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Sophie Ventadour, Marianne Jarzaguet, Simon S. Wing, Christophe C. Chambon, Lydie Combaret, et al.. A new method of purification of proteasome substrates reveals polyubiquitination of 20 S proteasome subunits. *Journal of Biological Chemistry*, 2007, 282 (8), pp.5302-5309. 10.1074/jbc.M610005200 . hal-02662552

**HAL Id: hal-02662552**

**<https://hal.inrae.fr/hal-02662552>**

Submitted on 31 May 2020

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# A New Method of Purification of Proteasome Substrates Reveals Polyubiquitination of 20 S Proteasome Subunits\*<sup>§</sup>

Received for publication, October 25, 2006, and in revised form, December 19, 2006. Published, JBC Papers in Press, December 21, 2006, DOI 10.1074/jbc.M610005200

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The 26 S proteasome is implicated in the control of many major biological functions but a reliable method for the identification of its major substrates, *i.e.* polyubiquitin (Ub) conjugates, is still lacking. Based on the steps present in cells, *i.e.* recognition and deubiquitination, we developed an affinity matrix-based purification of polyUb conjugates suitable for any biological sample. Ub-conjugates were first purified from proteasome inhibitor-treated C2C12 cells using the Ub binding domains of the S5a proteasome subunit bound to an affinity matrix and then deubiquitinated by the catalytic domain of the USP2 enzyme. This two step purification of proteasome substrates involving both protein-protein interactions and enzyme-mediated release allowed highly specific isolation of polyUb 26 S proteasome substrates, which were then resolved on two-dimensional gels post-deubiquitination. To establish our method, we focused on a gel area where spots were best resolved. Surprisingly, spot analysis by mass spectrometry identified  $\alpha 2$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ ,  $\beta 3$ ,  $\beta 4$ , and  $\beta 5$  20 S proteasome subunits as potential substrates. Western blots using an anti- $\beta 3$  proteasome subunit antibody confirmed that high molecular weight forms of  $\beta 3$  were present, particularly in proteasome inhibitor-treated cells. Sucrose gradients of cell lysates suggested that the proteasome was first disassembled before subunits were polyubiquitinated. Altogether, we provide a technique that enables large scale identification of 26 S proteasome substrates that should contribute to a better understanding of this proteolytic machinery in any living cell and/or organ/tissue. Furthermore, the data suggest that proteasome homeostasis involves an autoregulatory mechanism.

Over the past 20 years, proteasome-dependent proteolysis has proved to play a central role in many major biological functions. The complexity of this degradation pathway led to the

discovery of ubiquitin (Ub)<sup>3</sup>-independent (1, 2) and ubiquitin-dependent (3) proteolysis, the latter representing the vast majority of the target proteins discovered so far. While many proteins have been shown to be polyubiquitinated and degraded by the 26 S proteasome, evidence for the involvement of this proteolytic system is often indirect, *e.g.* the use of proteasome inhibitors of varying specificity. To establish firmly that a given protein is polyubiquitinated *in vivo*, it is absolutely necessary to possess good antibodies that are able to specifically immunoprecipitate the endogenous protein of interest. This requirement limits the potential discovery of proteasome substrates to proteins with well characterized antibodies directed against them.

The 26 S proteasome recognizes and captures ubiquitin conjugates through the 19 S regulatory complex that caps the 20 S proteolytic core. At least two subunits bind Ub-conjugates: S5a/RPN10 and the ATPase S6' (4, 5). Whereas the sites of polyUb recognition have been studied in depth for S5a (4, 6, 7), the mechanism of interaction between S6' and the polyUb chains remains unknown. Additionally, recombinant S5a can be easily produced and exhibits a polyUb binding activity indistinguishable from that of the proteasome with a very low affinity for mono-, di-, and tri-Ub conjugates which are usually not substrates of the proteasome. By contrast, recombinant S6' has virtually no polyUb binding activity, which is probably caused by conformational problems or missing partners.

Over the past 2 years, new proteomic approaches have emerged for identifying proteasome substrates. The most promising used Myc- or His-tagged Ub-transfected cells (8–10), but other *in vitro* approaches have been proposed (reviewed in Ref. 11). However, several drawbacks are linked to tagged Ub strategies. First, transfection of modified Ub can alter cell metabolism by increasing the stability of Ub-conjugates because of lowering their affinity for the proteasome and/or deubiquitinases (10). Whereas this technique is suitable for the purification of highly unstable Ub-conjugates, poor substrates (*e.g.* mono-Ub proteins) could be transformed artificially into polyUb proteins. Second, transfection is limited to cultured cells, while animal and human models represent the final targets. Third, it is impossible to distinguish between mono and polyUb substrates as the purification involves only the tag of the Ub moiety and the identification is based on the

\* This work was supported in part by grants (to D. T. and/or D. A.) from the Institut National de la Recherche Agronomique and the Association Française contre les Myopathies. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S3.

