Ubiquitinated proteins promote the association of proteasomes with the deubiquitinating enzyme Usp14 and the ubiquitin ligase Ube3c

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In mammalian cells, the 26S proteasomes vary in composition. In addition to the standard 28 subunits in the 20S core particle and 19 subunits in each 19S regulatory particle, a small fraction (about 10–20% in our preparations) also contains the deubiquitinating enzyme Usp14/Ubp6, which regulates proteasome activity, and the ubiquitin ligase, Ube3c/Hul5, which enhances proteasomal processivity. When degradation of ubiquitinated proteins in cells was inhibited, levels of Usp14 and Ube3c on proteasomes increased within minutes. Conversely, when protein ubiquitination was prevented, or when purified proteasomes hydrolyzed the associated ubiquitin conjugates, Usp14 and Ube3c dissociated rapidly (unlike other 26S subunits), but the inhibitor ubiquitin aldehyde slowed their dissociation. Recombinant Usp14 associated with purified proteasomes preferentially if they contained ubiquitin conjugates. In cells or extracts, adding Usp14 inhibitors (IU-1 or ubiquitin aldehyde) enhanced Usp14 and Ube3c binding further. Thus, in the substrate- or the inhibitor-bound conformations, Usp14 showed higher affinity for proteasomes and surprisingly enhanced Ube3c binding. Moreover, adding ubiquitinated proteins to cell extracts stimulated proteasome binding of both enzymes. Thus, Usp14 and Ube3c cycle together on and off proteasomes, and the presence of ubiquitinated substrates promotes their association. This mechanism enables proteasome activity to adapt to the supply of substrates.

proteasome subunits | Usp14/Ubp6 | Ube3c/Hul5 | proteasome-associated proteins | deubiquitinating enzyme inhibitors

In eukaryotic cells, the 26S proteasomes are composed of the 20S core particle, within which proteins are degraded, and one or two 19S regulatory particles (PA700), which catalyze the binding of ubiquitinated proteins, disassembly of the ubiquitin (Ub) chain, and unfolding and translocation of the substrate into the 20S particles (1–3). Increasing evidence indicates that 26S proteasomes in cells are quite heterogeneous in subunit composition and functional capacity, and a number of alternative forms are known. For example, there exist specialized forms of the 20S core particle that are induced by γ-IFN ("immunoproteasomes") (4–6), and a unique form is present in the thymus ("thymoproteasomes"), which enhances immune function (7). In addition, the 26S particles may contain one 19S regulatory particle plus an additional activator complex, either PA28αβ, the nuclear complexes PA28γ (8–10), or PA200 (11). Whereas the core 20S proteasome contains precisely 28 subunits (14 distinct ones), and the 19S complex contains 19 standard subunits that are present in nearly equimolar ("stoichiometric") amounts, a number of proteasome-interacting proteins are present in lower amounts (12, 13), and several can be easily released by a salt wash (14). In addition, certain proteins become associated with these particles under specific physiological conditions, such as arsenite exposure (15) or muscle atrophy (16), and they enhance proteasome activity.

It is also clear that the binding of ubiquitinated substrates to the proteasome (17, 18) and postsynthetic modifications, especially phosphorylation (19, 20), of specific subunits can stimulate the particles' peptidase and ATPase activities and capacity to degrade ubiquitinated proteins. Moreover, recent cryo-electron microscopy (cryo-EM) studies indicate that these particles exist in multiple states including a substrate-free state and an active form that catalyzes translocation the ubiquitinated substrate (21, 22). In intact neurons, only about 20% of the 26S proteasomes appear by structural criteria to be engaged in substrate degradation (23), and those may differ from most proteasomes in composition. These findings indicate that the enzymatic properties and composition of 26S proteasomes, especially those of the 19S complex, can vary within cells, between cells, and under different physiological conditions. Because the degradation of ubiquitinated proteins involves the sequential actions of different 26S subunits and multiple 26S-associated proteins, changes in proteasome composition can markedly influence rates of proteolysis. Despite their potential physiological importance, the influence of proteolysis rates on 19S composition have never been studied systematically. This heterogeneity of proteasomes is also of medical importance because proteasome inhibitors are now widely used to treat multiple myeloma (24). Pharmacological activation or inhibition of only a subpopulation of proteasomes might allow more selective therapies. In fact, selective inhibitors of immunoproteasomes are being developed as a potential treatment for inflammatory disease (25) and inhibitors of Usp14 for possible treatment for neurodegenerative diseases (26).

The various proteins that are loosely associated with the proteasome may function as cofactors that facilitate degradation of certain types of substrates, such as the "shuttling factors," Rad23 or ubiquilin/PLIC, which deliver ubiquitinated substrates to the 26S (15, 27). Among these proteasome-associated proteins are the deubiquitinating enzyme (DUB) Usp14, the homolog of the

Significance

The efficient degradation of ubiquitinated proteins by 26S proteasomes is critical in maintaining protein homeostasis. Although these particles contain many subunits and associated proteins, it was unclear if proteasome composition changes when rates of proteolysis vary. We studied the proteasome content of two enzymes important in regulating proteolysis: the deubiquitinating enzyme, Usp14, and the ubiquitin ligase, Ube3c. Experiments with cells and isolated proteasomes indicated that levels of Usp14 and Ube3c on these particles correlated with their content of ubiquitinated proteins. Addition of ubiquitin conjugates to extracts stimulated rapid association of both enzymes with the particles. Thus Usp14 and Ube3c continuously cycle on and off proteasomes, and the stimulation of their association by ubiquitin conjugates represents a new mechanism regulating proteasome function.

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yeast Ubp6 and the Ub ligase Ube3c, and the homolog of the yeast Hul5, all of which can promote degradation of certain substrates and regulate 26S function (14, 28–30). Usp14/Ubp6, by disassembling Ub chains and thus preventing proteasomal destruction of Ub molecules, is crucial in maintaining the cellular levels of Ub in yeast (14) and neurons (31, 32). A lack of Usp14/Ubp6 leads to Ub deficiency, which slows the growth of yeast (14) and causes neurological symptoms in mice (33). Recently, Usp14 has been shown to catalyze specifically the removal of Ub chains from substrates that bear multiple Ub chains (34).

Usp14/Ubp6 also has major roles in regulating protein degradation rates and multiple aspects of proteasome function. Depletion of Ubp6 (35) or treating cells with the selective inhibitors of Usp14 (IUI) (26) stimulates the degradation of various mutated proteins because Ub-chain removal by Usp14 can reduce the dwell time of Ub conjugates on proteasomes (34) and lead to substrate release without degradation (26). Usp14 inhibition seems to enhance conjugate degradation by providing the proteasome with greater time for degradation (26, 35). In addition, Usp14 has important noncatalytic actions that allosterically regulate several of the proteasome's catalytic activities. When ubiquitinated substrates bind to the 26S and interact with Usp14, they increase the 26S proteasome's capacity to hydrolyze peptides and ATP (17, 18). Thus, by binding to Usp14, the Ub conjugate promotes the likelihood of polypeptide destruction. Despite its importance, Ubp6 surprisingly has been reported to be present on only a small subset of yeast proteasomes (36), and like Ubp6, Usp14 exists in cells in both proteasome-bound and free forms (37, 38), and these two pools exchange readily (39).

Ube3c/Hul5 is believed to function on the proteasome as an "E4" that elongates Ub chains on 26S-bound substrates, thus increasing their chances of degradation (40). Because Hul5 mutations can suppress the phenotypic effects of Ubp6 deletions (40), Hul5 was proposed to counter substrate deubiquitination by Ubp6 (40). Ube3c/Hul5 also enhances the degradation of misfolded cytosolic proteins, especially upon heat shock (29). Presumably, these actions involve the proteasome-bound form of Ube3c/Hul5 because its expression enhances the processive degradation of difficult-to-degrade substrates (41). Surprisingly, Ube3c also plays an opposite regulatory role. When conjugate degradation is impaired by inhibition of proteolysis, ATP hydrolysis, or the DUB Rpn11 (the 19S metalloprotease that removes Ub chains en bloc) (42), Ube3c catalyzes ubiquitination of Rpn13, which prevents the binding of additional ubiquitinated proteins (43). Presumably, this mechanism prevents the association of a substrate with nonfunctioning particles (43).

