Osmotic Stress Inhibits Proteasome by p38 MAPK-dependent Phosphorylation*§

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Osmotic stress causes profound perturbations of cell functions. Although the adaptive responses required for cell survival upon osmotic stress are being unraveled, little is known about the effects of osmotic stress on ubiquitin-dependent proteolysis. We now report that hyperosmotic stress inhibits proteasome activity by activating p38 MAPK. Osmotic stress increased the level of polyubiquitinated proteins in the cell. The selective p38 inhibitor SB202190 decreased osmotic stress-associated accumulation of polyubiquitinated proteins, indicating that p38 MAPK plays an inhibitory role in the ubiquitin proteasome system. Activated p38 MAPK stabilized various substrates of the proteasome and increased polyubiquitinated proteins. Proteasome preparations purified from cells expressing activated p38 MAPK had substantially lower peptidase activities than control proteasome samples. Proteasome phosphorylation sites dependent on p38 were identified by measuring changes in the extent of proteasome phosphorylation in response to p38 MAPK activation. The residue Thr-273 of Rpn2 is the major phosphorylation site affected by p38 MAPK. The mutation T273A in Rpn2 blocked the proteasome inhibition that is mediated by p38 MAPK. These results suggest that p38 MAPK negatively regulates the proteasome activity by phosphorylating Thr-273 of Rpn2.

The 26 S proteasome plays a central role in ubiquitin-dependent proteolysis by regulating physiological processes such as cell cycle progression and signal transduction (1, 2). The 26 S proteasome consists of a 20 S core particle (CP)3 and a 19 S regulatory particle (RP, also known as PA700) (3). The 20 S CP has a barrel-shaped structure composed of two outer α rings and two inner β rings (4). The central β rings possess proteolytic active sites, whereas the outer α rings provide attachment sites for the 19 S RP and function as a gated channel that controls the access of substrates to the catalytic chamber (5, 6). Both the 20 S core and the 19 S RP are required for ATP-dependent degradation of ubiquitinated proteins. The 19 S RP is responsible for binding the substrate, removing the substrate-attached ubiquitin chain, unfolding the substrate, and translocating it into the catalytic chamber of CP (3).

The functions and activities of the proteasome are regulated in diverse ways. For instance, replacing catalytic subunits generates special proteasomes, immunoproteasomes, and thymoproteasomes, which have altered enzymatic activities (7–9). Besides the RP, other proteasome activators such as REGγ, PA28, and PA200 also bind to CP to form proteasome variants with modified functions (10–12). Recent mass spectrometric analysis of purified proteasome has identified novel proteasome subunits and proteasome-associated proteins (13–17). Many of these play regulatory roles in the ubiquitin-proteasome pathway. Post-translational modifications such as phosphorylation and glycosylation have been reported to regulate the proteasome activity. For example, modification of Rpt2 by O-GlcNAc inhibited proteasome activity (18), whereas the phosphorylation of Rpt6 by cAMP-dependent protein kinase or by calcium/calmodulin-dependent protein kinase II activated the proteasome (19, 20).

Osmotic stress causes profound perturbations of cell function, but little is known about how ubiquitin-dependent proteolysis is regulated by osmotic stress. In this study, we investigated the effects of osmotic stress on the ubiquitin-proteasome pathway. We showed that osmotic stress inhibited proteasome activity through p38 MAPK-dependent phosphorylation of Thr-273 of Rpn2.

EXPERIMENTAL PROCEDURES

Plasmids—The cDNAs encoding human proteasome subunit S1/Rpn2 (D44466) and S14/Rpn12 (BC065006) were obtained from MRC Geneservice. Plasmids expressing epitope-tagged proteins were constructed from cDNAs that were amplified by PCR with appropriate primers and ligated into pcDNA3.1 (Invitrogen) or pYR vectors (21). The MKK6 and p38α expression constructs were kindly provided by Kang-Yell Choi (Yonsei University, Seoul, Korea).

