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O-GlcNAc Modification Is an Endogenous Inhibitor of the Proteasome

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Summary

The ubiquitin proteasome system classically selects its substrates for degradation by tagging them with ubiquitin. Here, we describe another means of controlling proteasome function in a global manner. The 26S proteasome can be inhibited by modification with the enzyme, O-GlcNAc transferase (OGT). This reversible modification of the proteasome inhibits the proteolysis of the transcription factor Sp1 and a hydrophobic peptide through inhibition of the ATPase activity of 26S proteasomes. The Rpt2 ATPase in the mammalian proteasome 19S cap is modified by O-GlcNAc in vitro and in vivo and as its modification increases, proteasome function decreases. This mechanism may couple proteasomes to the general metabolic state of the cell. The O-GlcNAc modification of proteasomes may allow the organism to respond to its metabolic needs by controlling the availability of amino acids and regulatory proteins.

Introduction

The proteome is in a dynamic state of renewal and this metabolism must be coordinated with the needs not only of the cell, but the metazoan as a whole. The ubiquitin-proteasome system (UPS) plays a major role in the degradation of the intracellular protein (for reviews, see Glickman and Ciechanover, 2002; Voges et al., 1999; DeMartino and Slaughter, 1999). Not only does it remove damaged proteins, but it also regulates the concentration of short-lived proteins such as cyclins and transcription factors like β-catenin (Aberle et al., 1997) and p53 (Honda et al., 1997). Hence, the UPS is central to the basic mechanisms of cell division, differentiation or death. Proteins destined for degradation are usually tagged with ubiquitin in a temporally and protein-specific manner and then degraded by the proteasome using ATP.

The proteasome is composed of a cylindrical 20S, 670 kDa core particle and a 19S regulatory particle. The core particle is composed of 28 subunits, α and β in type, which are arranged in four stacked heptagonal rings ($\alpha_{(1-7)}\ \beta_{(1-7)}\ \beta_{(1-7)}\ \alpha_{(1-7)}$). It has three distinct peptidase activities: chymotrypsin-like, trypsin-like, and postglutamyl hydrolyzing activities with preference for different amino acid at the P1 position. These catalytic activities each require an N-terminal threonine residue as a nucleophile on their respective β subunit (β5, β1, and β2, respectively) to coordinately cleave proteins (DeMartino and Slaughter, 1999). The core particle alone cannot degrade ubiquitinylated protein substrates. The degradation of protein substrates requires the 19S regulatory particle (PA700), which is 900 kDa in size and is composed of about 17 subunits. The regulatory particle binds to one or both ends of the core particle. Besides opening the core particle channel, perhaps for both entry and exit (Köhler et al., 2001), the 19S cap recognizes and unfolds ubiquitin-conjugated proteins and thereby controls the access of the substrates into the cavity of the core particle. The hexameric ring that contacts the heptameric α ring of the core particle is composed of six different ATPases, which belong to the AAA protein family. Studies with the archaebacterial proteasome demonstrate that ubiquitin-conjugated proteins are unfolded or threaded in a C- to N-direction through the ATPase ring followed by translocation into the channel of the core particle (Navon and Goldberg, 2001) where degradation occurs. Evidence from yeast proteasomes suggests that the six ATPases work coordinately and are not functionally redundant (Rubin et al., 1998). Studies using mutations in the AAA ATPase, Rpt2, have suggested that this ATPase has an opening function for protein access to the core particle (Köhler et al., 2001; Rubin et al., 1998).

It has been suspected that the UPS is under metabolic control. The digestion of muscle fiber by the proteasome is stimulated during starvation (Jagoe et al., 2002), thereby providing the energy stored in amino acids to the animal. The degradation of the transcription factor, Sp1 has been demonstrated to be both proteasome and glucose dependent (Han and Kudlow, 1997; Su et al., 1999). Its proteasomal degradation in cultured cells is inhibited by LLnL and lactacystin and by culture in high glucose or glucosamine (GlcN) (Han and Kudlow, 1997) and conversely stimulated by glucose deprivation and forskolin. Nuclear extract (NE) from glucose-starved/forskolin-treated cells also degrades recombinant Sp1 (Su et al., 1999). An ATPase mutant of Sug1 (Su et al., 2000), one of the six ATPases in the regulatory particle, blocks Sp1 degradation. We have postulated that this coupling of Sp1 proteasomal degradation to metabolic intermediates is part of a mechanism relating macromolecular synthesis to substrate availability. Sp1 is modified posttranslationally by the monosaccharide N-acetylglucosamine (GlcNAc) through β-linkage to serine or threonine hydroxyl groups (Jackson and Tjian, 1988; Roos et al., 1997). O-linked GlcNAc (O-GlcNAc) occurs dynamically on many nuclear and cytoplasmic proteins (Comer and Hart, 2000; Wells et al., 2001; Hanover, 2001). Because the degradation of Sp1 could be blocked by glucosamine, a metabolite in the O-GlcNAc pathway,

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this modification pathway became a candidate for this coupling of the metabolic state to proteasomal function.

The addition of O-GlcNAc to proteins is catalyzed by O-GlcNAc transferase (OGT) (Krepple et al., 1997; Lubas et al., 1997) and its removal by O-GlcNAc-selective N-acetyl-β-D-glucosaminidase (O-GlcNAcase) (Gao et al., 2001). While it is now clearer that OGT plays a role in transcriptional regulation (Roos et al., 1997; Yang et al., 2001, 2002), its role in coupling the proteasome to metabolism has not been thoroughly investigated. In this paper, we show that proteasome functions could be reversibly inhibited by O-GlcNAc modification. The inhibition of the proteasome in susceptible cells may cause the accumulation of proapoptotic factors resulting in apoptosis much as exogenous proteasome inhibitors do (Lee and Goldberg, 1998; Qiu et al., 2000; Pasquini et al., 2000; Adams et al., 1999; Hideshima et al., 2001). The identification of a metabolically coupled endogenous regulatory pathway for proteasomes independent of the ubiquitin system may provide a means for globally controlling this organelle in conditions such as starvation, uncontrolled diabetes, cancer, and inflammation (Goldberg and Rock, 2002; Kisselev and Goldberg, 2001).