In yeast, Ubp6 was reported to bind to Rpn1 and Ube3c to Rpn2 (40), whereas in mammals, both Usp14 and Ube3c were reported to bind to Rpn1 (44). These two enzymes, which can be released from the proteasomes by high concentrations of NaCl (150–300 mM) (14, 45), are present in all cells, but information on their amounts on the proteasome under different conditions is lacking. For example, it is unclear how frequently and under what conditions these factors associate with or dissociate from proteasomes, and if this association is regulated in vivo. To further understand the regulation of proteasomal degradation, the present studies were undertaken to learn how proteasome composition, especially the association with Usp14 and Ube3c, may change when rates of proteolysis in cells vary and also if the composition of proteasomes may change during incubation under conditions commonly used to study their activities. We show here that Usp14 and Ube3c, but not other easily releasable subunits, rapidly cycle on and off a small fraction of the cell's 26S proteasomes and that this association depends on the levels of ubiquitinated proteins.

Results

A Small Fraction of Mammalian Cell Proteasomes Contains Usp14. In yeast, Ubp6 was present on only about 30% of the 26S proteasomes based on mass spectrometry (36). To determine the percentage of proteasomes containing Usp14 in several commonly studied human and mouse cell lines, we prepared soluble extracts by ultracentrifugation at 100,000 × *g* for 30 min and then measured by quantitative immunoblot the amounts of Usp14 that were expressed relative to the amounts of Rpn6, a stoichiometric 19S subunit. The fact that Rpn6 is present in stoichiometric amounts was confirmed in initial control studies by its presence in a constant ratio to the ATPase subunit Rpt5 after proteasome isolation. The absolute amounts of Usp14 and Rpn6 were determined by comparisons to increasing amounts of pure recombinant Usp14 and Rpn6 (Fig. S1*A*, *Bottom*). In these various cells, Rpn6 was three to eight times more abundant than Usp14; thus, in these cells, Usp14 content ranged from one-eighth to one-third that of the content of Rpn6 (Fig. S1*A*, *Top*). [These measurements differ considerably from a prior proteomic analysis of HeLa cells, where Usp14 appeared to be expressed at similar levels as standard 26S subunits (46)]. Upon further centrifugation at 350,000 × *g* for 2 h, nearly all of the proteasomes (i.e., all of Rpn6, Rpn11, and Rpt5) were collected in the pellets, where Rpn6 was five to nine times more abundant than Usp14. These measurements implied that only 11–20% of the 19S complexes contain Usp14 (Fig. 1*A*, *Left*). Also, virtually all of the 19S complexes in these extracts were present in 26S complexes, as shown by native PAGE and Western blot with no free 19S detected (Fig. S1*B*). Furthermore, only about 10–30% of the Usp14 and none of the Rpn6 in the extracts was recovered in the proteasome-free fractions (Fig. 1*A*, *Right*). Thus, although only a small fraction of 19S particles contain Usp14, the great majority of the Usp14 in the cells was bound to proteasomes.

Usp14 and Ube3c Dissociate Rapidly from Purified Proteasomes at 37 °C. Usp14's catalytic activity is activated upon binding to the proteasome (14); however, the rates of association of Usp14 and other loosely associated proteins with the 26S and their dissociation have not been studied. Therefore, we measured during incubation in vitro the proteasomes' content of Ub conjugates, several stoichiometric 26S subunits, and the salt-extractable proteins Usp14, Ube3c, and Ecm29 (14). HEK293 cells stably overexpressing the FLAG-tagged DSS1 (43), a 19S subunit (47–49), were lysed in a buffer widely used to isolate 26S proteasomes (*Materials and Methods*). The soluble extracts were incubated for up to 40 min at 37 °C, and at different times the DSS1-containing proteasomes were purified using a FLAG resin. The components were then analyzed by Western blotting. No clear change was detected in the subunit pattern on SDS/PAGE or in the contents of three standard subunits, Rpt5, Rpn11, and Rpn10 (a receptor for Ub conjugates that also exists free in the cytosol) (Fig. 1*B*). In addition, the amounts of proteasome-bound Usp14, Ube3c, and Ecm29, or the shuttling factor Rad23B, did not change significantly during incubation of these crude extracts (Fig. 1*B*).

Although proteasome composition in the extracts seemed constant, it was possible that the loosely associated proteins may have dissociated from the 26S complex, but were replaced by proteins present in the extracts (39). To test this possibility, we removed cytosolic proteins by affinity purification of the proteasomes and incubated them at 37 °C in the standard buffer (*Materials and Methods*). The proteasome content of the stoichiometric subunits Rpt5, Rpn10, and Rpn11 remained constant during incubation in buffer (Fig. 1*C*). By contrast, a large fraction of Usp14 and Ube3c dissociated from the proteasomes within only 10 min (Fig. 1*C*, *Left*). During this time, the ubiquitinated proteins that were initially bound to the particles had largely disappeared either through degradation or deubiquitination (Fig. S1*C*). By 20 min, half the Usp14 and 75% of the Ube3c had dissociated. Concomitantly, the shuttling factor Rad23B, which facilitates the binding of ubiquitinated substrates to proteasomes (50), also had largely (~75%) dissociated (Fig. 1*C*, *Right*). The decrease in the levels on the 26S of these loosely associated proteins was due to dissociation and not degradation because they accumulated in the buffer (Fig. S1*D*). By contrast,