Western Blot Analysis—Transfection was performed with Lipofectamine 2000 (Invitrogen). After 48 h, cells were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM PMSF, and 1.0% Nonidet P-40. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (Whatman), and visualized by Western blotting with enhanced chemiluminescence reagents (Amersham Bio-
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EM microplate spectrofluorometer (Molecular Devices) using 380-nm excitation and 460-nm emission filters. The activities were quantified by referring to an AMC calibration curve.

Peptide Labeling with the Isobaric Tag for Relative and Absolute Quantitation (iTRAQ) Reagents—The iTRAQ Reagent Multi-Plex kit (ABI) was used for trypsin digestion and subsequent peptide labeling. Two distinct reagents for iTRAQ with tandem mass spectrometry (MS/MS) signals at either 116 or 117 Da were resuspended in 70 μl of ethanol, and then each sample was mixed with one of the reagents for peptide labeling. The control samples were labeled with the iTRAQ reagent with the 116-Da signature ion signal. The test samples that had been treated with p38 MAPK/MKK6EE were reacted with the reagent that resulted in the 117-Da ion signal in MS/MS mode. After incubation for 1 h, the two labeled samples either were mixed and analyzed by liquid chromatography (LC)-MS/MS, or phosphopeptides were enriched by a TiO₂ column before analysis by LC-MS/MS.

TiO₂-based Phosphopeptide Enrichment—Purification of phosphopeptides from the peptide mixture was performed essentially as described previously (22). The TiO₂ 3-mm-long microcolumns were packed in GELoader tips. The peptide solution was diluted with 100 μl of loading buffer containing 1 M glycine in 80% acetonitrile and 5% trifluoroacetic acid (TFA) prior to loading onto the TiO₂ microcolumn. The microcolumn was washed with 5 μl of loading buffer followed by 20 μl of washing buffer that contained 80% acetonitrile and 5% TFA. The phosphopeptides were eluted with 20 μl of aqueous ammonia that consisted of 20 μl of 25% ammonia solution in 980 μl of H₂O, pH 11, acidified with 2 μl of 100% formic acid, and subsequently purified with a Poros R3 microcolumn.

Protein Identification and Quantitation by Mass Spectrometry—Tryptic digests of the proteasome or TiO₂-enriched phosphopeptides from the proteasome were analyzed by nanoelectrospray LC-MS/MS. High pressure liquid chromatography separation was performed on an Ultimate instrument equipped with a QSTAR Pulsar Q-TOF mass spectrometer (Applied Biosystems) in the information-dependent acquisition mode. The MS-MS spectra were used to search the National Center for Biotechnology Information nonredundant and expressed sequence tag databases using Mascot (Matrix Science). Relative quantitation of proteins was performed on the MS/MS scans and was given by the peak area ratio at m/z 116 and 117 Da.

Affinity Purification of the Proteasome—Cells derived from HeLa Tet-Off (Clontech) that stably expressed EBNA-1 were transfected with the episomal expression vector pYR-FLAG-Rpn12 or pYR-FLAG-Rpn2. These vectors contained the gene of interest under a tetacycline-regulated promoter, oriP, for episome replication and a selection marker for hygromycin B. The cells were selected and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 μg/ml G418 (Sigma), 300 μg/ml hygromycin B (Clontech), 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM l-glutamine, and 2 μg/ml tetracycline (Sigma). Cells were grown without tetracycline for 2 days to induce the expression of the FLAG-tagged proteins. The cells were lysed in buffer A containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM PMSF, 50 mM NaF, 0.1 mM Na₂VO₄, 10 mM β-glycerol 2-phosphate, and 0.5% Nonidet P-40. The lysates were centrifuged at 20,000 × g for 15 min to remove cell debris. The supernatant was incubated with anti-FLAG M2-agarose (Sigma) over night at 4 °C. After extensive washes with buffer A without Nonidet P-40, the proteins were eluted with 0.3 mg of FLAG peptide per ml in buffer A without Nonidet P-40.