<sup>1</sup> Recipient of fellowships from the French Ministry of Research and the Fondation pour la Recherche Médicale.

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<sup>3</sup> The abbreviations used are: Ub, ubiquitin; USP2, ubiquitin-specific processing protease 2; NEM, *N*-ethylmaleimide; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; UIM, ubiquitin-interacting motif.

signature of one Ub. Fourth, nonspecific interacting proteins are too numerous and false positive proteins cannot be readily distinguished. Others have used the ability of the 19 S proteasome subunit S5a to purify native Ub-conjugates (12). However, the number of Ub moieties for a given protein is highly variable, which considerably reduces the power of identification of the proteins by subsequent mass spectrometry.

To overcome these difficulties, we designed a technique that (i) is applicable to any kind of sample, and (ii) distinguishes between proteins linked covalently to polyUb (*i.e.* proteasome substrates) and proteins only interacting with polyUb (*i.e.* “contaminants”). To efficiently purify Ub-conjugates, we used the two steps existing in cells: capture of the Ub-conjugates and release of the substrates by subsequent deubiquitination. We used a partial clone of S5a (MLG) encompassing the two ubiquitin-interacting motifs (UIM), which retained the whole binding affinity (4). We produced an affinity matrix that allowed highly efficient purification of Ub-conjugates with low levels of contaminants. After deubiquitination, proteins were then resolved by two-dimensional gel electrophoresis, and specific spots were identified by mass spectrometry. Our methodology efficiently isolates polyUb conjugates and allows an easy and highly reproducible identification of proteasome substrates. Using this method in C2C12 myotubes, we report that 20 S proteasome subunits are polyubiquitinated.

## EXPERIMENTAL PROCEDURES

**Plasmids, Constructions, and Recombinant Protein Expression**—The His<sub>6</sub>-MLG in pET26b (gift from Prof. M. Rechsteiner, Salt Lake City, UT) expresses the two polyUb binding sites of S5a (4). The catalytic core region of USP2 (13) was subcloned in pGEX-5X1 using the XhoI and BamHI restriction sites to produce GST-USP2 core. *Escherichia coli* BL21(DE3) were transformed with pET26b-His<sub>6</sub>-MLG or with pGEX-5X1-USP2-core and expression was induced according to the manufacturer's instructions. The His<sub>6</sub>-MLG recombinant protein was purified using Ni-nitrilotriacetic acid beads (Qiagen) and then covalently bound to NHS-activated Sepharose® 4 Fast Flow resin (Amersham Biosciences) thereby forming the NHS-His<sub>6</sub>-MLG affinity matrix used throughout the experiments. The GST-USP2-core was purified using the glutathione-Sepharose® 4B kit (Amersham Biosciences), and the USP2-core was eluted following incubation with Xa factor. Plasmids encoding for the *Drosophila* GST-D19 and His<sub>6</sub>-p37A deubiquitinases were kindly provided by Profs. M. Bownes, (Edinburgh, UK) and W. Baumeister, (Martinsried, Germany). Recombinant proteins were produced in *E. coli* BL21(DE3) and purified using glutathione-Sepharose® 4B and Ni-nitrilotriacetic acid beads, respectively, according to the manufacturer's instructions.

**Cell Culture**—The C2C12 mouse myoblast cell line was obtained from American Type Culture Collection and routinely grown and differentiated as indicated by the supplier. Myotubes were used at day 6 of differentiation and, when necessary, treated with 50 μM proteasome inhibitor MG132 (Scientific Marketing Associates) for 12 h before cell harvesting.

**Ub Binding Assays**—Synthetic K48-Ub chains (gift from Prof. C. M. Pickart, Baltimore) were used to test the binding capacity

of the His<sub>6</sub>-MLG affinity matrix. We routinely labeled 50 μg of Ub chains with 1 mCi <sup>125</sup>I-Na using Iodogen tubes (Pierce). Labeled chains (0.45 μCi) were incubated with 50 μl of 50% slurry of the His<sub>6</sub>-MLG affinity matrix in 50 mM Tris, pH 7.5 for 20 min at room temperature. The slurry was then centrifuged at 1,500 × *g* for 1 min at room temperature, the supernatant was discarded, and the beads washed three times with 50 mM Tris, pH 7.5, 1% Triton X-100. Ub chains bound to the matrix were eluted with two volumes of 2× Laemmli and separated by SDS-PAGE followed by analysis using a Storm PhosphorImager (Molecular Dynamics).