Results

OGT Inhibits the Proteasomal Dependent Degradation of Sp1

Nuclear extract from glucose-starved/forskolin-treated NRK cells (activated extract) can replicate the in vivo (Han and Kudlow, 1997) degradation of Sp1 (Su et al., 1999, 2000). The in vivo and in vitro degradation can be blocked by proteasome inhibitors. In vitro degradation of Sp1 occurs as a two-step process (Su et al., 1999) beginning with an endopeptic cleavage between leu^{56} and leu^{57} in Sp1 (Su et al., 1999) (Supplemental Figure S1 available online at http://www.cell.com/cgi/content/full/115/6/715/DC1). A glycine-rich N-terminal peptide, SpX, is generated while the major downstream portion of Sp1 is further degraded. While forskolin-activated nuclear extract could cleave Sp1 (Figure 1A, lane 1), nuclear extract from cells treated with glucosamine was inactive at Sp1 cleavage (Figure 1A, lane 2) (Su et al., 1999). Pretreatment of the active nuclear extract with vaccinia-produced, glutathione bead-bound GST-OGT, but not GST, GST-OGTN286, or GST-OGTN485, completely blocked the generation of SpX (Figure 1B, Supplemental Figure S2 available at above URL). Only the full-length OGT was confirmed to be active using p62 (Han et al., 2000) as a substrate. The addition of exogenous UDP-GlcNAc was not required for the inhibition with vaccinia-produced GST-OGT (Figure 1B, lane 3), but the addition of UDP-GalNAc, an inhibitor of OGT (Haltiwanger et al., 1992), blocked the inhibitory effect of OGT on Sp1 cleavage (Figure 1B, lane 6). Bacterially expressed OGT, also enzymatically active, inhibited the cleavage of Sp1 and this effect was enhanced by the addition of UDP-GlcNAc (Figure 1C, lane 5). The TPR domain of OGT mediates specific protein-protein interactions but, without catalytic activity, failed to inhibit Sp1 cleavage (Figure 1B, lane 9; Figure 1C, lane 4). Catalytically active His-tagged OGT (His-OGT) in this assay also inhibited GST-Sp1 degradation by the active nuclear portion (Figure 1D, lane 2). These results, taken together, indicate that catalysis by OGT and not the TPR domain or tag, interferes with the degradation of GST-tagged Sp1.

The O-GlcNAc State of Sp1 Has No Effect on Its Proteolysis

The activated nuclear extract was pretreated with GST-OGT or His-OGT beads and then these beads were removed before the addition of GST-Sp1, suggesting that the OGT acted upon the proteasome or some component in the nuclear extract, but not on Sp1. Nevertheless, some of the OGT remained, thereby potentially modifying Sp1, an O-GlcNAc protein. To control for Sp1 glycosylation, the GST-Sp1 was expressed under different conditions to generate GST-Sp1 in various states of O-glycosylation. The level of Sp1 O-glycosylation was measured with RL-2, an anti-O-GlcNAc monoclonal antibody (Figure 2B). When the Sp1 is expressed in cells exposed to glucose-free medium, it is only barely detectable with RL-2 (Figure 2B, lane 1), whereas cells exposed to glucose, glucosamine, or streptozotocin (STZ), a non-competitive inhibitor of O-GlcNAcase (Roos et al., 1998; Hanover et al., 1999; Yang et al., 2001), express a similar amount of Sp1 protein that is detectable with RL-2 (Figure 2B, lanes 2 to 4). Nevertheless, the degradation of GST-Sp1 and generation of SpX by the activated nuclear extract were not significantly affected by the initial state of Sp1 O-glycosylation (Figure 2C). Furthermore, the degradation of the variously glycosylated forms of Sp1 could be inhibited equally by the pretreatment of the nuclear extract with GST-OGT on affinity beads (Figure 2D, lanes 1 to 4). The O-glycosylation state of Sp1 does control its entry into transcriptional complexes (Roos et al., 1997; Yang et al., 2001, 2002).

The Effect of OGT on the Peptidase Activity of the Proteasome

Inhibition of Sp1 degradation in nuclear extract by OGT could result from the modification of the proteasome itself, a component of the ubiquitin-conjugation system, or a modifier of the UPS. To simplify these studies, we made use of synthetic fluorogenic peptides that cannot be modified by ubiquitin or O-GlcNAc to measure the effect of OGT on the proteasome peptidases. The chymotrypsin-like activity was measured using the fluorogenic peptide suc-LLVY-AMC (Figures 3A and 3B). When this peptide was incubated with activated nuclear extract, a fluorescent signal was generated (Figures 3A and 3B). The peptidase activity of the nuclear extract was inhibited by multiple proteasome inhibitors, β-lactone, PSI, LLnL, and epoxomicin (Figure 3A), which implies this activity is proteasomal in origin. If the extract was made from cells incubated with glucosamine, only low fluorescence intensity was detected (Figure 3A). When the active extract was pretreated with full-length GST-OGT (Figure 3B) but not GST or GST-OGTN485, the fluorescence intensity was significantly reduced. The inhibitory effect of OGT is as potent as the proteasome inhibitor, epoxomicin (Figure 3B). Because the peptide cannot be modified, the inhibition of its cleavage must

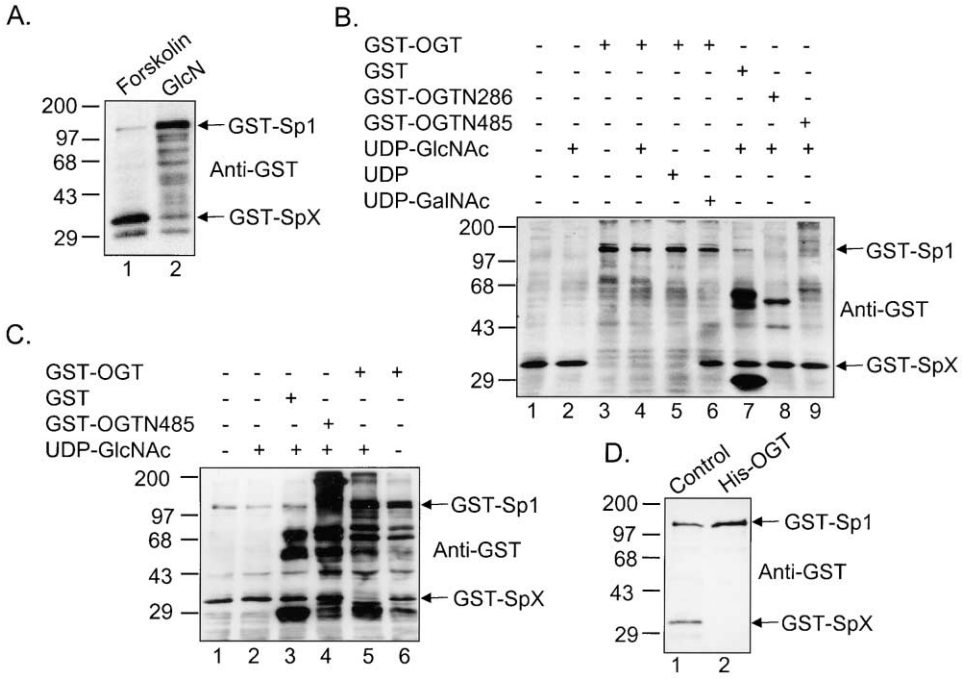

Figure 1. Generation of GST-SpX from GST-Sp1 (degradation) by Active NE Is Inhibited by GST-OGT

(A) Recombinant GST-Sp1 is degraded by activated nuclear extract from NRK cells treated with forskolin and glucose starvation and not degraded by extract from glucosamine-treated cells.

(B) GST-Sp1 degradation, assessed by GST Western blot, is inhibited by GST-OGT but not its truncation mutants produced by vaccinia virus. Nucleotide-sugars (5 μM) were added as indicated.

(C) GST-Sp1 degradation is inhibited by *E. coli*-produced GST-OGT.

(D) GST-Sp1 degradation is inhibited by His-tagged OGT produced with vaccinia virus.

result from modification by OGT of the proteasome or another modifier.