In Vitro Kinase Assay—A total of 1 μg of purified 26 S proteasome was incubated with 50 ng of activated p38 MAPK in kinase buffer that contained 50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol (DTT), 10 mM MgCl₂, and 50 μM ATP. Reaction mixtures totaling 25 μl were incubated for 30 °C and then digested with SDS-PAGE after adding the SDS sample buffer. For the p38 MAPK assay using radiolabeled ATP, 10 μCi of [γ-²³P]ATP was included in the reaction mixture. The phosphorylation of substrate proteins was analyzed by autoradiography and quantitated with a Fuji BAS 1000.

Peptidase Activity Assays—All peptidase assays of the 26 S proteasome were performed in 200-μl reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 40 mM KCl, 5 mM MgCl₂, 0.5 mM ATP, 1 mM DTT, 100 μM fluorescent substrates, and 1 μg of 26 S proteasome. Proteasome samples were assayed for peptidase activities by using succinyl-LLVY-AMC for chymotrypsin-like activities, benzamidoxycarbonyl-LLE-AMC for trypsin-like activities, and benzyl-VGR-AMC for caspase-like activities. Peptidase activity was measured at 37 °C for 30 min by continuously monitoring AMC production with a Gemini
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FIGURE 1. Polyubiquitinated proteins are accumulated under osmotic stress. 

A. Polyubiquitinated proteins were immunoblotted (IB) after sorbitol treatment. HeLa cells were either treated for 1 h with the indicated concentrations of sorbitol (lanes 1–5) or with 400 mM sorbitol for the indicated times (lanes 6–12). Cell lysates were subjected to SDS-PAGE and then Western blot analysis with anti-ubiquitin (α-Ub), anti-phospho-p38 MAPK (Thr-180/Tyr-182, abbreviated as α-p-p38), anti-phospho-JNK (Thr-183/Tyr-185, abbreviated as α-p-JNK), and anti-actin antibodies (α-β actin). B. p38 MAPK inhibitor affected sorbitol-induced accumulation of polyubiquitinated proteins. HeLa cells were treated for 1 h with SB202190 (5, 10, and 20 μM in lanes 3–5, respectively), a p38 MAPK inhibitor, or with SP600125 (10 and 20 μM in lanes 8 and 9, respectively), a JNK inhibitor. The cells were then incubated with 400 mM sorbitol. Cell lysates were separated by SDS-PAGE and immunoblotted with the antibodies indicated.

RESULTS

Osmotic Stress Causes Accumulation of Polyubiquitinated Proteins—The effect of hyperosmotic stress on the ubiquitin proteasome system was investigated by treating HeLa cells with varying concentrations of sorbitol. Polyubiquitinated proteins were analyzed with anti-ubiquitin Western blotting. Sorbitol treatment increased the level of polyubiquitinated proteins in HeLa cells in a concentration- and time-dependent manner (Fig. 1A). These data suggest that hyperosmotic stress affects the ubiquitin-dependent protein turnover in HeLa cells.

Hyperosmotic stress initiates an array of cellular responses, including reorganization of the cytoskeleton, cell growth arrest, and activation of a variety of protein kinases (23, 24). Treatment with sorbitol activated JNK and p38 MAPKs (Fig. 1A), which play important roles in regulating stress-induced cellular responses. The possible involvement of these MAPKs in the accumulation of polyubiquitinated proteins in response to high osmolarity was investigated by treating cells with MAPK inhibitors before exposing them to osmotic stress. The osmotic stress-associated increase in polyubiquitinated proteins was lowered by pretreating cells with SB202190, a p38 MAPK inhibitor in a dose-dependent fashion, but not with SP600125, a JNK inhibitor (Fig. 1B). This suggests that p38 MAPK is involved in osmotic stress-induced accumulation of polyubiquitinated proteins.