**Deubiquitination**—Deubiquitination was performed on <sup>125</sup>I-Ub chains or muscle homogenates using purified deubiquitinases. Incubation conditions are specified for each enzyme and assay in the figure legends. For USP2-core, unless otherwise specified, we routinely incubated 20 ng of the purified enzyme per 30 μg of C2C12 protein homogenates (see below) in deubiquitination buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol) for 2 h at 37 °C. UCH-D19 was incubated in 50 mM Tris, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin for 4 h at 37 °C. UCH-p37A was incubated in 50 mM Tris, pH 7.5, 0.5 mM EDTA, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin for 4 h at 25 °C.

**Purification of Native Ub-conjugates**—Myotubes were washed and scraped off the plate into 1× phosphate-buffered saline and then sonicated for 30 s at maximum power in lysis buffer (5 mM Tris, pH 7.5, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM NEM, 1% Triton X-100/anti-proteases (Protease Inhibitor Mixture/Sigma)). Cell extracts were centrifuged at 10,000 × *g* (10 min, 4 °C), and the soluble proteins were quantified using the Bradford protein assay (Bio-Rad). Soluble proteins were then incubated with the MLG matrix for 2 h at room temperature with gentle agitation (1 mg of protein per 1.6 ml of 50% slurry). The beads were washed twice with lysis buffer and then twice in 5 mM Tris, pH 7.5, 5 mM EDTA. Elution was performed either by Laemmli buffer (1× final) or by the USP2-core enzyme for subsequent Western blot or two-dimensional gel analysis. For two-dimensional gel assays, the beads were separated prior to elution into two equal fractions. One fraction was incubated with the active USP2-core (0.4 μg/ml of C2C12 homogenate) at 37 °C for 3 h with gentle agitation in deubiquitination buffer. The other fraction was treated identically except that USP2-core was heat-inactivated (boiled 10 min) and NEM (10 mM, final concentration) was added to the assay. The beads were centrifuged at 1,500 × *g* for 5 min at 4 °C, and the supernatants saved. Beads were then washed in two volumes of incubation buffer (50 mM Tris, pH 7.5, 1% Triton X-100). The eluate and the washes were combined.

Alternatively, series of sucrose gradients were performed using the soluble proteins from C2C12 myotubes treated or not with MG132, except that sonication was avoided. 5 mg of the soluble proteins were directly loaded on top of a 10–40% sucrose gradient (12 ml, 25 mM Tris, pH 7.5, 0.05% Triton X-100, 2.5 mM NEM) centrifuged at 100,000 × *g* in a Kontron TST 41.14 rotor for 18 h at 4 °C and aliquoted. Samples were either directly used to perform Western blot analysis or subjected to prior deubiquitination with the native or heat-denatured USP2-core enzyme as described above. When necessary,

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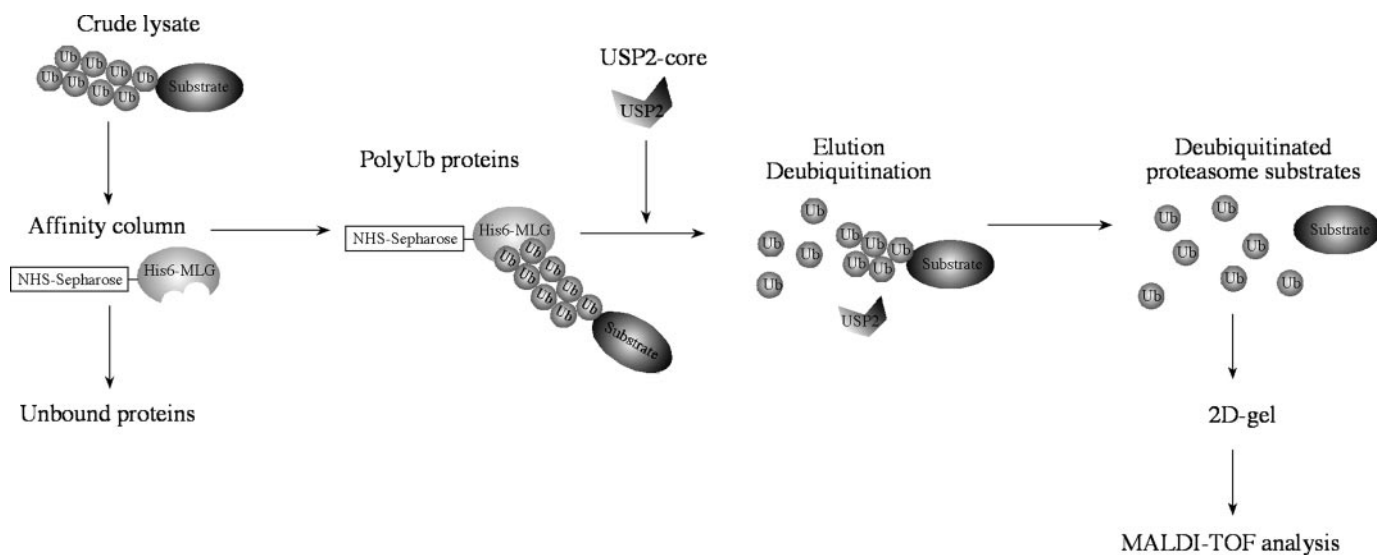