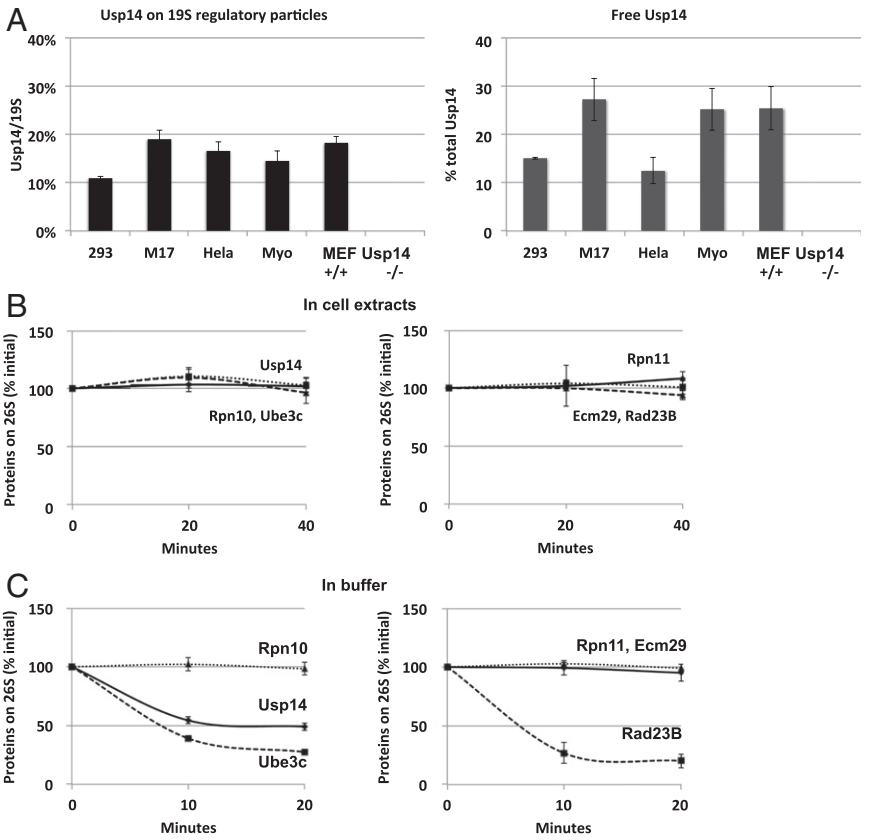

Fig. 1. Only a small fraction of proteasomes contained Usp14 although the majority of Usp14 was recovered in the proteasome-rich fractions. The levels of Usp14 and Ube3c on the particles did not change during incubation of crude cell extracts, but decreased rapidly during incubation in the Hepes buffer containing 10% glycerol, 5 mM $MgCl_2$, and 1 mM ATP at 37 °C. (*A*) Extracts of different cell lines including human embryonic kidney (HEK293), neuroblastoma (M17), epithelial (HeLa), mouse myoblast (Myo), mouse embryonic fibroblast (MEF), and MEF with Usp14 knockout ($Usp14^{-/-}$) lines were centrifuged at 100,000 × *g* for 30 min, and the supernatants ultracentrifuged further at 350,000 × *g* for 2 h to collect proteasome-rich fractions in the pellets. The pellets were then solubilized in buffer containing 25 mM Hepes (pH 7.5), 10% glycerol, 1 mM ATP-Mg, and 5 mM $MgCl_2$ by sonication with four rounds of a 10-s burst and a 50-s rest on ice. Levels of Usp14 and Rpn6 in cell extracts (100,000 × *g*, 30 min) and proteasome-rich or proteasome-free fractions obtained by ultracentrifugation were analyzed by infrared Western blot using Odyssey CLx Imager (LI-COR). Quantification was carried out using Image Studio Software. The amounts of Usp14 and Rpn6 were determined using different concentrations of recombinant Usp14 (rUsp14) and Rpn6 as standards. (*B*) Soluble cell extracts from HEK293 stably overexpressing DSS1-FLAG after ultracentrifugation at 100,000 × *g* for 30 min were incubated at 37 °C for the indicated times. FLAG-tagged proteasomes were then affinity-purified, and levels of proteasome subunits and associated proteins on proteasomes were analyzed as described above. The amounts of individual proteins were normalized to the amounts of Rpt5, a stoichiometric ATPase subunit. Amounts of samples at time 0 were set at 100%. (*C*) Resin-bound FLAG-tag purified proteasomes were incubated at 37 °C in the Hepes buffer for the indicated times. The levels of selected proteasome subunits and associated proteins at each time point were measured as in *A* and *B*. The error bars represent SEMs of four samples in *A* and *C* or three samples in *B*. Similar results were obtained in three independent experiments.

although Ecm29 can also be released readily from 26S (14), in our assay it did not dissociate. Thus, Usp14 and Ube3c are the only loosely bound 26S components that rapidly dissociate under conditions typically used to study proteasome activities (Fig. 1 *B* and *C*). In contrast to the 37 °C incubation, when the affinity-purified proteasomes were incubated at 4 °C in the same buffer for 30 min, the levels of these proteins, as well as Ub conjugates, did not decrease significantly (Fig. S1*E*). Therefore, the dissociation of Usp14 and Ube3c is probably linked to the functioning of the proteasomes. Because no similar decrease in their levels on the 26S occurred upon incubating cell extracts at 37 °C, these enzymes in vivo must continually cycle on and off proteasomes and exchange with cytosolic pools.

The Amounts of Usp14 and Ube3c Bound to 26S Correlate with Levels of Ub Conjugates. An attractive explanation for the rapid loss of Ube3c, Usp14, and Rad23B during incubation in buffer at 37 °C would be that their levels on proteasomes depend on the presence also of Ub conjugates, which are rapidly lost from the particles under these conditions through proteolysis or deubiquitination. To test if the content of these proteins depends on 26S function, HEK293 cells were briefly treated with the proteasome inhibitor, bortezomib (BTZ), which, by blocking degradation, causes ubiquitinated proteins to accumulate on the particles or with an inhibitor of the Ub-activating enzyme E1 (MLN7243) to deplete the cells of Ub conjugates. Both treatments suppress protein degradation dramatically, but they have opposite effects on the levels of Ub conjugates. After BTZ treatment for 30 min, the total levels of Ub conjugates in the cell and on the proteasomes reached twice that of control levels (Fig. 2). By contrast, treatment with the E1 inhibitor for 1 h reduced the 26S-bound Ub conjugates to 5–10% the levels in untreated cells (Fig. 2). As expected, from its role as a shuttling factor (50–52), the amount of Rad23B on the proteasome increased two- to threefold after BTZ treatment, but when Ub conjugates were depleted, no Rad23 was present on the particles (Fig. 2 and Fig. S2). More interestingly, when proteasome activity was inhibited, and Ub conjugates accumulated, about 60–70% more Usp14 and Ube3c ($P < 0.05$) were copurified with the 26S particles. A similar increase in Usp14 content on the 26S had been found upon overnight treatment with a proteasome inhibitor by mass spectrometry (43), but the present finding after 30 min of

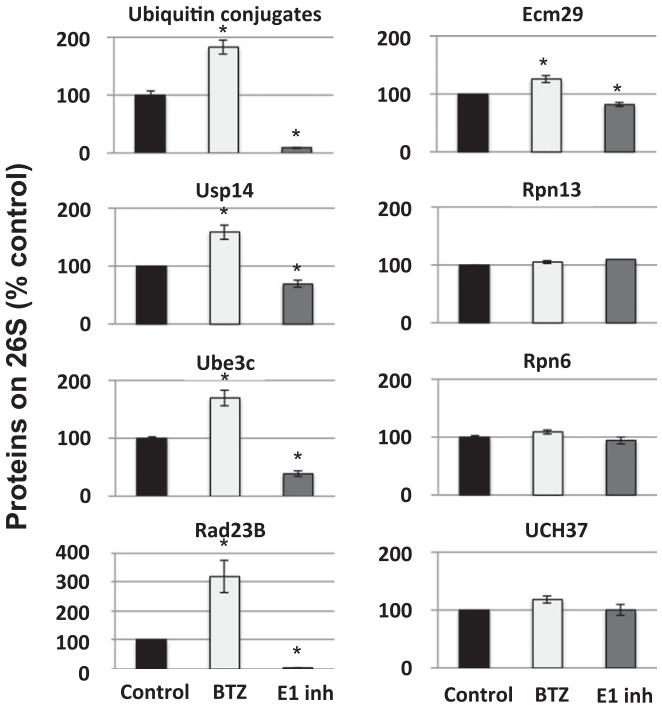

Fig. 2. Inhibition of proteasomal proteolysis in HEK293 cells with BTZ increased, whereas blocking ubiquitination with an inhibitor of the Ub-activating enzyme (E1) decreased rapidly the levels of Usp14, Ube3c, and Rad23B without affecting the levels of stoichiometric subunits (Rpn6, Rpn13, and UCH37) on proteasomes. Cells were treated with 1 μM BTZ for 30 min or 5 μM of the E1 inhibitor (E1 inh: MLN7243) for 1 h. Proteasomes bearing FLAG-tagged DSS1 were then affinity-purified, and levels of selected 26S proteasome subunits or 26S-associated proteins were analyzed and quantified as in Fig. 1. The amounts of proteins in control samples from untreated cells were set at 100%. The error bars represent SEMs of four samples, and similar results were obtained in three independent experiments. *$P < 0.05$ when compared samples of control with BTZ or E1 inhibitor treatment.

BTZ treatment indicate that this recruitment of Usp14 occurs rapidly. Proteasome inhibition also increased slightly (~20%), but reproducibly, the amount of bound Ecm29 and Uch37, whereas there was no significant change in the proteasomal content of the standard subunits Rpn6 or Rpn13 (Fig. 2 and Fig. S2). Conversely, when Ub conjugates were depleted with the E1 inhibitor for 1 h, the amount of Usp14 on the 26S fell by ~30% ($P < 0.05$) and of Ube3c by ~60% ($P < 0.005$). Because both of these inhibitors prevented proteolysis, but had opposite effects on the levels of Ub conjugates on the particles, the binding of Usp14 and Ube3c reflects the levels of Ub conjugates on the particles rather than the rates of conjugate degradation (Fig. 2 and Fig. S2).

Ub Aldehyde Inhibited Usp14 Dissociation and Enhanced Its Binding to Proteasomes. Because Usp14 and Ube3c dissociated rapidly from purified proteasomes at 37 °C, when Ub conjugates were degraded or deubiquitinated, we tested if three different factors known to affect proteasomal activities might influence their dissociation: (*i*) Blocking ATP hydrolysis with ATPγS, which prevents substrate translocation and degradation, slowed the loss of Ub conjugates and reduced partially this dissociation at an early time point (Fig. 3*A*, *Left*, and Fig. S3*A*). (*ii*) Inhibiting deubiquitination by Rpn11 with the Zn-chelator 8-Quinolinethiol slowed the loss of Ub conjugates from the particles (Fig. S3*A*), but it did not prevent the dissociation of Usp14 (Fig. 3*A*, *Right*). (*iii*) Inhibiting the proteasomal peptidase activities with BTZ prevented neither the loss of Ub conjugates nor the dissociation of Usp14 (Fig. 3*A*, *Right*, and Fig. S3*A*). These results suggest that merely maintaining the amount of Ub conjugates on the proteasomes by preventing their degradation or deubiquitination is not sufficient to prevent Usp14 dissociation. By contrast, the presence of Ub aldehyde, an inhibitor and substrate analog of Usp14 (Fig. 3*A*, *Left*), almost completely blocked the dissociation of Usp14. Ub aldehyde not only inhibits Ub conjugate deubiquitination, but also binds to Usp14 and causes a conformational change similar to that induced by substrates (53). Thus, the conformation of Usp14 with the inhibitor Ub aldehyde and presumably with a bound substrate appears to have a higher affinity for the 19S particle (see below). Accordingly, we also found that exposure at 4 °C to buffer containing 300 mM NaCl, which released more than half of the Usp14 from proteasomes, dissociated only 25% in the presence of Ub aldehyde (Fig. S3*C*). In addition to blocking the dissociation of Usp14, Ub aldehyde slowed the loss of Ube3c, but had no effect on the rapid