p38 MAPK Stabilizes Proteasome Substrates—The accumulation of polyubiquitinated proteins could be due to enhanced ubiquitination by E3 ligases, impaired delivery of polyubiquitinated proteins to the proteasome, or direct inhibition of the proteasome. We ruled out some possibilities by first testing whether p38 MAPK is involved in the negative regulation of proteasome-dependent protein degradation. Plasmids expressing p38 MAPK and a constitutively active form of its upstream activator MKK6 (M KK6EE) were cotransfected into HeLa cells along with plasmids expressing GFP-based proteasome substrates that contain various degradation signals, or degrons. Western blotting analysis revealed that the activation of p38 MAPK by MKK6EE resulted in increased expression of both ubiquitination-dependent (UbG76V-GFP, Ub-R-GFP, and GFPu) and ubiquitination-independent (GFP-ODC) proteasome substrates, although the expression of the unmodified GFP remained unchanged (Fig. 2A). Transfected cells were treated with the protein synthesis inhibitor cycloheximide prior to analysis of the stability of the expression of proteasome substrates by Western blotting. The expression of p38 MAPK/MKK6E significantly prolonged the half-lives of UbG76V-GFP and GFP-ODC (Fig. 2B). We also analyzed the effect of p38 MAPK/MKK6EE expression on IκBα, an endogenous proteasome substrate. As shown in Fig. 2C, reduction of IκBα following treatment with tumor necrosis factor α (TNFα) was significantly blocked by expression of p38 MAPK/MKK6EE, suggesting that activated p38 MAPK stabilizes endogenous proteasome substrates. In addition, p38 MAPK/MKK6EE expression increased the level of polyubiquitinated proteins in HeLa cells, although not as high as that after the sorbitol treatment (Fig. 2D). Taken together, these results indicate that activated p38 MAPK stabilized the proteasome substrates and regulated a specific step common to both ubiquitinated and nonubiquitinated substrates in the ubiquitin-proteasome pathway.

p38 MAPK Inhibits Proteasome Activity—We next examined whether activated p38 MAPK affects proteasome activity. The purification of the proteasome was facilitated by establishing a HeLa-derived cell line that conditionally expressed FLAG-hRpn12, a subunit of the 19 S regulatory complex. After the induction of FLAG-hRpn12, we transfected the cells with p38 MAPK/MKK6EE expression constructs or with an empty vector, and the proteasome containing FLAG-hRpn12 was purified with anti-FLAG antibody-conjugated beads. Analyses of the proteasome preparations by SDS-PAGE and mass spectrometry revealed that expression of p38 MAPK and MKK6EE did not grossly affect the proteasome composition (Fig. 3A and supplemental Fig. 1). We then...
used fluorogenic peptide substrates to measure chymotrypsin-like, trypsin-like, and caspase-like peptidase activities of the proteasome to evaluate the effects of p38 MAPK/MKK6EE expression on the proteasome. The activities of all three peptidases from the 26 S proteasome from cells transfected with the p38 MAPK and MKK6EE expression con-

FIGURE 2. Proteasome substrates were stabilized by p38 MAPK. A, expression of GFP-based proteasome substrates was increased by activated p38 MAPK. HeLa cells were transfected with an expression construct for GFP, Ub<sup>G76V</sup>-GFP, Ub-R-GFP, G Fu, or GFP-ODC and also the expression vectors for both the FLAG-p38 MAPK and the HA-tagged MKK6EE or for the empty vector, as indicated. The expression levels of GFP and its derivatives, p38 MAPK, MKK6EE, and actin, were determined by immunoblots (IB). B, Ub<sup>G76V</sup>-GFP and GFP-ODC were stabilized by activated p38 MAPK. HeLa cells were transfected with the expression construct of Ub<sup>G76V</sup>-GFP or GFP-ODC along with the expression vectors for FLAG-p38 MAPK and HA-MKK6EE as indicated. Thirty six hours after transfection, cycloheximide was added to all samples. Cells were harvested at the indicated time points. The expression levels of reporters were determined by Western blots with anti-GFP antibody. In all cases, actin levels were a loading control. The GFP signals were analyzed by densitometry (right panel). All experiments were repeated at least three times.