26S but Not 20S Proteasomes Were Inactivated by OGT treatment

To narrow our search for the OGT substrate, we tested if the degradation of the LLVY peptide by purified proteasomes is inhibited by OGT. 26S proteasomes (65% or >95% pure (HP-26S)) (Figure 3C) or 20S proteasomes (> 95% purity) were pretreated with the beads carrying GST alone, GST-OGT, or GST-OGTN485. Treatment of the 65% pure 26S proteasomes with the full-length GST-OGT beads, but not GST or GST-OGTN485, inhibited the degradation of the peptide (Figure 3D). This effect of catalytically active OGT required its substrate UDP-GlcNAc (Figure 3E). However, no inhibition of the 20S proteasome core particles was observed with OGT (Figure 3F), implying that it is not a functional target of OGT.

The Degradation of Tryptic Peptide Substrate Boc-LSTR-AMC Was Not Affected by OGT Treatment

The observation that OGT can inhibit the degradation of complex proteins like Sp1 and simple peptides like LLVY suggests that the effect of the O-GlcNAc modification is very general on 26S proteasome function. To test this generality, the effect of OGT on the trypsin-like activity of proteasomes was examined using a tryptic fluorogenic peptide substrate Boc-LSTR-AMC. Treatment of nuclear extract with GST-OGT was not inhibitory (Figure 4A) nor was it inhibitory for partially purified 26S proteasomes (Figure 4B) and purified 20S proteasomes (Figure 4C). These results suggest that OGT does not inhibit global proteasomal function but modifies some subunit(s) to impart a differential effect on peptide substrate digestion.

The Degradation of Chymotryptic Peptide Substrate Z-GGL-AMC Was Not Affected by OGT Treatment

The experiments indicate that the LLVY chymotryptic activity of the 26S proteasome is inhibited by OGT but the LSTR tryptic activity is not. Furthermore, the activity of the core particle is not modified by OGT. Since the three peptidase activities of the proteasome reside in the core particle, it is unlikely that the proteolytic activity of chymotryptic subunit β5 is functionally modified by OGT. Thus, OGT may alter the chymotrypsin-like activity through another property of LLVY, namely its hydrophobicity (Figure 5A). To test this idea, another chymotryptic peptide was used as a substrate, Z-GGL-AMC. This peptide is as hydrophilic as LSTR (Figure 5A). When exposed to GST-OGT beads, cleavage of GGL by either partially purified 26S proteasomes (Figure 5B) or active nuclear extract (Figure 5C) was unaffected, while its cleavage was blocked by epoxomicin (Figures 5B and 5C), indicating that the chymotryptic cleavage of the GGL peptide can be blocked by exogenous proteasome inhibitors but not by OGT. These results suggest that

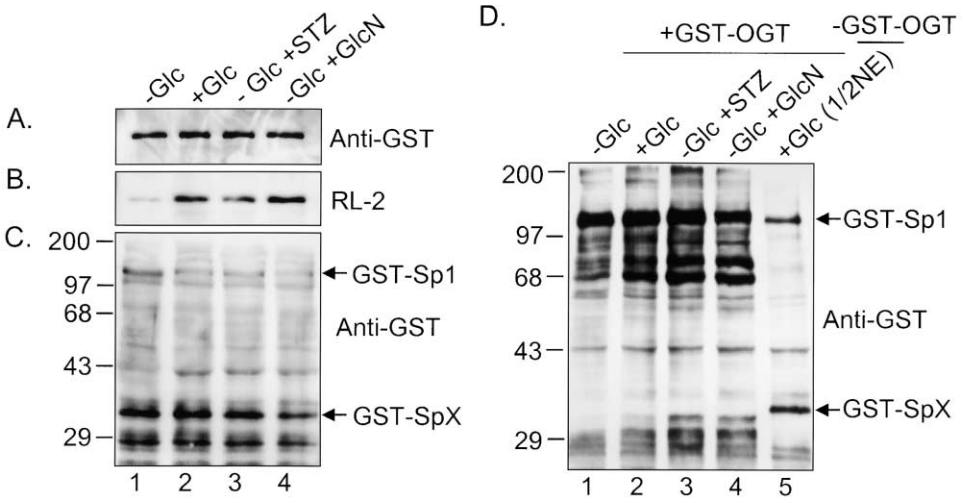

Figure 2. O-GlcNAc State of Sp1 Has No Effect on Its Proteasomal Degradation

The GST-Sp1 was analyzed by Western blot with the indicated antibodies.

(A) Anti-GST blot shows equal loading of GST-Sp1.

(B) RL-2 blot shows O-GlcNAc state of GST-Sp1.

(C) The indicated preparations of Sp1 were added to activated nuclear extract. The appearance of GST-SpX, the first step in Sp1 degradation, was independent of the starting Sp1 O-GlcNAc level.

(D) Degradation of GST-Sp1 with different O-glycosylation levels can all be inhibited by OGT.

the chymotryptic proteolytic activity, which resides in the core particle of the proteasome, is not inhibited by OGT. Rather, the inhibition is peptide specific and appears to correlate with the hydrophobicity of these peptides. Since OGT appears to inhibit the hydrophobic peptidase activity of the 26S proteasome, then we predict that OGT modifies one or more subunits in the 19S cap which might block access of the hydrophobic peptide LLVY into the cavity of the core particle.

The ATPase Activity of the Proteasome Was Inhibited by GST-OGT

The 19S cap of the proteasome contains 6 ATPases. To test whether OGT regulates proteasome function by interfering with the ATPase activity, partially purified 26S proteasomes were exposed to GST-OGT on beads. The release of inorganic phosphate from ATP was measured before and after OGT exposure. While the proteasomes continued to release inorganic phosphate from ATP after exposure to beads, or beads with bound GST, the beads bearing GST-OGT markedly inhibited the ATPase activity of the 26S proteasomes (Figure 5D). To eliminate the possibility of contamination from other more active ATPases in the partially purified proteasomes, more highly purified 26S proteasomes (Figure 3C, lane 2) were exposed to OGT beads. OGT inhibited the LLVY cleavage by this proteasome preparation (Figure 5E). Purity was also confirmed by measuring the relative NTPase activity of the proteasomes before and after OGT treatment. The hierarchy of Vmax was UTP, GTP, CTP, and ATP as previously observed (Hoffman and Rechsteiner, 1996). Again, inhibition of the ATPase activity was observed (Figure 5F), indicating that the inhibitory effect of OGT on the ATPase activity copurified with the proteasomes.

OGT treatment of 26S proteasomes has a differential effect on peptide substrates that correlates with the effect of OGT on the ATPase activities of the 19S cap. To determine if an independent means of inhibition of the ATPase activity results in the same selectivity, the activity of the proteasomal ATPases were inhibited using a noncleavable ATP analog, AMP-PNP. The cleavage of LLVY (Figure 6A), but not that of GGL (Figure 6B) and LSTR (Figure 6C) substrates was sensitive to AMP-PNP treatment, similar to OGT pretreatment. These results suggest that the 19S ATPases control access of hydrophobic but not hydrophilic peptides to the core particle of the proteasome.