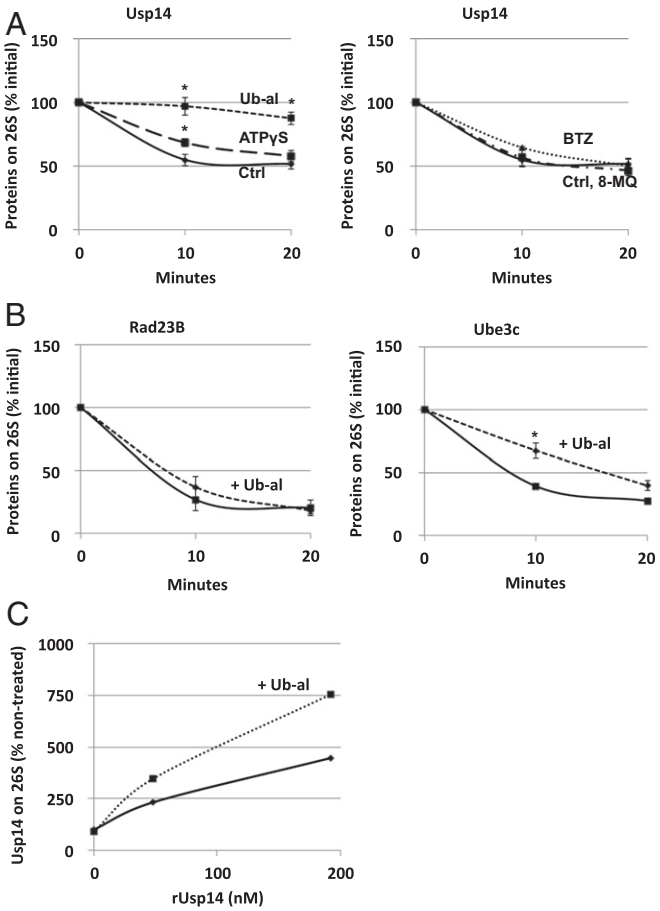

Fig. 3. Ub aldehyde slowed the dissociation of Usp14 and Ube3c, but not Rad23B, from purified proteasomes incubated in buffer at 37 °C and increased the binding of rUsp14 at 4 °C. (*A* and *B*) Resin-bound FLAG-tag purified proteasomes were incubated at 37 °C in buffer for the indicated times. Inhibitors of Usp14 (1 μM Ub aldehyde), of Rpn11 (5 μM 8-MQ), of the proteolytic sites (1 μM BTZ), or 0.5 mM ATPγS, which blocks ATP hydrolysis, were added at time 0. The levels of selected proteins remaining on the particles were measured as in Fig. 1. Protein levels on the proteasome at time 0 were set at 100%. (*C*) Increasing concentrations of rUsp14 were incubated with the FLAG-tag–purified proteasomes with or without 1 μM Ub aldehyde in the reaction buffer for 20 min at 4 °C. After four washes, proteasome-bound Usp14 was analyzed as in Fig. 1. Usp14 content without added rUsp14 was taken as 100%. Similar results were obtained in three independent experiments. The error bars shown are the SEMs of three samples. For Usp14, $P < 0.005$ for compared samples of control and Ub-al at 10 min and 20 min; $P < 0.01$ for compared samples of control and ATPγS at 10 min; and, for Ube3c, $P < 0.005$ for compared samples of control and Ub-al at 10 min.

loss of Rad23B at 37 °C (Fig. 3*B*). Interestingly, ATPγS and 8-Quinolinethiol, which slowed the loss of Ub conjugates, also decreased the dissociation of Ube3c robustly at 37 °C (Fig. S3 *A* and *B*) but not Usp14. Therefore, the dissociation of Usp14 and Ube3c is not tightly linked (Fig. 3 *A* and *B* and Fig. S3*B*).

To test whether Ub aldehyde may also promote Usp14 binding to proteasomes, we assayed the binding of rUsp14 at 4 °C to study binding independently of conjugate degradation. Upon incubation of affinity-purified proteasomes in buffer with increasing concentrations of rUsp14, the amount of 26S-associated Usp14 increased up to fivefold (Fig. 3*C*). Thus, although only a small fraction of proteasomes normally contains Usp14 (Fig. 1*A*), most of these particles can bind Usp14 if the cellular content of Usp14 is increased. If Ub aldehyde is also present, the amount of Usp14 that bound to 26S increases even further, such that more than 75% of the 19S complexes contained Usp14 (Fig. 3*C*). These findings provide further evidence that Usp14 in its inhibitor-bound conformation has a higher affinity for the 26S. Because we found that Ub aldehyde did not bind to Usp14 in the absence of proteasome (Fig. S3*D*), the increase by Ub aldehyde in 26S-bound Usp14 was due to the stabilization of the enzyme on the particles.

Because Ub aldehyde inhibits Usp14, Uch37, and many cytosolic DUBs, its presence maintained higher levels of Ub conjugates in the extracts and on the particles even at 4 °C (Fig. 4). Higher levels of Usp14, Ube3c, and Rad23B were also detected on the proteasomes, whereas the amounts of proteasome-bound Ecm29, Rpn10, and Uch37 were unchanged (Fig. 4). Thus, the association with proteasomes of Usp14 and Ube3c, like Rad23B, again correlated with the levels of ubiquitinated substrates on the particles. Although protein degradation did not occur and BTZ treatment did not cause conjugate accumulation at 4 °C, some deubiquitination of the conjugates occurred and was slowed by Ub aldehyde. Therefore, the amounts of proteins on proteasomes were mostly unchanged in the presence of BTZ (Fig. 4). The relatively greater level of ubiquitinated proteins on the 26S again correlated with the amounts of Usp14 and Ube3c.

Inhibiting Ubiquitination in Cells Reduced the Proteasome's Capacity to Bind Usp14. To clarify the reason for the decreased levels of Usp14 on proteasomes when ubiquitination was blocked, we compared the binding of rUsp14 to proteasomes in cell extracts or purified from control cells or from cells treated with either BTZ for 30 min or the E1 inhibitor for 1 h. When the 26S proteasomes lacking Ub conjugates were isolated, not only did they contain less Usp14 than those from untreated cells (Figs. 2 and 5*A*), but also when these particles were incubated with increasing concentrations of rUsp14, they had a lower capacity to bind rUsp14 than proteasomes from control cells (Fig. 5*A*). Nevertheless, Ub aldehyde increased the amounts of Usp14 that bound to these purified conjugate-deficient particles (Fig. 5*C*). By contrast, proteasomes isolated from BTZ-treated cells, although containing higher amounts of Ub conjugates (Figs. 2 and 5 *A* and *B*), had a greater capacity than those from control cells to bind rUsp14 added to the cell extracts or purified proteasome (Fig. 5 *A* and *B*). These results provide further evidence that Usp14 preferentially binds to proteasomes bearing ubiquitinated substrates and that Ub aldehyde, presumably by mimicry of the substrate, induces a Usp14 conformation with a higher affinity for the particles.