C, TNFα-induced degradation of IκB was inhibited by activated p38 MAPK. HeLa cells were transfected with expression vectors for FLAG-p38 MAPK and HA-MKK6EE as indicated. Thirty six hours after transfection, HeLa cells were stimulated with 45 ng/ml TNFα for the indicated times. The expression levels of IκB were determined by Western blotting with anti-IκB antibody. D, polyubiquitinated proteins were accumulated by activated p38 MAPK. HeLa cells were treated with 0.4 M sorbitol (2nd lane) or transfected with the expression vectors for both the FLAG-p38 MAPK and the HA-tagged MKK6EE (4th lane) or for the empty vector (3rd lane) for 36 h. Cell lysates were subjected to SDS-PAGE followed by Western blot analysis using anti-ubiquitin antibody.
structs were reduced to ~60% of levels in cells transfected with the control plasmid (Fig. 3, B–D). These results indicate that activated p38 MAPK inhibits the proteasome.

**Identifying and Quantifying p38 MAPK-dependent Phosphorylation Sites in the Proteasome**—The ability of p38 MAPK to inhibit proteasome activity by phosphorylating the proteasome was evaluated by analyzing changes in relative phosphorylation of the proteasome by p38 MAPK through a quantitative proteomic technique. Proteasome preparations were affinity-purified from cells transfected either with the p38 MAPK and MKK6EE expression constructs or with the empty vector. A total of 10 μl of each of the FLAG-hRpn12 samples was purified with 2 mM ATP, separated by SDS-PAGE, and visualized by Coomassie staining. B–D, activated p38 MAPK affected the peptidase activities of the proteasome. Peptidase activities were analyzed with 1 μg of each of the proteasome samples from 4 with the fluorogenic substrates of Suc-LLVY-AMC for chymotrypsin-like activity, benzoyloxycarbonyl-LLE-AMC for trypsin-like, and Bz-VGR-AMC for caspase-like. Data presented are the mean ± S.D. from three independent experiments.

**Activated p38 MAPK increased the Rpn2-derived peptide with phosphorylation at Thr-273 by 4.0-fold (Fig. 4A) or the one with phosphorylation at Thr-311 by 5.7-fold (Fig. 4D), although relatively minor changes in phosphorylation levels were observed at other sites of the proteasome (Table 1 and supplemental Fig. 2). If the phosphorylation of a proteasome subunit blocked the activity of the proteasome complex containing the phosphorylated subunit, then the observed 40% inhibition of the proteasome activity by p38 MAPK would result from a similar level of phosphorylation in the proteasome pool. Thus, the degree of phosphorylation was assessed by determining the relative abundance of unmodified cognate peptides from the peak areas of iTRAQ reporter ions. The unmodified peptide containing Thr-273 of Rpn2 was reduced to 65% by p38 MAPK and MKK6EE expression (Fig. 4B). In contrast, the peptide containing nonphosphorylated Thr-311 of Rpn2 was decreased only slightly (Fig. 4E), suggesting that a minute portion of Thr-311 of Rpn2 was phosphorylated by activated p38 MAPK. For most of the other peptides listed in Table 1, the ratio was close to 1 for the reporter ions (supplemental Fig. 2). Because an increase in the phosphorylated peptide by p38 MAPK caused a corresponding decrease in the unmodified peptide, we could estimate the increase of p38 MAPK-mediated phosphorylation at Thr-273 of Rpn2 from 10 to 40% in the cellular proteasome population (Fig. 4C).
These data suggest that p38 MAPK-dependent phosphorylation at Thr-273 of Rpn2 likely contributed to decreased proteasome activity.