Knockdown of OGT with an OGT siRNA Increases Proteasome Function in NRK Cells

To determine if OGT regulates proteasome function in vivo, we used a GFP-tagged proteasome reporter (GFP-degron) (Bence et al., 2001). Cells cotransfected with OGT siRNA, confirmed to suppress OGT expression (Supplemental Figures S3 and S4), displayed 10% stimulation of GFP synthesis (Figure 6D) over cells cotransfected with a scrambled sequence. Since GFP expression is driven by the CMV promoter that contains Sp1 binding sites, it is not unexpected that depletion of OGT would result in less O-GlcNAc modification of Sp1 and therefore more Sp1 activity (Yang et al., 2001, 2002). Nevertheless, a loss of GFP-degron fluorescence was observed in cells transfected with the OGT siRNA, resulting in a net 30% loss of the GFP-degron synthesis over degradation. This observation is compatible with the idea that only a fraction of proteasomes are inactivated by OGT in NRK cells cultured in standard medium. The reduction of OGT would increase the degradation of the reporter GFP-degron in proportion to the starting relative number of OGT-inactivated proteasomes, in this case about 30%.

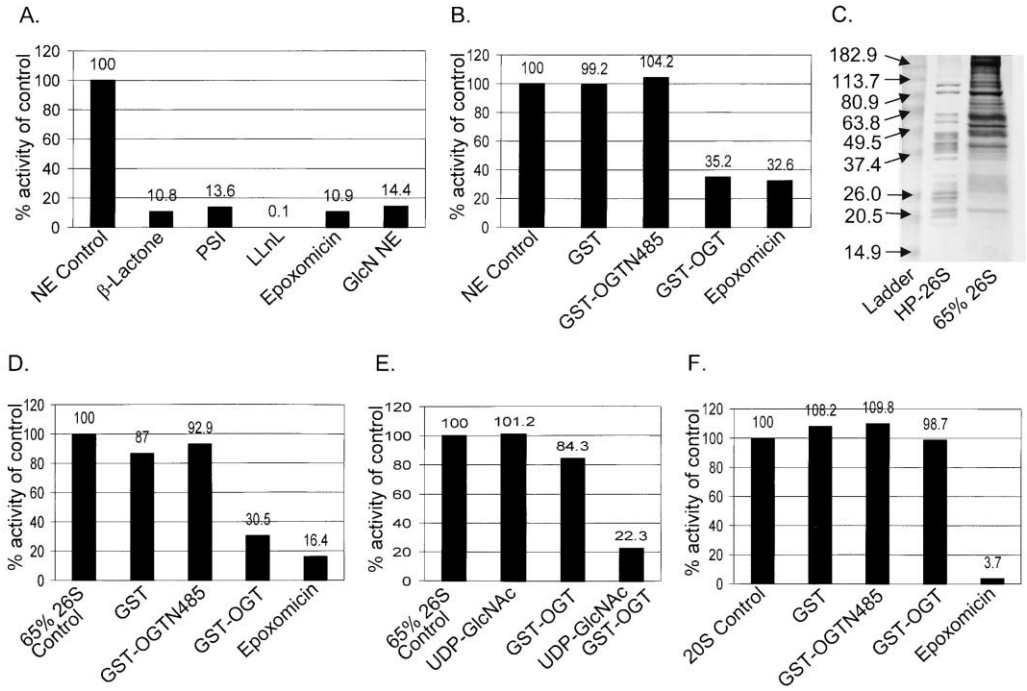

Figure 3. The Proteasome Chymotrypsin-like Peptidase Activity Is Inhibited by OGT Plus UDP-GlcNAc

The fluorescence from the cleavage of suc-LLVY-AMC peptide was measured by fluorometry. The graphs show relative fluorescence with 100% occurring in the absence of inhibitor.

(A) Chymotrypsin-like peptidase activity in active NRK nuclear extract was inhibited by various proteasome inhibitors (50 μM).

(B) Chymotrypsin-like peptidase activity in the active NE was inhibited by GST-OGT and epoxomicin but not inactive OGT truncation mutants.

(C) Commassie blue staining of SDS-PAGE 26S proteasome proteins. The proteasomes were purified to the indicated degree. The HP-26S proteasomes were >95% pure.

(D) Chymotrypsin-like peptidase activity of 65% pure 26S proteasomes was inhibited by catalytically active GST-OGT.

(E) Chymotrypsin-like peptidase activity of 65% pure 26S proteasomes required 5 μM UDP-GlcNAc for inhibition by GST-OGT.

(F) Chymotrypsin-like peptidase activity of 20S proteasome core particles was not affected by GST-OGT.

O-GlcNAcase Activates Proteasome in Inactive NRK Nuclear Extract

Treatment of NRK cells with glucose or glucosamine results in a nuclear extract with a significantly diminished ability to cleave LLVY (Figure 3A). To support the notion that OGT inhibition of this activity is catalytic, physiological, and reversible, we determined if nuclear extract, inactivated in vivo by culturing cells with glucosamine, could be reactivated by O-GlcNAcase, the enzyme that specifically removes O-GlcNAc from proteins. GST-O-GlcNAcase, expressed using vaccinia virus and confirmed to be active (Roos et al., 1998), reactivated the in vivo inactivated nuclear extract while GST did not (Figure 6E). The reactivated chymotryptic activity was sensitive to proteasome inhibitors (Figure 6E), suggesting that the enzyme was activating proteasomes in the nuclear extract.

Further substantiation of this notion was observed on 95% pure proteasomes. These proteasomes were first inactivated by OGT, then treated with the O-GlcNAcase (Figure 6F). The LLVY cleaving activity was restored to 135% of the starting level, indicating not only that the inhibition by OGT is reversible, but also suggesting that the purified proteasomes contained an O-GlcNAc modified inactive fraction prior to OGT treatment. The biochemistry shown below furthers this point.

Subunits of the 26S Proteasome Including Rpt2 Are Modified by O-GlcNAc

Evidence points to the functional substrates of OGT in the 19S cap and the ATPases are reasonable candidates. To determine which of the subunits in the proteasome were modified by O-GlcNAc, we treated the >95% pure 26S proteasomes with OGT and UDP-GlcNAc. The proteins were then resolved by two-dimensional gel electrophoresis and Western blotted with RL-2. While RL-2 detected only GST-OGT (recombinant vaccinia) in the blot of OGT alone (control, data not shown), several spots were apparent in both treated (Figure 7B) and untreated (Figure 7A) 26S proteasome samples. However, OGT treatment caused some spots to become darker. Most spots were proteasomal in origin as they were detectable by Coomassie staining of pure proteasomes run on gels (Supplemental Figure S5). At the 55 kDa, 4.8 isoelectric point coordinate, one spot (perhaps a doublet) changed most dramatically (arrows, Figures 7A and 7B). Its size and predicted isolectric point (http://us.expasy.org/tools/pi_tool.html) correspond to Rpt2, one of the proteasomal ATPases. The blot was stripped and reprobed with an Rpt2 antibody. The spot(s) at this coordinate, identified by alignment markers, appeared as a close doublet; no other spots were detected (Figure 7C). The doublet identified as Rpt2 implies not only that