The Usp14 Inhibitors IUl and IU1-47 Promote Usp14 and Ube3c Binding to 26S. Because Ub aldehyde also inhibits Uch37, it is possible that its stimulation of Usp14 binding to 26S (Figs. 3*C* and 5*C*) occurs not simply by its changing the conformation of Usp14, but by an indirect mechanism. Therefore, we tested the effects of the specific small-molecule Usp14 inhibitors, IU1 and its derivative IU1-47, which do not inhibit Uch37 or other DUBs and are cell-permeable and not substrate analogs (26). After treatment of cells with IU1-47 for 2 h or 24 h, the levels of proteasome-bound

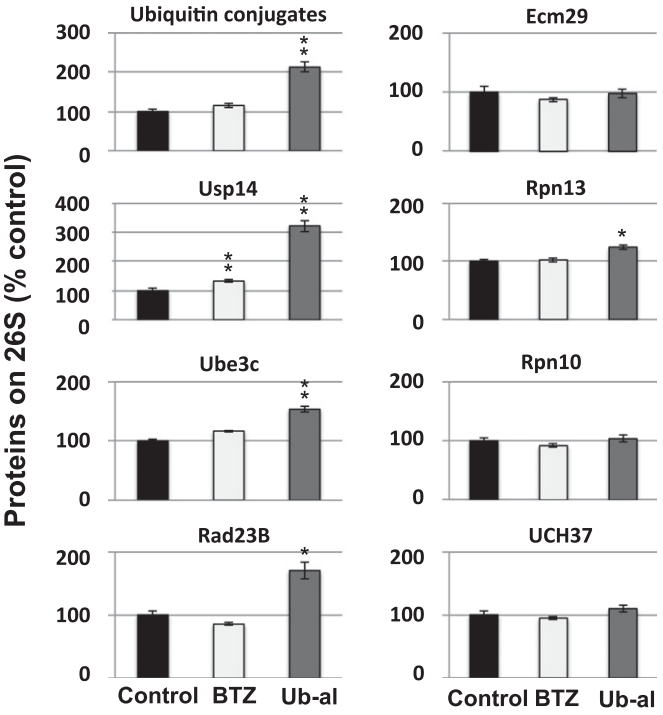

Fig. 4. Addition of the DUB inhibitor Ub aldehyde but not inhibitor of proteasomal peptidase to the cell extracts increased the levels of Usp14, Ube3c, and Rad23B bound to proteasomes; 1 μM Ub aldehyde or 1 μM BTZ was added to the cell extracts after centrifugation at 100,000 × *g* for 30 min and was present during affinity purification of the proteasomes. Select proteasome subunits and associated proteins were detected and analyzed as in Fig. 1. $*P < 0.05$ and $**P < 0.01$ for samples incubated with Ub-al or BTZ compared with controls.

Usp14 increased about twofold (Fig. 6*A*). However, this treatment had no effect on the levels of Uch37 on the 26S (Fig. 6*A*). Furthermore, when IU1 (10 μM) was added to cell extracts depleted of Ub substrates during 26S purification at 4 °C, it increased Usp14 on the proteasomes by 50% within 1 h. Similarly, addition of IU1-47 or Ub aldehyde increased the bound Usp14 by ∼150% (Fig. 6*B*, *Upper*). Surprisingly, under these conditions IU1-47 also increased the amount of proteasome-bound Ube3c twofold, but did not affect Ecm29 (Fig. 6*B*, *Lower*). Thus, cytosolic Usp14 and Ube3c appear to associate with proteasomes in some kind of linked process, depending on Usp14's conformation (see below).

IU1 and IU1-47, like Ub aldehyde, increased the binding of Usp14 and Ube3c to proteasomes several fold (Fig. 6 *B* and *C*). Unlike Ub aldehyde, IU1 and IU1-47 did not slow the dissociation from the 26S of Usp14 or Ube3c at 37 °C (Fig. 6*D*). Presumably, the conformation changes of Usp14 upon binding to Ub aldehyde or IU1 are different. Thus, the factors affecting Usp14 association and disassociation differ and have distinct structural requirements. Although the similar effects of these inhibitors on the binding of Usp14 and Ube3c strongly suggest some linkage of these processes, Usp14 and Ube3c can dissociate separately (Figs. 3 and 6 and Fig. S3*B*). Also, increasing the binding of Usp14 by addition of excess rUsp14 to the extracts did not further enhance Ube3c binding (Fig. S4*A*). By contrast, in yeast, simply supplying Ubp6 stabilized the binding of Hul5 to the proteasome (40). Conversely, IU1-47 not only increased the binding of rUsp14 on proteasomes (Fig. 6*C*), but also enhanced the association of Ube3c with the particles in a rUsp14-concentration–dependent manner (even in the absence of Ub conjugates) (Fig. S4*A*). Prior data showed that the catalytically inactive Usp14 mutant, unlike WT Usp14, tends to bind Ub molecules with high affinity (17). Interestingly, when this rUsp14

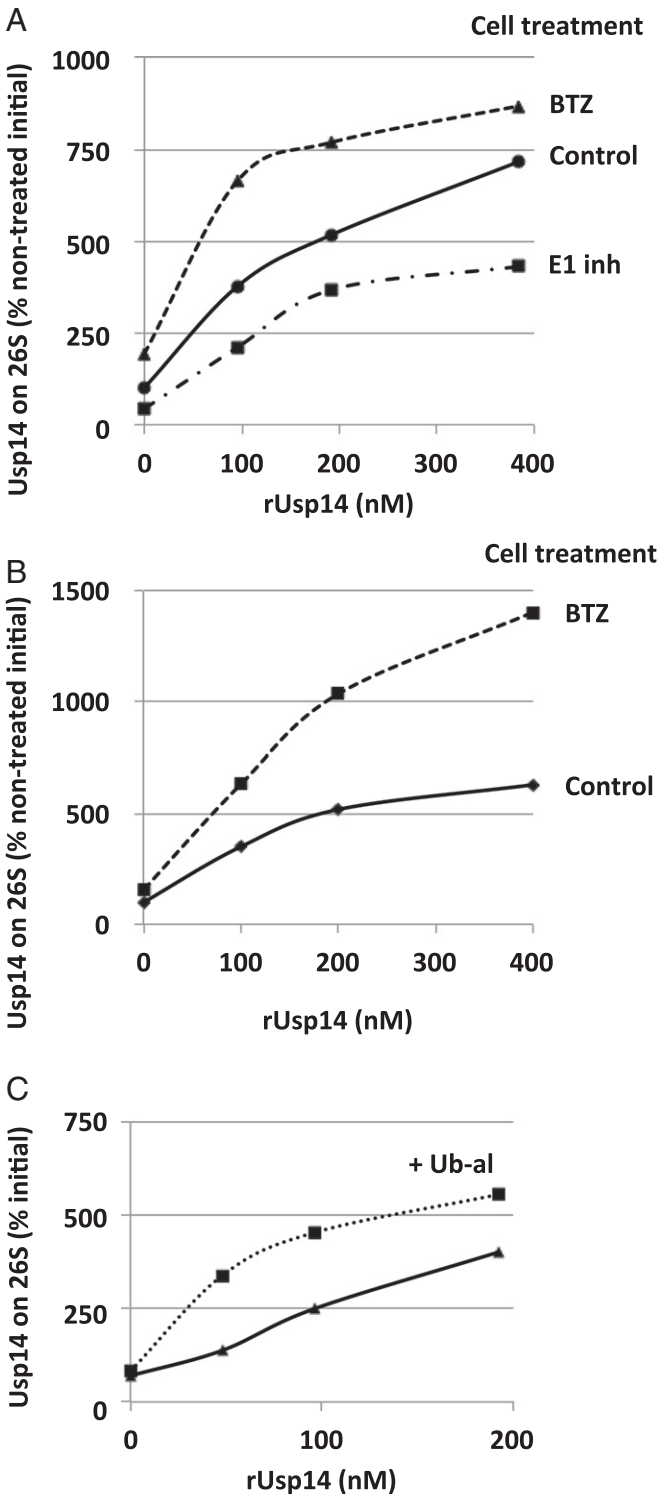

Fig. 5. Proteasomes lacking ubiquitinated proteins (E1 inhibitor treated) showed decreased capacity to bind Usp14 whereas those in cells accumulating ubiquitinated proteins showed increased capacity to bind Usp14, and Ub aldehyde still increased its binding in the absence of Ub conjugates. To deplete proteasomes of Ub conjugates, cells were treated with the E1 inhibitor (5 μM MLN7243) for 1 h, to increase conjugates on 26S, cells were treated with 1 μM BTZ for 30 min, and controls received an equal volume of the vehicle DMSO. (*A*) Increasing amounts of rUsp14 were added to the cell extracts during affinity purification of 26S with FLAG resin at 4 °C. (*B*) Proteasomes were first affinity-purified by the FLAG-tag and then incubated with increasing amounts of rUsp14 in buffer for 20 min at 4 °C. After four washes, proteasome-bound Usp14 was analyzed as in Fig. 1. Control samples without any added rUsp14 were set at 100%. (*C*) Cells were pretreated with E1 inhibitor to deplete them of Ub conjugates. Increasing amounts of Usp14 were then added to the affinity-purified proteasomes as in *B*, and Ub aldehyde (1 μM) was present in the reactions where indicated. Samples without added rUsp14 were set at 100%. Similar results were obtained in three experiments.

mutant was added to the cell extracts, it markedly enhanced the levels of Ube3c on 26S (Fig. S4*B*). Thus, Usp14, when in a substrate or inhibitor-bound form, promotes the association of Ube3c with proteasomes, and the binding of Usp14 and Ube3c seem to occur in a sequential manner. In other words, these observations imply that Usp14 associates rapidly and reversibly with proteasomes, and this association is stabilized by the presence of a substrate or an inhibitor, which alters Usp14's conformation. In this conformation, Usp14 then promotes the association of Ube3c.