p38 MAPK Phosphorylates Thr-273 of Rpn2—Direct evidence of p38 MAPK-mediated phosphorylation at Thr-273 of Rpn2 was obtained by generating polyclonal antibodies against the phosphorylated Thr-273 of Rpn2 by immunizing rabbits with the synthetic phosphopeptide QNLRTVGpTPI-ASVP, which was derived from residues 266–279 of Rpn2. The lysate of cells transfected with the p38 MAPK and MKK6EE expression constructs had a strong 110-kDa protein band, the expected size for Rpn2, when analyzed with the affinity-purified anti-phospho–Thr-273–Rpn2 antibodies, whereas the signal was almost undetectable with the control lysate (Fig. 5A). The specificity of the anti-phospho-Rpn2 (Thr(P)-273) antibodies was further demonstrated by analyzing the purified proteasome with the T273A mutation in Rpn2 by Western blotting. Although the proteasome with the wild-type Rpn2 produced an increased signal with activated p38 MAPK, the proteasome containing the T273A mutant Rpn2 did not generate any signal, even when expressing activated p38 MAPK (Fig. 6A). Taken together, these results indicate that the anti-phospho–Rpn2 (Thr(P)-273) antibodies specifically recognized phosphorylated Thr-273 of Rpn2 and confirm that p38 MAPK phosphorylates Thr-273 of Rpn2 in the cell.

We next investigated whether p38 MAPK could directly phosphorylate Thr-273 of Rpn2 in vitro. Proteasome preparations were incubated with affinity-purified, activated p38 MAPK in the presence of ATP and then analyzed by Western blotting with anti-phospho–Rpn2 (Thr(P)-273) antibodies. The phosphorylation of Thr-273 increased in the sample treated with activated p38 MAPK, indicating that Thr-273 of Rpn2 is a direct target of p38 MAPK (Fig. 5B). We also examined in vitro phosphorylation of proteasome by activated p38 MAPK using radiolabeled ATP (Fig. 5C). Two protein bands with apparent molecular sizes of 110 and 47 kDa in the purified proteasome preparations were phosphorylated by activated p38 MAPK. The T273A mutation in Rpn2 reduced phosphorylation of the 110-kDa band to 25%, indicating that Thr-273 is the major phosphorylation site of Rpn2 affected by p38 MAPK. We have not determined the identity of the 47-kDa phosphorylated protein band.

We used anti-phospho–Rpn2 (Thr(P)-273) antibodies to examine the induction of Rpn2 phosphorylation by osmotic

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Proteasome samples that were purified from cells transfected with the p38 MAPK and MKK6EE expression constructs were labeled with iTRAQ-117 and those with an empty vector were labeled with iTRAQ-116. These samples were subsequently mixed in a 1:1 ratio, digested with trypsin, and enriched in phosphopeptides by a TiO2 column. Then the proteolytic phosphopeptide mixture was analyzed by ESI-MS/MS. The lower panel displays a magnified view of the indicated m/z region of the MS/MS, where iTRAQ reporter ions appear. The asterisk denotes ions after a single neutral loss (−H, PO4, 98Da). B, MS/MS of the nonphosphorylated Rpn2 peptide TVGTPIASVPSTNTGP VPEK from iTRAQ-labeled proteasome is shown. Note that the nonphosphorylated Rpn2 peptide containing Thr-311 is shorter than the phosphopeptide shown in D because phosphorylation at Thr-311 blocks further digestion of the peptide by trypsin.
stress. Increased Thr-273 phosphorylation in Rpn2 was detected within 20 min after treatment with 0.4 M sorbitol (Fig. 5D). The induction of Rpn2 phosphorylation was effectively blocked by SB202190, a p38 MAPK-specific inhibitor, indicating that osmotic stress induced Rpn2 phosphorylation via activation of p38 MAPK.

