA, 293 cells were transfected with wild type His-Xpress-Rpt6. The transfected 293 cells were treated with forskolin or H-89 before harvest to increase cAMP activa-
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A.

B.

C.

D.

E.

FIGURE 7. Rpt6 was phosphorylated at Ser120 in vivo. A, endogenous Rpt6 was phosphorylated. Highly purified 26S proteasome was resolved with SDS-PAGE and blotted with anti-phosphoserine (anti-PS) antibody. After the blot was stripped, it was re-probed with anti-Rpt6 antibody. B, mutation S120A eliminated phosphorylation of Rpt6 in vivo. His-Xpress-Rpt6, His-Xpress-Rpt6S120A, or pcDNA3.1 vector were transfected into 293 cells. His tagged proteins were isolated and resolved with SDS-PAGE followed by Western blotting. The arrow in the top panel indicates phosphorylated His-Xpress-Rpt6. C, phosphorylation of recombinant His-Xpress-Rpt6 was increased by forskolin treatment. D, phosphorylation of recombinant His-Xpress-Rpt6 was reduced by H-89 treatment. Wild type His-Xpress-Rpt6 was transfected into 293 cells. The transfected cells were treated with or without 100 μM forskolin (For) or 5 μM H-89 45 min before harvest. The recombinant proteins were isolated and resolved with SDS-PAGE followed by anti-phosphoserine (anti-PS) Western blotting. E, the reactivity of the mutant His-Xpress-Rpt6S120A with phosphoserine antibody was not stimulated by forskolin treatment. Mutant His-Xpress-Rpt6S120A was transfected into 293 cells. 100 μM forskolin (For) was added to the cell 45 min before the cells were harvested. WT, wild type.

tion of PKA. Forskolin treatment increased the reactivity of recombinant His-Xpress-Rpt6 with the phosphoserine antibody compared with the untreated control (Fig. 7C), whereas H-89 treatment greatly reduced the reactivity of the recombinant protein with the same antibody (Fig. 7D). As a control, reactivity of the recombinant His-Xpress-Rpt6S120A mutant was not stimulated by forskolin treatment (Fig. 7E). Together with the data in Fig. 7B, these results demonstrate that phosphorylation of Rpt6 at Ser120 occurs in vivo both in the basal state in the absence of external stimulation of PKA activity and in the stimulated state as a result of PKA activation by a foreign substance, forskolin, a stimulator of adenylate cyclase in all cell types.

Forskolin Stimulated Proteasome-dependent Degradation of GFP-CL1 in Living Cells—The above in vitro experiments suggest that PKA stimulates proteasome function. To determine whether this stimulatory effect occurs in vivo, we used GFP fused to a short degron, CL1 (ACKNWFSSSLHFIHVHL) (32), as a reporter (GFP-CL1) for the proteasome activity in vivo (20, 33). Because forskolin treatment elevates cAMP levels in the cell and activates PKA, we tested the effect of forskolin on the degradation of GFP-CL1 in the cell. Cycloheximide, an inhibitor of translation, was used to block protein synthesis so only protein degradation could be observed in the treated cells. The result showed that forskolin greatly increased the degradation rate of GFP-CL1 compared with the untreated control (Fig. 8A). It took about 80 min in the untreated control for the majority of GFP-CL1 to be degraded, whereas it took only about 40 min in the forskolin-treated cells. The stimulatory effect of forskolin on GFP-CL1 degradation was partly reversed by treating the cells at the same time with PKA-specific inhibitor H-89 (Fig. 8B). This result further confirms that PKA is essential for forskolin to stimulate the proteolytic activity of proteasome. Other signals using the cAMP/PKA system like forskolin might also stimulate proteolytic activity of the proteasome in vivo.