Adding Ubiquitinated Proteins Stimulates the Binding of Usp14 and Ube3c to Proteasomes. Because the amounts of Usp14 and Ube3c on the proteasome correlated under all conditions studied with the levels of Ub conjugates on them (Figs. 2 and 4), we tested if providing Ub conjugates to cell extracts could stimulate this association. To prepare ubiquitinated proteins, we allowed the GST-linked Ub ligase E6-Associated Protein (E6AP) (54) or Neuronal precursor cell expressed developmentally down-regulated (Nedd4) (55) to autoubiquitinate (17) by incubating with E1, E2, Ub, and ATP. Then ubiquitinated or nonubiquitinated proteins were added at 4 °C to cell extracts that lacked Ub conjugates (due to E1 inhibitor treatment of the cells). Both ubiquitinated E6AP, which forms K48 chains, and Nedd4, which forms K63 chains, enhanced the amount of Usp14 and Ube3c on the proteasomes two- to threefold within 1 h (Fig. 7). Both ubiquitinated E6AP and Nedd4 bind to proteasomes with high affinity (18, 56). By contrast, nonubiquitinated E6AP and Nedd4 had no effect. Thus, the capacity of 26S to bind both proteins depends on the supply of ubiquitinated proteins even at 4 °C. This stimulation of Usp14 and Ube3c binding was specific, as the level of Ecm29 on the 26S was not altered (Fig. 7). Therefore, the supply of ubiquitinated proteins, which also promotes peptide entry into the 20S (57) and a realignment of the 19S ATPases to an active state (21), promotes the recruitment of these key enzymes to the proteasomes and stabilizes their presence on the particles.

Further experiments explored whether the actions of the Usp14 inhibitor IU1-47 and Ub conjugates were independent. The maximal Usp14 binding was seen in the presence of the IU1-47, and adding the inhibitor and Ub conjugates together did not further increase Usp14 binding (Fig. S5*A*). However, together, this inhibitor and the conjugates had additive effects on the binding of Ube3c (Fig. S5*A*). Thus, Ube3c binding seems to depend both on the conformation of Usp14 and on the presence of a ubiquitinated protein. We therefore tested if Ub conjugates can promote the association of Ube3c in the absence of Usp14. After depleting cells of ubiquitinated proteins, we reduced Usp14 levels in the cell extracts using an anti-Usp14 antibody. The immune-precipitation step lowered total Usp14 by 70% and the amounts of Usp14 bound to proteasomes by 70%, but the levels of Ube3c on 26S were not affected (Fig. S5*B*). When Ub conjugates were added to these extracts, only a small stimulation of Usp14 binding was detected (Fig. S5*B*, *Upper*), but a robust stimulation of Ube3c still occurred (Fig. S5*B*, *Lower*). Thus, the binding of Ube3c to proteasomes can be stimulated by Ub conjugates alone or by Usp14 in its substrate- or inhibitor-bound conformation, and these two stimuli have additive effects.

Discussion

A large number of proteins have been reported to be associated with the 26S proteasome (39, 58), and this study systematically analyzes the conditions that influence this association. We focused

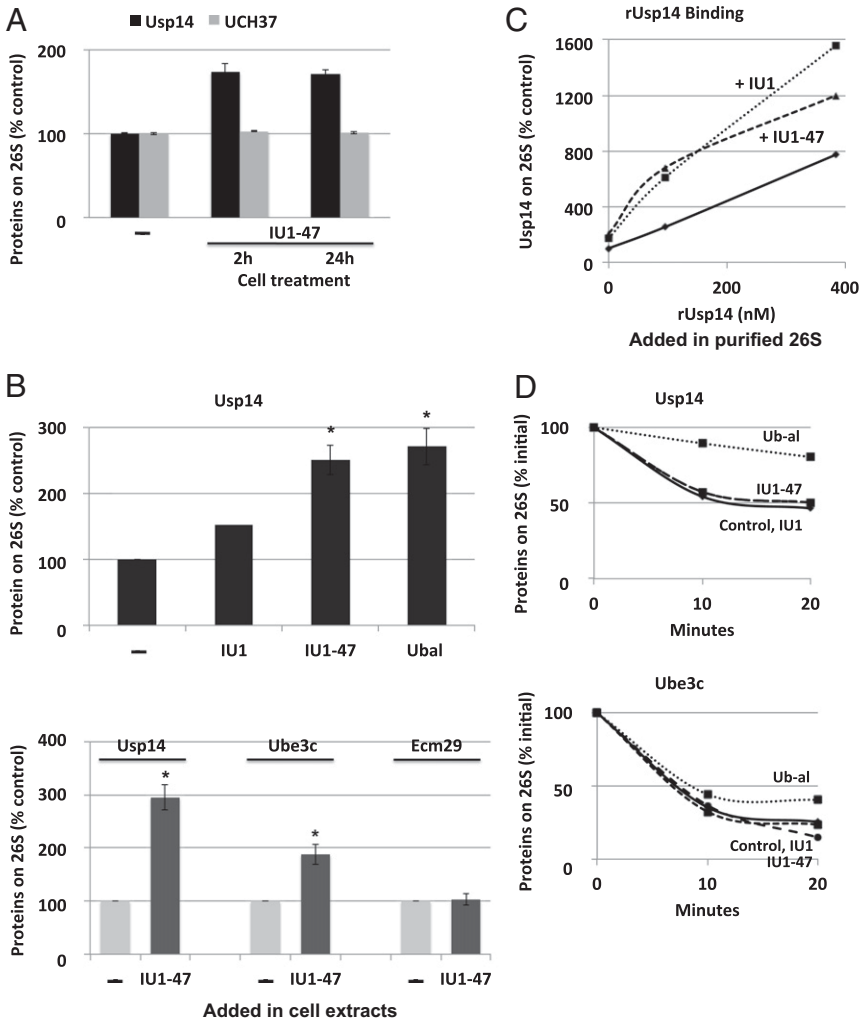

Fig. 6. The specific Usp14 inhibitors IU1 and IU1-47 increased the amount of Usp14 on proteasomes both in cells and in vitro, but did not affect Usp14 dissociation from the proteasomes at 37 °C. (*A*) Cells were treated with 25 μM IU1-47 for 2 h or 10 μM for 24 h (control: DMSO for 24 h) before proteasome purification. (*B*) During proteasome purification at 4 °C, 10 μM IU1, 10 μM IU1-47, or 1 μM Ub aldehyde was added to the cell extracts. (*C*) rUsp14 was incubated with resin-bound FLAG-tag proteasomes in the presence of 10 μM IU1 or 10 μM IU1-47 for 20 min (control: DMSO for 20 min). (*D*) A total of 10 μM IU1, 10 μM IU1-47, or 1 μM Ub aldehyde was added to resin bound FLAG-tag–purified proteasomes for 5 min at 4 °C before incubation at 37 °C in buffer for the indicated time. Levels of proteins on purified proteasomes were analyzed as in Fig. 1. Samples from untreated control cells were set at 100% in *A* and *B*. In *C*, samples without added rUsp14 were set at 100%, and in *D* samples at time 0 were set at 100%. The error bars shown in *A* and *B* are the values of SEMs of four samples. Amounts of Usp14 after addition of IU1 in *B* were the average of duplicates. Similar results were obtained in three independent experiments.

on the binding to the 19S regulatory particle of Usp14 and Ube3c because of their major roles in removing or extending Ub chains (14, 30) and because of Usp14's important allosteric effects in controlling the proteasome's degradative capacity (17, 18). In addition, the presence of Usp14 on the proteasome and its interaction with substrates markedly influences the conformation of the 19S particles (36, 59). As shown here, these enzymes, unlike the stoichiometric 26S components and the easily extractable 26S proteins ECM29 or Uch37, continuously cycle on and off these particles. Although the proteasomal levels of several other interacting proteins, including Ecm29 and four E3 ligases, increase after prolonged inhibition of proteolysis (43), these proteins were not found to rapidly cycle on and off the proteasomes.