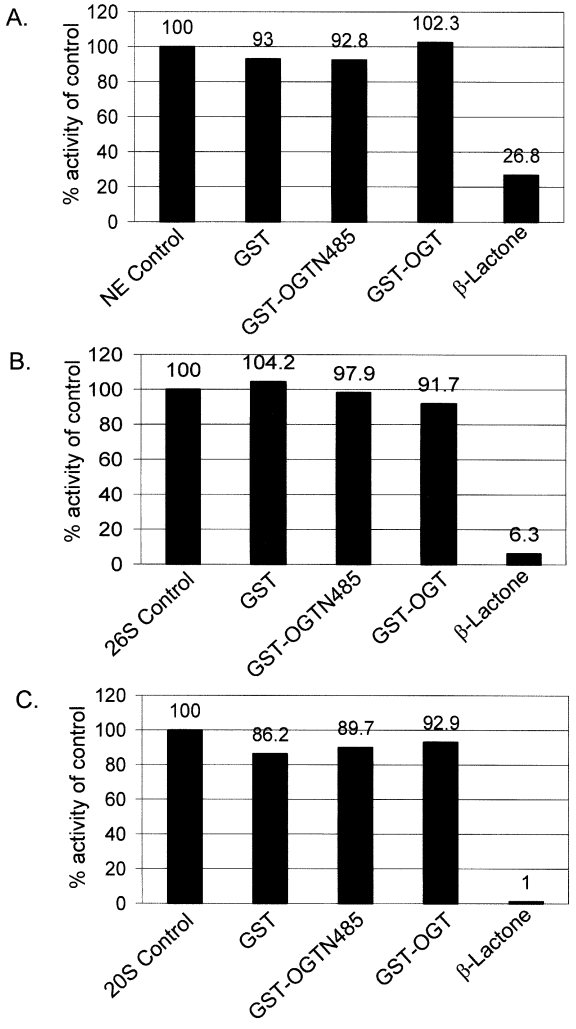

Figure 4. The Proteasome Trypsin-like Peptidase Activity, Measured by Fluorescence from the Cleavage of boc-LSTR-AMC, Was Not Affected by OGT Plus UDP-GlcNAc Treatment

(A) Trypsin-like peptidase activity in the active nuclear extract was not affected by GST-OGT.

(B) Trypsin-like peptidase activity of the 26S proteasomes was not affected by GST-OGT.

(C) Trypsin-like peptidase activity of the 20S proteasomes was not affected by GST-OGT.

it is modified in vitro by OGT in association with a loss of proteasomal functions, but that it may be modified in another way, giving rise to the doublet.

A one-dimensional blot also detected O-GlcNAc modification of a 55 kDa protein (Figure 7D), which was increased following exposure to OGT. The modification of the protein prior to recombinant OGT exposure implies that the modification occurs in vivo prior to proteasome isolation and corresponds to the idea addressed above that proteasomes contain a mixture of inactive O-GlcNAc and active proteasomes. This blot was stripped and reprobed with the anti-Rpt2 serum (Figure 7E). The Rpt2 band aligned with the band detected by RL-2. The Rpt2 antibody was cross-reactive with a bacterial protein in the GST and GST-OGT preparations (Figure 7E, lanes 3 and 4).

To determine if Rpt2 O-GlcNAc modification occurs in vivo under the conditions that inactivate the proteasomal functions, we analyzed the O-GlcNAc state of Rpt2 in activated (forskolin) and inactivated (glucosamine) NRK cell extract. Rpt2 was immunoprecipitated from the cell extracts and analyzed by Western blotting with RL-2. While Rpt2 protein quantity was similar under both conditions (Figure 7G), the Rpt2 from the glucosamine-treated cells showed significantly greater O-GlcNAc modification than that from the forskolin-treated cells (Figure 7F). Again, this modification of Rpt2 correlated inversely with the activity of the nuclear extract to degrade Sp1 and the LLVY peptide.

To determine if Rpt2 is a substrate for OGT in vitro, it was cloned and expressed as a fusion protein with GST. Incubation of this fusion protein with OGT and UDP-GlcNAc indicated that it was a substrate for the enzyme (Figure 7H). Recombinant GST-Sug1 was confirmed not to be a substrate (Su et al., 2000) for OGT nor was Sug2 (data not shown). The O-glycosylation state of the other ATPases is still unclear.

Discussion

The specificity of the proteasome for substrate degradation is classically controlled by the ubiquitin system. Under this model, the proteasome passively degrades ubiquitin-flagged substrates that are presented to it. Here, we describe a global control mechanism at the level of the proteasome itself that couples intermediary metabolism to the activity of the proteasome. Exposure of 26S, but not 20S, proteasomes to OGT reduces the ATPase activity of the intact proteasome, the processing of a hydrophobic, but not hydrophilic peptides, and the proteolysis of at least one of its protein substrates, the transcription factor Sp1. This inhibition of proteasomal function requires the catalytic activity of OGT. Enzymatically dead truncation mutants of OGT containing the TPR domains do not inhibit, UDP-GlcNAc is necessary with pure proteasome preparations, and O-GlcNAcase reverses the inhibition. The modification is present on untreated proteasomes and knock-down of OGT with siRNA in intact cells results in more degradation of a proteasome GFP reporter, suggesting a physiological role for modification. However, only modification of subunits within the19S regulatory particle, which includes the modified AAA ATPase, Rpt2, has a functional effect. This mode of proteasomal inhibition differs from the mechanism by which previously described inhibitors block function. These proteasome inhibitors all attack the proteasome proteolytic subunits within the 20S core particle. OGT, an endogenous inhibitor of proteasomes, acts on the 19S regulatory particle, having no effect on the 20S proteasome. Nevertheless, the action of OGT may be just as global in that the ATPases of the proteasome are vital for its function. The degradation of the ubiquitinylated proteins requires the opening of the 20S particle, probably by Rpt2 (Köhler et al., 2001) and ATP hydrolysis to provide energy to unfold protein and translocate it into 20S core particle (DeMartino and Slaughter, 1999; Rubin et al., 1998; Navon and Goldberg, 2001). The six ATPases form a hexameric ring that abuts the 20S core particle. These ATPase are not functionally