Mutant Rpt6S120A Inhibited the Degradation of GFP-CL1 in Living Cells—The effect of PKA phosphorylation of Rpt6 on proteasome function was assessed in living cells. Mutant His-Xpress-Rpt6S120A was cotransfected into 293 cells with GFP-CL1. Using the proteasome affinity beads conjugated with GST-UbLH9262, the recombinant proteins were shown to be incorporated into 26S proteasome (Fig. 8C). The mutant His-Xpress-Rpt6S120A when expressed in the transfected cells (Fig. 7B) increased the fluorescence intensity derived from the GFP-CL1 by about 4-fold over that of empty vector and that of wild type His-Xpress-Rpt6 control (Fig. 8D), suggesting that mutant Rpt6S120A acted as a dominant negative and blocked the proteasome function. The mutant Rpt6S120A, like the proteasome-specific inhibitor MG132, blocked the stimulatory effect of forskolin on the degradation of GFP-CL1 (Fig. 8E) in cells treated with cycloheximide, a translation inhibitor. Interestingly mutant Rpt6S120A also caused the accumulation of ordinary GFP by about 2-fold over that of control and wild type Rpt6 (Fig. 8F). This implies that the mutation blocked not only the degradation of degron-destabilized protein but also the turnover of the global protein in the cell. These data demonstrate that phosphorylation at Ser120 of Rpt6 by PKA is necessary for the PKA (forskolin)-mediated increase in proteasome function in vivo.

OGT Inhibition of Proteasome Function Dominated over PKA Stimulation—OGT has been demonstrated to inhibit proteasome. This inhibition can be detected by the failure of OGT-treated proteasomes to cleave the Suc-LLVY-AMC peptide. The subunit that is modified is Rpt2 (20), one of the AAA ATPases. The modification of Rpt2 by OGT blocks the ATPase activity (20) that is critical for proteasome function (18, 40). Rpt6 is also one of the ATPases. Modification of Rpt6 by PKA, however, did not alter the overall ATPase activity of proteasomes (data not shown). To determine which of the modifications dominates over proteasome dependent proteolysis of Suc-LLVY-AMC, the O-GlcNAcylation, or PKA phosphorylation, we co-treated the NE with PKA and OGT. Whether OGT or PKA was applied first, the cleavage of the peptide stimulated by PKA was inhibited (Fig. 9). These results suggest that the inhibitory effect of OGT dominates over the stimulatory effect of PKA in proteasome regulation. For PKA to

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stimulate proteasome activity, the regulatory O-GlcNAc modification has to be removed.

**DISCUSSION**

Proteasome and cAMP both have multiple effects on cells, and they may act together in unanticipated ways. They are both ubiquitous and distributed in cytoplasm and nucleus. But their actions might play different roles, depending on the circumstances of the particular cell type. One example of these roles may be in glucose homeostasis where the effects of PKA and OGT on proteasomes are decipherable. Under certain conditions that will be elaborated below, the activity of OGT is mutually exclusive to that of PKA. During starvation, the plasma glucose concentration is maintained by the liver so that tissues that depend on glucose utilization can receive this nutrient. The liver cells first engage in glycogenolysis. But when the stores of glycogen are depleted, the liver engages in gluconeogenesis from glycogenic amino acids. The principle source of the amino acids is muscle. Thus, the muscle serves two purposes, locomotion and calorie storage (41). How the muscle is signaled to release glycogenic amino acids to the liver is just beginning to be understood. During starvation, insulin levels are suppressed, and counter-regulatory hormones such as glucagons and epinephrine are elevated. In muscle, the suppression of insulin results in less glucose transport. With less glucose, there is less substrate for the enzyme, glutamine:fructose-6-phosphate trans-ferase to convert to glucosamine-6-phosphate. Furthermore the counter-regulatory hormones elevate cAMP levels in muscle; this increases the activity of PKA. PKA in turn inactivates glutamine:fructose-6-phosphate transferase to convert to glucosamine-6-phosphate.
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![Graph showing the inhibitory effect of OGT dominated over the stimulatory effect of PKA.](image)

**FIGURE 9. The inhibitory effect of OGT dominated over the stimulatory effect of PKA.** OGT and PKA were added to the nuclear extract sequentially. Either OGT (OGT/PKA) or PKA (PKA/OGT) was applied first. The other one was added at 30 min of incubation at 30 °C. After another 30 min, Suc-LLVY-AMC was added to the reaction. The incubation was continued for 1 h at 37 °C before the fluorescence intensity was measured (mean ± S.D., n = 3). Student’s t-test was performed (**, *p < 0.01*). INE, untreated NRK cell INE. Error bars indicate mean ± S.D.

proteins. Proteins (37), including those in the proteasome (26, 27), become less O-GlcNAcylated. Without O-GlcNAc on Rpt2, the proteasome can be activated (20) by PKA through the phosphorylation of Rpt6 at Ser120. The increased proteasome activity allows it to degrade ubiquitinated proteins in muscle (19, 42–46), providing the glycogenic amino acids used by the liver for gluconeogenesis. The converse applies in the fed state.