Mutating Rpn2 Thr-273 to Ala Impairs the Inhibition of Proteasome by p38 MAPK—The functional significance of p38 MAPK-mediated phosphorylation at Thr-273 of Rpn2 was investigated by mutating Thr-273 to alanine, which cannot be phosphorylated. We established cell lines that conditionally express activated p38 MAPK by transfecting HeLa cells with the p38 MAPK and MKK6EE expression constructs or with the empty vector as indicated. B, purified proteasome was analyzed for in vitro phosphorylation by activated p38 MAPK. Activated p38 MAPK was obtained by transfecting HeLa cells with FLAG-p38 MAPK and MKK6EE expression constructs. The FLAG-p38 MAPK was purified with anti-FLAG antibody-conjugated beads. A total of 1 μg from each of the proteasome samples was incubated with affinity-purified FLAG-p38 MAPK for 30 min at 30 °C in a kinase reaction buffer with 50 μM ATP. Reaction mixtures were analyzed by immunoblotting with the indicated antibodies. C, Rpn2 is a major proteasome subunit phosphorylated by activated p38 MAPK. In vitro kinase assays were performed with 26 S proteasome samples using radiolabeled ATP. One μg each of proteasome samples was incubated with affinity-purified FLAG-p38 MAPK for 30 min at 30 °C in kinase reaction buffer containing 50 μM ATP and 10 μCi [γ-32P]ATP. Phosphorylated proteasome was visualized by autoradiography. The asterisk indicates a phosphorylated protein band derived from purified p38 MAPK. Reaction mixtures were analyzed by immunoblotting with the indicated antibodies. D, phosphorylated hRpn2 was analyzed by immunoblot after treatment of HeLa cells with 400 mM sorbitol for the indicated times. p38 MAPK was blocked by treating the cells with SB202190 for 1 h before treatment with sorbitol. Cell lysates were separated by SDS-PAGE and analyzed with anti-phospho-Rpn2 (Thr(P)-273) antibodies.
Rpn2 nor expression of p38 MAPK and MKK6EE altered the proteasome composition (Fig. 6A and data not shown). We then examined the effect of activated p38 MAPK expression on peptidase activities of wild-type and mutant proteasome. Three peptidase activities of the 26 S proteasome containing T273A substitution in Rpn2 were slightly higher than those of the 26 S proteasome containing wild-type Rpn2 (Fig. 6, B–D). Although peptidase activities were ~60% for the 26 S proteasome that expressed p38 MAPK and MKK6EE and contained wild-type Rpn2 as compared with those from cells transfected with the control plasmid, peptidase activities were lowered by 10% for the 26 S proteasome containing the T273A mutant Rpn2 and expressing p38 MAPK and MKK6EE (Fig. 6, B–D). These data demonstrate that inhibition of the proteasome activity by p38 MAPK is mainly mediated by phosphorylation at Thr-273 of Rpn2.
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We also investigated the effect of osmotic stress on peptidase activities of wild-type and mutant proteasome. Sorbitol treatment did not grossly affect the proteasome composition (Fig. 6E). Sorbitol treatment reduced three peptidase activities to ~60% for the 26 S proteasome that contained wild-type Rpn2 as compared with those from untreated cells (Fig. 6, F–H). The T273A mutation of Rpn2 protected the peptidase activities of mutant proteasomes from reduction by osmotic stress (Fig. 6, F–H). However, the inhibition of mutant proteasome by osmotic stress was greater than that by activated p38 MAPK. These data suggest that phosphorylation at Thr-273 of Rpn2 plays an important role in inhibition of the proteasome by osmotic stress, and additional factors may be involved in osmotic stress-induced inhibition of the proteasome.

**DISCUSSION**

The p38 MAPK is activated by a variety of cellular stresses and cytokines and influences cellular processes that include inducing inflammation, regulating the cell cycle, and affecting cell survival, differentiation, and senescence (26, 27). We demonstrate in this study that the 26 S proteasome is a direct target of p38 MAPK. Several lines of evidence presented here indicate that p38 MAPK negatively regulates proteasome activity by phosphorylating Thr-273 of Rpn2. First, activated p38 MAPK stabilized both ubiquitination-dependent and ubiquitination-independent model substrates of the proteasome and induced the accumulation of polyubiquitinated proteins in the cell (Fig. 2). Second, proteasome preparations purified from cells expressing activated p38 MAPK had substantially lower peptidase activity levels than the control proteasome samples (Figs. 3 and 6). Third, Thr-273 of Rpn2 was the major phosphorylation site affected by p38 MAPK (Figs. 4 and 5). Finally, the T273A mutation in Rpn2 blocked the p38 MAPK-mediated inhibition of proteasome (Fig. 6). Our data suggest that p38 MAPK-dependent inhibition of the proteasome may play an important role in different biological phenomena involving the p38 MAPK signaling pathway, including the response to osmotic stress. Although the functional significance of proteasome inhibition by hyperosmolarity is not yet clear, the important function of p38 MAPK in adaptive responses to osmotic stress suggests that the p38 MAPK-mediated inhibition of proteasome may protect against osmotic stress by stabilizing one or more currently unidentified regulators.