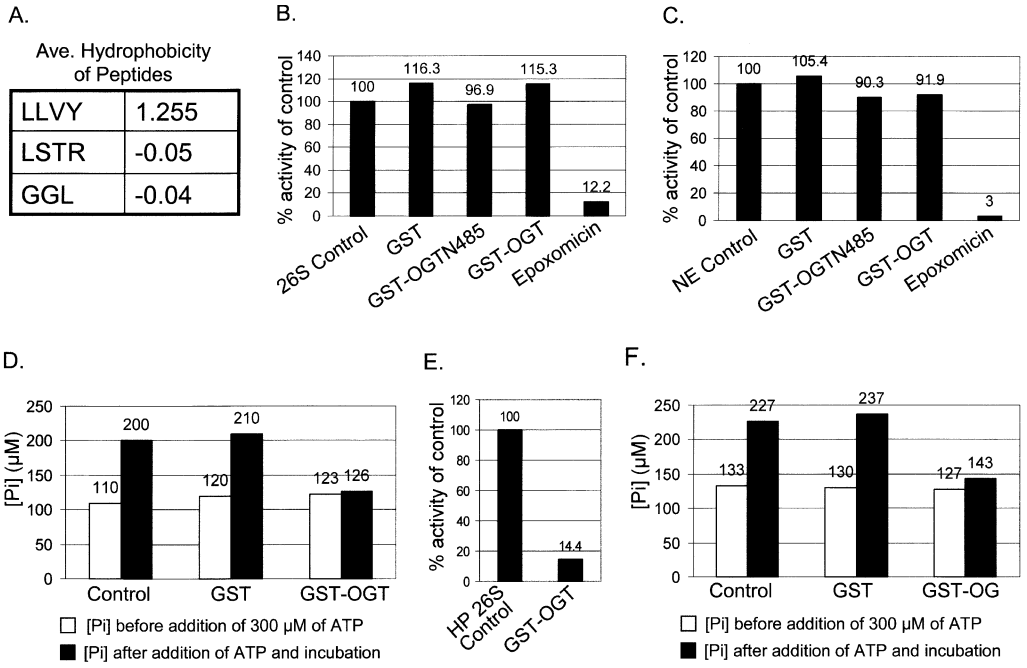

Figure 5. The ATPase activity of the 26S proteasome was inhibited by OGT

(A) Average hydrophobicity score of the proteasome peptide substrates (Sweet and Eisenberg, 1983).

(B) The degradation of chymotryptic fluorogenic peptide substrate Z-GGL-AMC by 65% pure 26S proteasome was not affected by OGT treatment.

(C) The degradation of chymotryptic fluorogenic peptide substrate Z-GGL-AMC by the active nuclear extract was not affected by OGT treatment.

(D) The ATPase activity of 65% pure 26S proteasomes was inhibited by OGT treatment.

(E) Chymotrypsin-like peptidase activity of >95% pure 26S proteasomes was inhibited by GST-OGT.

(F) The ATPase activity of >95% pure 26S proteasomes was inhibited by OGT treatment.

redundant and must act coordinately at the different steps in the degradation process (Rubin et al., 1998; Russell et al., 2001). Since inactivation of any ATPase by mutation (Rubin et al., 1998) or chemical will inhibit proteasome function, OGT inhibition of the ATPase activity of the proteasome predictably inhibits the proteolytic function of the entire organelle. Recently, yeast proteasomes were also shown to be metabolically responsive. In starving yeast cells, the 19S cap with its ATPases dissociates from the core particle, blocking proteasomal function to conserve the energy stored in synthesized proteins (Bajorek et al., 2003). Yeast cells differ from mammalian cells in that they have no OGT and glucose-starvation activates mammalian proteasomes. Furthermore, OGT modification of proteasomes did not result in dissociation (data not shown). Nevertheless, these studies further indicate the importance of the proteasomal cap, with its ATPases, in its function.

While the cleavage of the LLVY peptide was altered by OGT, the effect of this treatment was not observed on another chymotryptic peptide, GGL, nor on a tryptic peptide, LSTR. This observation suggests that OGT does not alter core proteasome function at the level of the specific peptidases. Rather, the common feature appears to be peptide hydrophobicity. One explanation for this selectivity is that the more hydrophilic peptides do not require the opening function of Rpt2 (Köhler et al., 2001) to gain access to the core particle. Another possibility is that Rpt2 serves as a master ATPase. While cooperation between AAA ATPase is arguable (Rubin et al., 1998; Hattendorf and Lindquist, 2002), at least two of the ATPases are not substrates for OGT yet near total loss of proteasomal ATPase function was observed. Since the energy required to unfold proteins is partially applied to the dissolution of intramolecular hydrophobic interactions, the loss of ATPase function due to OGT may affect this unfolding property. Indeed, the same peptide selectivity was observed after broad ATPase inhibition with a nonhydrolyzable ATP analog. Taken together with the finding that Rpt2 is among the OGT modified proteins, the data suggest that the ATPase activity of the proteasome, and particularly that of Rpt2, is inhibited by OGT and this effect blocks the energy exerted in the dissolution of hydrophobic protein folding prior to entry into the proteasome core particle.

OGT has at least 80 substrates (Wells et al., 2003) making generalizations about its cellular function difficult to ascribe. Nevertheless, in certain cell types, the level of modification of several of its substrates appears to be subject to the ambient glucose concentration (Roos et al., 1998; Sayeski and Kudlow, 1996). For example, pancreatic β-cells in vivo increased O-GlcNAc content in response to glucose (Liu et al., 2000). These cells express a glucokinase isoform that limits the entry of glucose into metabolism until its concentration exceeds 5 mM (Matschinsky, 1996). The entry of glucose into muscle and adipose cells is insulin dependent, limiting the availability of glucose to these cells during starvation when insulin levels are low. Other tissues, however, have high affinity hexokinases and transport glucose consti-

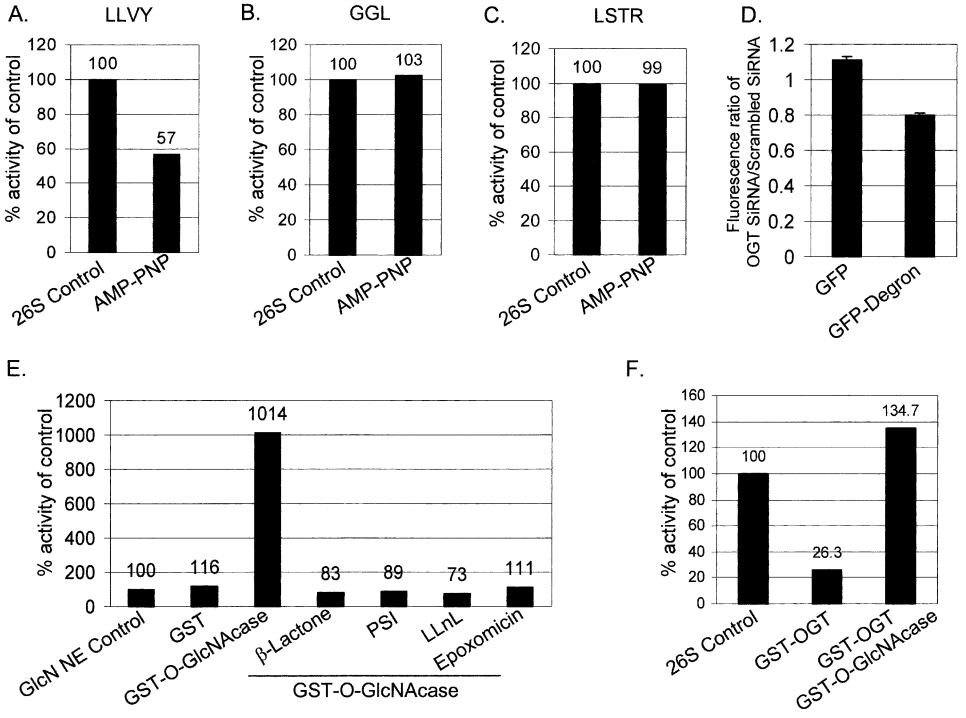

Figure 6. Physiologic Role of O-GlcNAc in Proteasome Function

(A–C) AMP-PNP inhibited the degradation of LLVY (A), but not that of GGL (B) nor LSTR (C). The fluorescence signal was measured as before.