Both O-GlcNAc and PKA-induced modifications so far studied occur in the ATPases in the 19 S cap. This suggests that modifications of the ATPases might play important roles in the regulation of the proteasome in response to the changing environment or signals from outside the cell. How O-GlcNAcyla-
ation of Rpt2 operates to inhibit proteasomes and preclude their activation associated with phosphorylation of Rpt6 is unknown. The proteolysis of the peptide substrates suggests different mechanisms by which these modifications affect proteasome function. O-GlcNAcylation of Rpt2 inhibits the overall ATPase activity of the proteasome, but it inhibits only the proteolytic process of the hydrophobic peptide LLVY (20). The ATP-dependent breakage of hydrophobic bonds is required in all folded protein substrates, and we have postulated that Rpt2 might be the "chief" ATPase that controls this process. Phosphorylation of Rpt6 by PKA, however, was associated with the stimulation of the proteasome to process all the tested peptides. How phosphorylation of Rpt6 by PKA affects proteasome function is unknown. We assume it causes conformational changes so that even the diffusion of the small peptide substrate into the proteolytic core of the proteasome is enhanced.

Malfunction of the proteasome has been documented in a variety of major human diseases such as neurodegenerative disorders (6, 7), cataracts (8), and muscle atrophy (9, 10, 47). Dysregulation of the posttranslational modifications might contribute to the causes of the malfunction. However, although the modifications are universal, the role of these modifications of the proteasomes may be different in diseases of different tissues. For example, although proteasome activity is elevated in muscle atrophy, its function is depressed in Alzheimer disease and cataracts. Protein aggregates, which are often seen in neurodegeneration, inhibit the function of the proteasome (20, 33). Perhaps the cAMP mechanism described here may overcome this inhibition. The proteasome is a complicated complex, and as such, its regulation must be as complicated. It is expected that more modifications affecting its function will be described in the future. The physiological and pathological roles of the proteasome will thus be understood more completely.