Most importantly, unlike other 26S-associated proteins tested, Usp14's and Ube3c's association with the 19S depends on the supply of ubiquitinated proteins. In both cells and extracts at 4 °C, increasing the amount of ubiquitinated substrates on the 26S by treating cells with BTZ not only led to greater amounts of Usp14 and Ube3c on the particles (Fig. 2), but also enhanced their capacity to bind additional Usp14 molecules (Fig. 5). Moreover, this effect is due to the presence of more Ub conjugates on the proteasomes (Fig. 4). Accordingly, two prior studies had noted increased proteasome content of Usp14 and Ube3c (43) and more Usp14 activity (37) after prolonged proteasome inhibition. Conversely, when ubiquitination was inhibited, proteasomal Ube3c decreased rapidly, and these particles, nevertheless, showed a reduced capacity to bind Usp14. However, depleting Ub conjugates in cells or loss of bound conjugates from incubated proteasomes only partially reduced the association of Usp14 with the 26S (Figs. 1*C* and 2). Thus, the levels of Usp14 and Ube3c on the 26S are probably regulated by additional factors aside from the supply of ubiquitinated proteins.

Because Ub conjugates induce peptide entry into the 20S (57) and activate the 19S ATPases (18), the ability of ubiquitinated proteins to stimulate the association of these key enzymes with the particles most likely serves to enhance the efficiency of proteolysis. Prior studies had indicated that the presence of ATPγS stimulates substrate entry (60), promotes Ub conjugate binding (57), and

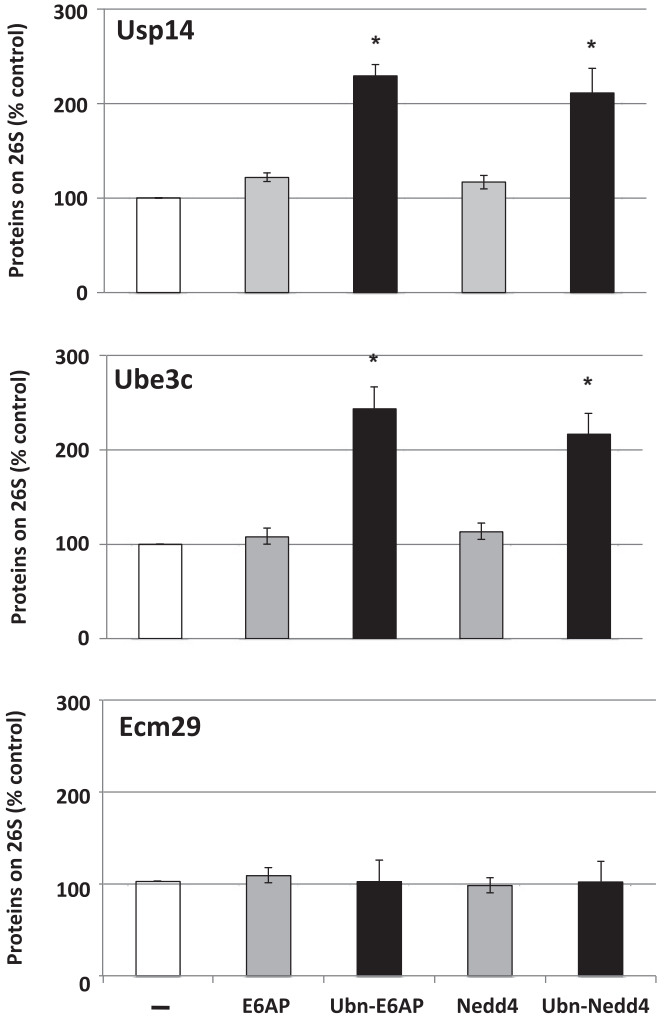

Fig. 7. Addition of Ub conjugates to cell extracts at 4 °C increased the amounts of Usp14 and Ube3c but not of Ecm29 on proteasomes. During affinity purification of FLAG-tagged proteasomes at 4 °C, 105 nM GST-E6AP, ubiquitinated GST-E6AP, GST-Nedd4, or ubiquitinated GST-Nedd4 was added to study the effects of added Ub conjugates. All cell extracts were prepared from cells pretreated with 5 μM of the E1 inhibitor for 1 h as in Fig. 2. Levels of proteins on purified proteasomes were analyzed as in Fig. 1. Control samples with no addition were set at 100%. The error bars shown were the values of SEMs from four experiments. $*P < 0.05$ when samples with or without added conjugates were compared.

induces large structural changes in the 19S that mimic the active state of the particles (21). With ATPγS present, we also found higher levels of Usp14 and Ube3c on the proteasomes (Fig. S6).

The similar effects of Ub aldehyde, IU1, and Ub conjugates indicate that Usp14 has a higher affinity for the proteasomes when in the substrate- or inhibitor-bound conformation. Accordingly, the active site (C to A) Usp14 mutant, which binds Ub tightly and thus structurally mimics the active form of Usp14 (17), bound to proteasomes more readily than WT Usp14. Also adding Ub aldehyde decreased the ability of high salt buffers to release Usp14 from the particles (Fig. S3*C*). These observations are consistent with our finding that Ub aldehyde slows the dissociation of Usp14 and enhances the binding of rUsp14. Recently, two groups using cryo-EM and cross-linking also noted that Ub aldehyde could stabilize the association of Ubp6 with proteasomes (36, 59). They confirmed that Ubp6 binds to Rpn1 (61), but when a substrate or analog occupies its active site, it also interacts directly with Rpt1, one of the six 19S ATPases. In this substrate-engaged, translocation-competent state, Ubp6 contacts the ATPase ring, which becomes coaxially aligned with the gated entry channel into the 26S particle. This interaction with Rpt1 may stabilize the association of 19S and 20S complexes (59) and probably explains how Ub conjugate or Ub aldehyde binding to Usp14/Ubp6 stimulates substrate entry into the 20S and also ATP hydrolysis, provided a loosely folded protein is also present (18). More extensive high-resolution cryo-EM studies of human 26S show that when Usp14 binds Ub aldehyde, it interacts with both Rpt1 and Rpt2 (62). Like Ub aldehyde, IU1 and its derivative IU1-47 enhance the association of Usp14 and Ube3c with the 26S, but, unlike Ub aldehyde, the small molecules (26) increase Usp14 binding, but do not slow its dissociation from the particles. Thus, IU1 and IU1-47, which are not substrate analogs, must influence Usp14 conformation differently from Ub aldehyde.

It is unclear exactly how these changes in proteasomal content of Usp14 and Ube3c alter the degradation of different types of ubiquitinated proteins. This movement of Usp14 onto the proteasomes may have other regulatory effects in the cell in addition to altering 26S function. Surprisingly, Usp14 has recently been shown to also influence rates of autophagy (63), and cytosolic Usp14 was found to be phosphorylated and activated by Akt (64) [although we have not seen significant changes in Usp14 distribution upon Akt inhibition (Fig. S7)].

Our results in several mammalian cells, like those in yeast (36), show that typically only about one or two Usp14 molecules are found on every ten 19S complexes isolated by differential centrifugation. The findings that Usp14 preferentially binds to the proteasomes that carry Ub conjugates and that Usp14 is present on only a small fraction of 19S particles strongly suggests that the proteasomes bearing Usp14 and Ube3c are the ones actively digesting ubiquitinated substrates. The simultaneous accumulation on the proteasomes of Ub conjugates, Usp14, Ube3c, and Rad23B when proteolysis was inhibited and their dissociation when conjugates are digested are further indications that all these proteins are present together on the substrate-engaged particles. The presence of Usp14/Ubp6 on only a small fraction of 26S is difficult to reconcile with its major role in Ub recycling and preventing Ub deficiency in yeast and neurons (14, 31, 32). However, these results are anticipated if the Usp14-bearing particles are in fact the ones most active in degrading ubiquitinated proteins. Recently, Baumeister and coworkers reported that only about 20% of the 26S proteasomes in hippocampal neurons are in the substrate-processing conformation and thus appear to be engaged in proteolysis (23) and that most likely these proteasomes correspond to the fraction bearing substrate, Usp14 and Ube3c.