Post-translational modifications of the mammalian proteasome have been extensively analyzed by mass spectrometry (15, 28–30). These studies identified a number of phosphorylation sites in various subunits of the proteasome. Although several sites are reported to be phosphorylated by specific protein kinases and involved in regulation of the proteasome (19, 30), the function of most phosphorylation sites on the proteasome remains to be determined. Because the proteasome is abundant in the cell and the 20 S CP constitutes ~0.6% of total cellular protein, modifying it to a small extent may not significantly affect the activity of the total pool of proteasomes. We have measured changes in the extent of phosphorylation of the proteasome in response to p38 MAPK activation by combining the iTRAQ method with TiO₂-based selective enrichment of phosphopeptides. Our study shows that a significant fraction of the proteasome pool is modified by phosphorylation at Thr-273 of Rpn2, which is consistent with its importance in the inhibition of proteasomal activity.

The 26 S proteasome degrades protein substrates through a multistep process, which includes recognizing, deubiquitinating, unfolding, and translocating the substrate, and finally hydrolyzing its peptide bonds (3). Because we measured the activity of purified proteasome using small fluorogenic peptide substrates, we could not properly evaluate the effects of p38 MAPK-mediated phosphorylation on the steps of recognizing, deubiquitinating, and unfolding the substrate (31). Inhibiting the 26 S proteasome-dependent degradation of the fluorogenic peptide substrates suggests that phosphorylating Rpn2 may impair downstream steps of the degradation process. These steps may include opening the ATP-dependent gate, which regulates the entry of even small peptide substrates to the catalytic chamber of CP (6, 33). The Rpn2 subunit resides in the base subcomplex of 19 S RP, which contains six distinct ATPases and two other non-ATPase subunits, Rpn1 and Rpn13, in addition to Rpn2. The six proteasomal ATPases form a hexameric ring with a defined arrangement (34), through which unfolded substrates pass. The proteasomal ATPase complex is dynamic and transitions its conformations, although ATP is hydrolyzed (35). The proteasomal ATPases are directly involved in gate opening by transiently associating some of their C termini with the intersubunit pockets present on the α ring of 20 S CP. The conserved C-terminal hydrophobic amino acid-tyrosine-Xaa motif of Rpt2, Rpt3, and Rpt5 appears to serve an important function in the gate opening (31). Because of the lack of information on the atomic scale structure of the 19 S RP, the precise location of the non-ATPase subunits in the base of 19 S RP is not currently known. However, recent studies by purifying biochemically, cross-linking, and using electron microscopy indicate that Rpn2 interacts with Rpt3, Rpt4, and Rpt6 of the proteasomal ATPase complex (32, 36–39). The phosphorylation at Thr-273 of Rpn2 by p38 MAPK may cause a conformational change of Rpn2 in response, which might be transmitted to its interacting Rpt subunits, leading to their C termini inhibiting the gate opening.

In conclusion, we have demonstrated that osmotic stress activates p38 MAPK, which in turn phosphorylates Thr-273 of Rpn2 to inhibit proteasome activity. These results provide new insights into the function of p38 MAPK in response to osmotic stress. Further work will be required to understand the mechanism of proteasome inhibition by Rpn2 phosphorylation and to clarify the role of proteasome inhibition in function of the p38 MAPK signaling pathway.

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