REFERENCES

1. Hershko, A. (2005) *Cell Death Differ.* 12, 1191–1197
2. Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hershko, J., Luca, F. C., Ruderman, J. V., and Hershko, A. (1995) *Mol. Biol. Cell.* 6, 185–197
3. Richter-Ruoff, B., and Wolf, D. H. (1993) *FEBS Lett.* 336, 34–36
4. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) *EMBO J.* 16, 3797–3804
5. Maki, C. G., Hubregtse, J. M., and Howley, P. M. (1996) *Cancer Res.* 56, 2649–2654
6. Bossy-Wetzel, E., Schwarzenbacher, R., and Lipton, S. A. (2004) *Nat. Med.* 10, (suppl.) S2–S9
7. Ding, Q., and Keller, J. N. (2003) *J. Alzheimer’s Dis.* 5, 241–245
8. Andersson, M., Sjostrand, J., and Karlsson, J. (1998) *Exp. Eye Res.* 67, 231–236
9. Tawa, N. E., Jr., Odessy, R., and Goldberg, A. L. (1997) *J. Clin. Investig.* 100, 197–203
10. Mitch, W. E., and Goldberg, A. L. (1996) *N. Engl. J. Med.* 335, 1897–1905
11. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* 67, 425–479
12. Varshavsky, A. (2005) *Trends Biochem. Sci.* 30, 283–286
13. Liu, C. W., Corboy, M. J., DeMartino, G. N., and Thomas, P. J. (2003) *Science* 299, 408–411
14. Kierszenbaum, A. L. (2000) *Mol. Reprod. Dev.* 57, 109–110
15. Myung, J., Kim, K. B., and Crews, C. M. (2001) *Med. Res. Rev.* 21, 245–273
16. Navon, A., and Goldberg, A. L. (2001) *Mol. Cell.* 8, 1339–1349
17. Kohler, A., Cascio, P., Leggett, D. S., Woo, K. M., Goldberg, A. L., and Finley, D. (2001) *Mol. Cell.* 7, 1143–1152
18. Rubin, D. M., Glickman, M. H., Larsen, C. N., Dhruvakumar, S., and Finley, D. (1998) *EMBO J.* 17, 4909–4919
19. Jago, R. T., and Goldberg, A. L. (2001) *Carr. Opin. Clin. Nutr. Metab. Care* 4, 183–190
20. Zhang, F., Su, K., Yang, X., Bowe, D. B., Paterson, A. J., and Kudlow, J. E. (2003) *Cell* 115, 715–725
21. Bose, S., Stratford, F. L., Broadfoot, K. I., Mason, G. G., and Rivett, A. J. (2004) *Biochem. J.* 378, 177–184
22. Marambaud, P., Wilk, S., and Checler, F. (1996) *J. Neurochem.* 67, 2616–2619
23. Bardag-Gorce, F., Venkatesh, R., Li, J., French, B. A., and French, S. W. (2004) *Life Sci.* 75, 585–597
24. Satoh, K., Sasajima, H., Nyomura, K. I., Yokosawa, H., and Sawada, H. (2001) *Biochemistry* 40, 314–319
25. Murray, E. J., Bentley, G. V., Grisanti, M. S., and Murray, S. S. (1998) *Exp. Cell Res.* 242, 460–469
26. Su, K., Roos, M. D., Yang, X., Han, L., Paterson, A. J., and Kudlow, J. E. (1999) *J. Biol. Chem.* 274, 15194–15202
27. Han, I., and Kudlow, J. E. (1997) *Mol. Cell. Biol.* 17, 2550–2558
28. Krebs, E. G. (1989) *J. Am. Med. Assoc.* 262, 1815–1818
29. Collins, S., and Surwit, R. S. (2001) *Recent Prog. Horm. Res.* 56, 309–328
30. Cohen, P. T. (2002) *J. Cell Sci.* 115, 241–256
31. Su, K., Yang, X., Roos, M. D., Paterson, A. J., and Kudlow, J. E. (2000) *Biochem. J.* 348, 281–289
32. Gilon, T., Chomsky, O., and Kukla, R. G. (1998) *EMBO J.* 17, 2759–2766
33. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) *Science* 292, 1552–1555
34. Seamon, K. B., and Daly, J. W. (1986) *Adv. Cyclic Nucleotide Protein Phos-
phorylation Res. 20, 1–150
35. Seamon, K. B., and Daly, J. W. (1981) J. Cyclic Nucleotide Res. 7, 201–224
36. Daly, J. W., Padgett, W., and Seamon, K. B. (1982) J. Neurochem. 38, 532–544
37. Chang, Q., Su, K., Baker, J. R., Yang, X., Paterson, A. J., and Kudlow, J. E. (2000) J. Biol. Chem. 275, 21981–21987
38. Hu, Y., Riesland, L., Paterson, A. J., and Kudlow, J. E. (2004) J. Biol. Chem. 279, 29988–29993
39. Russell, S. J., Gonzalez, F., Joshua-Tor, L., and Johnston, S. A. (2001) Chem. Biol. 8, 941–950
40. Glickman, M. H., Rubin, D. M., Fu, H., Larsen, C. N., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Vierstra, R., Baumeister, W., Fried, V., and Finley, D. (1999) Mol. Biol. Rep. 26, 21–28
41. Owen, O. E., Reichard, G. A., Jr., Patel, M. S., and Boden, G. (1979) Adv. Exp. Med. Biol. 111, 169–188
42. Dahlmann, B., Rutschmann, M., Kuehn, L., and Reinauer, H. (1985) Biochem. J. 228, 171–177
43. Dahlmann, B., Kuehn, L., Rutschmann, M., and Reinauer, H. (1985) Biochem. J. 228, 161–170
44. Temparis, S., Asensi, M., Taillandier, D., Aurousseau, E., Larbaud, D., Obled, A., Bechet, D., Ferrara, M., Estrela, J. M., and Attaix, D. (1994) Cancer Res. 54, 5568–5573
45. Wing, S. S., Haas, A. L., and Goldberg, A. L. (1995) Biochem. J. 307, 639–645
46. Medina, R., Wing, S. S., Haas, A., and Goldberg, A. L. (1991) Biomed. Biochim. Acta 50, 347–356
47. Price, S. R., and Mitch, W. E. (1998) Curr. Opin. Clin. Nutr. Metab. Care 1, 79–83