(D) Degradation versus synthesis of GFP-degron in cells cotransfected with OGT siRNA. A net increase in GFP fluorescence was observed in cells cotransfected with OGT siRNA compared to cells cotransfected with a scrambled sequence. When GFP was tagged for proteasome degradation, a net decrease in GFP-degron fluorescence was observed in the OGT siRNA over the scramble transfectants.

(E) The degradation of chymotryptic peptide substrate suc-LLVY-AMC by glucosamine inactivated NE was stimulated by GST-O-GlcNAcase. Ten microliters of inactive nuclear extract was pretreated as indicated. After the GST-O-GlcNAcase on beads was removed, the supernatant was treated with or without 50 μM of the indicated proteasome inhibitor and the peptidase activity was measured.

(F) Chymotryptic activity of purified proteasomes, inhibited by OGT, can be recovered with O-GlcNAcase. The recovered activity was 35% greater than the starting material.

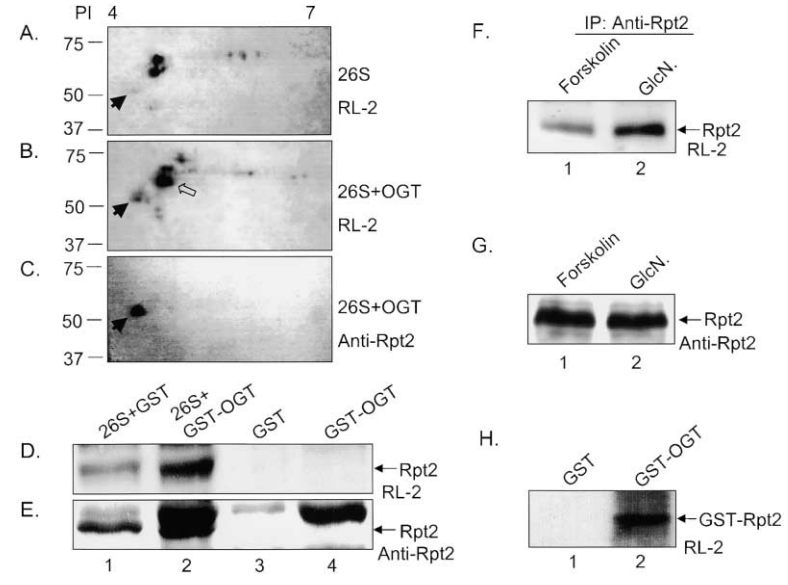

Figure 7. Subunits of Pure Proteasomes Including Rpt2 Were Modified by O-GlcNAc

(A) Untreated proteasome proteins were resolved by two-dimensional gel electrophoresis and then Western blotted with RL-2. All detectable spots are shown.

(B) Proteasomes were treated with OGT and confirmed inactivated (LLVY assay) prior to two-dimensional gel electrophoresis and RL-2 blot. The filled arrow indicates the putative position of Rpt2.

(C) The blot in (B) was stripped and reprobed with an Rpt2 antibody. Rpt2 appeared as a close doublet (filled arrow) at the putative position while the other spots vanished (hollow arrow). Shown here is the same area of the gel shown in (A) and (B).

(D) Partially purified 26S proteasomes were labeled by OGT, and the protein was resolved by SDS-PAGE and blotted with RL-2. No proteasomes were loaded on lanes 3 and 4.

(E) Anti-Rpt2 blot of the stripped membrane from (D).

(F) Rpt2 was immunoprecipated from the cell lysates of forskolin- or glucosamine-treated NRK cells, and the immunoprecipitate was subjected to SDS-PAGE and Western blotting with RL-2.

(G) The Western blot, probed for Rpt2, indicates equal amounts of Rpt2 in the lysate.

(H) Recombinant GST-Rpt2 is a substrate for OGT in vitro. Recombinant Rpt2 was exposed to OGT plus UDP-GlcNAc, then analyzed by Western blot with RL-2.

tutively so that this metabolite is not limiting at normal concentrations of glucose. In tissues that are glucose-sensitive in these ways, this nutritional sensing mechanism of OGT may come into play.

Muscle tissue plays a dual role; one for locomotion and the other for energy storage. The proteasome may play an important role by degrading myofibrils to provide amino acids for energy (Lecker and Goldberg, 2002). Starvation, denervation, sepsis, glucocorticoids, and certain cytokines stimulate the UPS to degrade muscle (Wing and Goldberg, 1993) while insulin inhibits this pathway (Price et al., 1996; Mitch and Goldberg, 1996). The post-absorptive insulin-stimulated flux of glucose into skeletal muscle would provide substrate for OGT, and the substrate-dependent O-GlcNAc inhibition of proteasome function. The cessation of proteasome-mediated amino acid release from muscle receiving the insulin signal would coincide with the decreased need for the gluconeogenesis of the intact organism.

The concentration of several proteins is controlled by proteasomal degradation including oxidized and aggregated proteins as well as several proapoptotic factors like p53 (Chen et al., 2000; Lopes et al., 1997) and bax (Li and Dou, 2000). Because proteasomal function can be modulated by OGT, then those tissues that are glucose-sensitive may display nutritionally controlled proteasome function which could impinge on the apoptotic pathways. Glucose-induced apoptosis has been observed in pancreatic β-cells of certain animal models (Donath et al., 1999). When the O-GlcNAcase enzyme in β-cells is blocked noncompetitively by streptozotocin, glucose- and glucosamine-dependent apoptosis ensues with a failure to reverse the O-GlcNAc modification (Liu et al., 2000). The inhibition of proteasome function would also be associated with the accumulation of oxidized or aggregated proteins. The relationship between this inhibition of proteasome function by OGT and diseases associated with apoptosis and the accumulation of oxidized or aggregated proteins needs further definition to establish causal relationships.

Experimental Procedure

Materials

The partly purified mammalian 26S proteasome fraction (estimated purity > 65%), rabbit 20S proteasome (estimated purity > 95%), and the proteasome inhibitors, Z-Ile-GLu(OtBu)-Ala-Leu-aldehyde (PSI) and Ac-leucine-leucine-norleucinal (LLnL) were purchased from A.G. Scientific, San Diego, CA. The proteasome inhibitor, clasto-lactacystin β-lactone, and substrates N-succinyl-Leu-Leu-Val-Tyr 7-amido-4-methylcoumarin (suc-LLVY-AMC), N-T-Boc-Leu-Ser-Thr-Arg 7-amido-4-methylcoumarin (Boc-LSTR-AMC), forskolin, and UDP-[^{3}H]GlcNAc were purchased from Sigma-Aldrich Inc, St. Louis, MO. The high purity 26S proteasome (>95% pure), the substrate, N$^{\alpha}$-Benzyloxycarbonyl-Gly-Gly-L-Leu 7-amido-4-methylcoumarin (Z-GGL-AMC), and the anti-Rpt2 antibody were purchased from Affinity Research Products, Exeter, UK. The EnzChek Phosphate Assay Kit was purchased from Molecular Probes Inc., Eugene, OR.