The present findings imply further that Usp14 and Ube3c remain on the proteasomes as long as they are in their active conformation, i.e., until the Ub chain is released or is hydrolyzed, as was also suggested recently (59). After the initial reversible binding of Ub conjugates to the 19S particles, there is an ATP-dependent transition to a tighter binding, which appears to commit the substrate to proteolysis (57). In our studies, supplying ubiquitinated proteins increased the binding of Usp14 and Ubc3c at 4 °C, where this transition to tighter binding cannot occur. Therefore, the Ub conjugates must be promoting proteasomal binding of Usp14 and Ube3c when the conjugates are in the loosely bound state before they are committed to degradation.

Like the levels of Usp14, the amount of Ube3c on the proteasomes correlated with their content of Ub conjugates and Rad23. Accordingly, in yeast, the association of Hul5 with proteasomes is stabilized by Ubp6 (40). Surprisingly, we found here that the binding of Ube3c was stimulated in two ways: (*i*) its association with the 26S depended on Usp14's conformation because it increased in the presence of Usp14 inhibitors and (*ii*) the binding of Ube3c can also be directly promoted by Ub conjugates [e.g., in cells deficient in Usp14 (Fig. S5)]. Most likely, after a ubiquitinated

substrate binds to the proteasome, these two mechanisms function together to stabilize the association with Ube3c. Together, these results also imply that Usp14 and Ube3c bind to proteasomes sequentially. Most likely, Ub conjugates bind first and then Usp14's weak (readily reversible) association with the proteasome is stabilized by its interaction with the bound substrate (or a Usp14 inhibitor). Then, in this substrate- or inhibitor-bound conformation, Usp14 promotes the association with Ube3c.

Inhibitors of Usp14, like IU1, were proposed to stimulate the degradation of certain substrates by inhibiting their deubiquitination and thus reducing substrate dissociation from the particle (26). However, by slowing Ub-chain digestion, such agents also maintain Usp14 in the substrate-bound conformation longer and thus prolong the activated state of the 26S, which favors conjugate degradation (17, 18). In addition, our data suggest that IU1 and IU1-47 may enhance degradation of certain substrates indirectly by increasing rapidly the amount of Ube3c on the particles. Ube3c/Hul5 enhances the processivity of the proteasome (30, 41), which must be particularly important to promote degradation of difficult-to-unfold, multidomain substrates and heat-damaged aggregated proteins (29, 65). Whatever its pharmacologic implications, the unexpected ability of ubiquitinated proteins to stimulate Usp14 and Ube3c binding represents a mechanism controlling the proteasome's degradative capacity in cells and linking proteasome function to substrate supply.

Materials and Methods

Cell Culture, Lysis, and Extract Preparation. Cells were cultured in Dulbecco's Modified Eagles Medium (Westnet) with 10% FBS (Sigma). Cell treatments were performed when needed. Ninety-percent confluency cells received BTZ (1 μM) for 30 min, E1 inhibitor MLN7243 (5 μM) for 1 h (Active Biochem; another E1 inhibitor, ML00603997, was also used at 10 μM and similar results were obtained), IU1-47 (25 μM) for 2 h, or IU1-47 (10 μM) for 24 h. After a PBS (Lonza) wash, cells were resuspended in lysis buffer that was widely used for proteasome purification with some modifications (43). The modified lysis buffer contains 25 mM Hepes (pH 7.5), 10% glycerol, 1× protease inhibitor mixture (Roche), 1 mM ATP-Mg, and 5 mM $MgCl_2$. Cells were sonicated with four rounds of a 10-s burst and a 50-s rest on ice. After sonication, cell debris was cleared by centrifugation at 10,000 × *g* for 15 min, and the supernatants were further centrifuged at 100,000 × *g* for 30 min. The resulting supernatants (S100) were then used as cell extracts for proteasome preparations.

Proteasome Purification by FLAG Resin. FLAG resin (ANTI-FLAG M2 Affinity Gel, Sigma) was added to cell extracts after centrifugation at 100,000 × *g* for 30 min and incubated at 4 °C for 1 h to pull down proteasomes with FLAG-DSS1. After four washes with standard buffer (same as lysis buffer with protease inhibitor mixture omitted), resin-bound proteasomes were eluted with 200 ng/mL of 3× FLAG peptide (Sigma) in the standard buffer. When indicated, 1 μM Ub aldehyde (Boston Biochem), 1 μM Bortezomb, 0.5 mM ATPγS, 10 μM IU1, or 10 μM IU1-47, ∼105 nM GST-E6AP or Ub(n)-GST-E6AP and GST-Nedd4 or Ub(n)-GST-Nedd4 was added to the cell extracts during proteasome pull down at 4 °C.

Protein Dissociation from the Proteasomes. Cell extracts (after centrifugation at 100,000 × *g*, 30 min) were incubated at 37 °C for up to 40 min before proteasome purification with FLAG resin. In buffer, resin-bound proteasomes were incubated at 37 °C in standard buffer for 0–20 min. After 4 washes, proteasomes were eluted, and proteins on the proteasomes were analyzed by Western blotting. Protein contents were normalized to the amounts of Rpt5 (a stoichiometric 19S subunit) in each sample, and the amounts of samples at time 0 were set at 100%. When indicated, 1 μM Ub aldehyde (Boston Biochem), 5 μM 8-Quinolinethiol hydrochloride (8-MQ, Sigma), 1 μM Bortezomib, 0.5 mM ATPγS, 1 mM NEM (Sigma), 10 μM IU1 or 10 μM IU1-47 was added to proteasomes 5 min before incubation at 37 °C.

Recombinant Usp14 Binding to Proteasomes. Cells were pretreated with 5 μM E1 inhibitor for 1 h or with 1 μM Bortezomib for 30 min (control: DMSO for 1 h) before proteasome purification to reduce or increase Ub conjugate levels on the proteasome. Increasing concentrations of rUsp14 (0–400 nM) were added to the cell extracts (1 h) or were incubated with resin-bound proteasomes (20 min) at 4 °C. After four washes, proteasomes were eluted and Usp14 was analyzed by Western blotting. When indicated, 1 μM Ub aldehyde, 10 μM IU1, or 10 μM IU1-47 was present in the reaction.

Western Blotting. Purified proteasomes were applied to NuPAGE Novex 4–12% Bis-Tris Protein Gels (Life Technologies) and transferred to nitrocellulose membranes (VWR). Bound Ub conjugates, proteins, and proteasome subunits were analyzed by Western blotting using specific antibodies: FK2, Rpt5, and MCP231 (Enzo Life Sciences); P4D1, Rpn2, and Usp14 (Santa Cruz); Uch37 (Epitomcis); Rpn13 and Usp14 (Abcam); Ecm29 (Thermo Fisher Scientific); β5, Usp14, Ube3c, and Rad23B (Bethyl Laboratories); Rpn11 and Rpn6 (Cell Signaling); and IRDye 800CW donkey anti-rabbit IgG and IRDye 680LT goat anti-mouse IgG (H + L) (LI-COR). Signals were analyzed by using Odyssey CLx Imager (LI-COR). Quantification was performed using Image Studio Software.

Protein Purification and Ubiquitination. *Escherichia coli* BL21 expressing GST-E6AP or GST-Nedd4 was cultured in LB/ampicillin at 37 °C with agitation overnight. Bacteria were then subcultured at a ratio 1:200 in 1 L of LB/ampicillin at 25 °C, 220 × *g* until optical density equaled 0.6–0.8, and then protein expression was induced by adding 1 mM Isopropyl β-D-1-thiogalactopyranoside. Bacterial cultures were transferred to 16 °C and cultured for 16 h. Bacteria cells were lysed in PBS, and supernantants were collected. GST-E6AP or GST-Nedd4 was pulled down using Glutathione Sepharose 4B (GE Healthcare) according to the manufacturer's instructions. Purified GST-E6AP or GST-Nedd4 was dialyzed in buffer containing 25 mM Hepes and 10% glycerol and stored at −20 °C. For ubiquitination, 50 nM of His-E1 (Boston Biochem), 600 nM of His-UbcH5b, 600 nM of GST-E6AP or GST-Nedd4, and 12.5 μM of Ub was incubated in Ub conjugation buffer containing 20 mM Tris·Cl, pH 7.6, 20 mM KCl, 5 mM MgCl2, 1 mM DTT, and 1 mM ATP at 37 °C. After 2 h, the reaction mixtures were dialyzed in buffer containing 25 mM Hepes and 10% glycerol. E1 and E2 were then removed by Ni-NTA Agarose (Qiagen) according to the manufacturer's instructions.

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