Cell Culture and Preparation of Nuclear Extract (NE)

NRK cells were cultured in Dulbecco's modified medium (DMEM) with 10% fetal bovine serum and BSC-40 cells were cultured in DMEM containing 10% newborn calf serum and antibiotics. The forskolin-activated and the glucosamine-inactivated NRK cell nuclear extracts (NE) were prepared as described (Su et al., 1999).

Expression and Purification of Proteins

The following GST fusion proteins were expressed in *E. coli* or in the vaccinia virus system (Moss, 1991; Han and Kudlow, 1997; Su et al., 1999, 2000; Yang et al., 2002) as indicated and purified by affinity chromatography on glutathione beads: OGT, its truncation mutants, O-GlcNAcase, Sp1, p62, hRpt2, hSug1, and hSug2. To alter the glycosylation state of GST-Sp1, it was expressed using the vaccinia system in DMEM containing no glucose, 5mM glucose, 5 mM glucose and 5 mM streptozotocin (STZ), or 5 mM glucose and 5 mM glucosamine.

His-tagged OGT was subcloned into pcDNA3.1 and expressed using vTF7-3 vaccinia virus in BSC-40 cells. The protein was purified on His-Mag agarose beads (Novagen).

p62 was cleaved off the glutathione beads with thrombin. GST-Rpt2 was eluted with glutathione and concentrated with a Microcon 30 (Millipore).

siRNA and GST-Degron Plasmids

To generate OGT siRNA, a double-stranded oligonucleotide with the following upper strand sequence was cloned into pSuper (Brummelkamp et al., 2002): top strand 5′-GATCCCCTGGCATCGACCTC AAAGCATTCAAGAGATGCTTTGAGGTCGATGCCATTTTTGGAAA-3′. The bottom strand began with the sequence 5′-AGCTT…. The scrambled sequence with a top strand as follows was cloned into the same plasmid: 5′-GATCCCCGACATAGCGTAAGCCTATCTTC AAGAGAGATAGGCTTACGCTATGTCTTTTTGGAAA-3′. The GFP-degron plasmid was constructed with the proteasome targeting sequence (Bence et al., 2001) between the XhoI and BhmHI sites of the pEGFP-C1 plasmid (Clontech, Palo Alto, CA). NRK cells were electroporated at 250V, 500 μF with 15 μg of the two plasmids (pEGFP-C1 or pEGFP-degron plus siRNA OGT or scambled in pSuper), then plated on a 15 cm plate for 48 hr. Fluorescence (excitation 470 nm, emission 535 nm) was measured on the microfuge-cleared cell extract (Su et al., 1999) (900 μl) using a Turner Qantech Digital Fluorometer (Barnstead International). The GFP (synthesis) or GFP-degron (synthesis plus degradation) fluorescence of the OGT siRNA transfected cells was divided by the fluorescence from cells transfected with the plasmid containing the scrambled sequence. The assay was performed 6 times.

Sp1 Degradation Assay

The reconstituted Sp1 degradation assay was performed as described (Su et al., 1999). To test the effect of OGT on the degradation of Sp1, 10 μl of forskolin-activated NRK cell nuclear extract was first treated with 10 μl of beads bearing GST-OGT in the presence of 5 μM UDP-GlcNAc at 22°C for 30 min. After the beads were removed, the reaction mixture was incubated at 22°C for another 45 min after the addition of 10 ng of purified GST-Sp1. The degradation of Sp1 was monitored for the generation of GST-SpX by SDS-PAGE and Western blotting using anti-GST antibody (Su et al., 1999).

Proteasome Peptidase Activity Assay

To measure the proteasome peptidase activity in the nuclear extract, 100 μM of one of the fluorogenic peptide substrates, suc-LLVY-AMC, Boc-LSTR-AMC, or Z-GGL-AMC, was added into 10 μl nuclear extract and incubated at 37°C for 90 min. For the purified proteasomes, 5 μg of partially purified 26S, 2 μg of high purity 26S, or 0.1 μg of 20S proteasome was incubated with the peptide substrate in proteasome activity assay buffer (50 mM Tris-HCl, [pH 7.5], 5 mM $MgCl_2$, 10% glycerol, 1 mM ATP, and 1 mM DTT) in a total volume of 30 μl at 37°C for 90 min. The reactions were then stopped with 900 μl of 1% SDS. The fluorescence intensity was measured (excitation 360 nm, emission 450 nm) using a Turner Qantech Digital Fluorometer in triplicate. To test the effect of OGT on the proteasome activity, the nuclear extract or purified proteasomes were first treated with 10 μl GST-OGT beads in the presence of 5 μM UDP-GlcNAc at 22°C for 30 min. After the beads were centrifuged away, the peptidase activity in the supernatant was measured as above. The concentration of proteasome-specific inhibitors was 50 μM whenever they were used. The functional effectiveness of OGT on proteasomes was pretested with suc-LLVY-AMC peptide.

Proteasome ATPase Activity Assay

To test the effect of OGT on the ATPase activity of proteasomes, they were incubated with glutathione beads bearing nothing, GST, or GST-OGT in proteasome assay buffer containing 5 μM UDP-GlcNAc at 22°C for 30 min. After the beads were removed, the reaction mixture was then split into two parts. One part was subjected to phosphate measurement immediately using the EnzChek phosphate assay kit (Molecular Probes) as instructed. The other part was supplemented with 300 μM ATP and the further release of phosphate was measured after an additional 60 min. incubation. For measurement of the Vmax NTPase activity of the proteasomes, 1 mM of each of ATP, CTP, GTP, and UTP was added and phosphate generation was measured from >95% pure proteasomes treated with or without OGT.

Identification of O-GlcNAc Modified Proteins

26S proteasomes (10 μg 65% pure or 6 μg > 95% pure) or 0.1 μg of GST-Rpt2 recombinant protein was incubated with GST-OGT beads and 5 μM UDP-GlcNAc in the OGT activity assay buffer (50 mM Tris-HCl [pH 7.5], 12.5 mM $MgCl_2$, and 1 mM DTT) at 22°C for 90 min. The functional effectiveness of OGT to inhibit suc-LLVY-AMC cleavage by proteasomes was tested. After the beads were spun down, the supernatant was resolved with SDS-PAGE or 2D gel electrophoresis (Proteomics Laboratory, UAB) and subjected to Western blotting.

Immunoprecipitation

After indicated treatments, 2×10^7 NRK cells were collected and resuspended in 100 μl denaturing lysis buffer (1% SDS, 50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 10 mM DTT, 1 mM PMSF, and 15 u/ml DNAase I) and boiled for 5 min. The suspension was then diluted with 0.9 ml nondenaturing lysis buffer (1% Triton X-100, 50 mM Tris-HCl [pH 7.4], 300 mM NaCl, 5 mM EDTA, 0.02% sodium azide,10 mM DTT, and 1 mM PMSF) and incubated on ice for 5 min. Rabbit anti-Rpt2 antibody (8 μl) was added to this lysate and incubated at 4°C for 1 hr. The immunoprecipitate was collected on protein A beads (Amersham) for 1 hr followed by centrifugation, washing 3 times with nondenaturing buffer, and Western blotting using RL-2 antibody.

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