FIGURE 1. **Schematic purification procedure of cellular polyUb conjugates using a MLG affinity matrix.** Cells are resuspended in a protease and DUB inhibitor-containing buffer, lysed by sonication, and centrifuged. The supernatant is then loaded onto NHS-His<sub>6</sub>-MLG beads, and unbound proteins are discarded. Purified matrix-bound polyUb proteins are then deubiquitinated by USP-core to elute proteasome substrates. Proteins in the eluate are separated by two-dimensional gel analysis. Spots appearing specifically after deubiquitination are analyzed by MALDI-TOF mass spectrometry.

NEM was neutralized as described (13). Protein standards (GE Healthcare) were systematically used to calibrate series of gradients. Equal amounts of each fraction were loaded onto 12% SDS-PAGE. Western blots were performed using anti-polyUb (1:1,000) (clone FK1, BioMol), anti-Ub (1:650) (Sigma) or anti- $\beta$ 3 (1:1,000) (BioMol) proteasome subunit and analyzed by chemiluminescence (ECF) or autoradiography (ECL) (Amersham Biosciences).

**Isoelectrofocusing and Gel Analysis**—Samples were concentrated and desalted using Centricon-10 filtration units (Millipore) and isoelectrofocusing (IEF) was performed using nonlinear IPG strips (pH 3–10/Bio-Rad) according to the manufacturer's instructions. The second dimension was performed using gradient SDS-PAGE (8–16%). Analytical gels were stained using a silver staining procedure, as described (14), scanned with a Image Scanner II Densitometer (GE Healthcare), and analyzed with the ImageMaster Platinum two-dimensional software (v. 6.0, GE Healthcare).

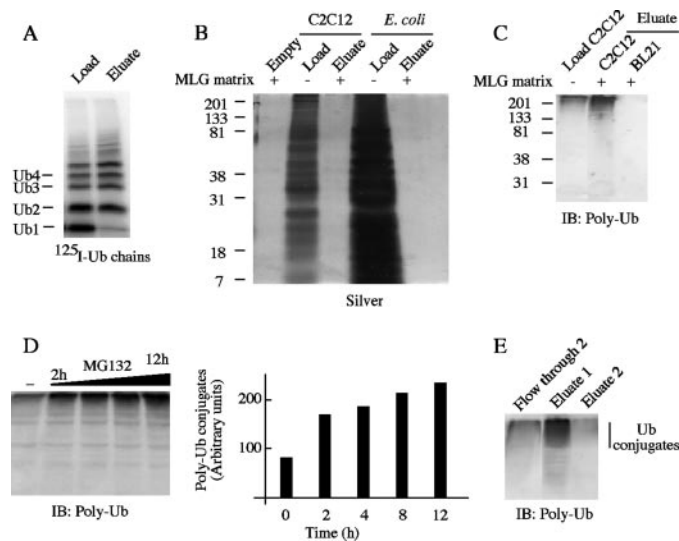
Two types of control gels were used. Control gels including samples incubated with the inactivated USP2-core allowed the detection of contaminants mainly arising from recombinant protein purification. However, as proteins possessing a high affinity for Ub chains could represent potential contaminants when Ub-conjugates were eluted by the USP2-core, we added a second control. In another assay, Ub-conjugates bound to the affinity matrix were divided into three parts and eluted with either USP2, heat plus NEM-denatured USP2 or SDS. Spots appearing repeatedly and specifically in gels treated with the active USP2-core and not in the control gels were considered as target proteins, *i.e.* proteasome substrates. Two independent experiments including four repeats per experiment were performed under the same conditions except that gels used for mass spectrometry analysis were loaded with twice the amount of proteins.

**Mass Spectrometry**—Target spots were excised from preparative gels and proteins were identified using a Voyager-DE Pro

MALDI-TOF mass spectrometer (Perceptive Biosystems), as described (14). The identification of proteins was performed according to the Pro-Found and MASCOT probabilistic scores together with the accuracy of the experimental to theoretical  $pI$  and  $M_r$ .

## RESULTS AND DISCUSSION

**The S5a (MLG Clone) Affinity Matrix Binds Potently to Ub and Endogenous Ub-conjugates**—To identify Ub-conjugates in any biological sample, we set up a complete purification technique based on the very steps used by the proteasome to process *in vivo* Ub-conjugates, *i.e.* the recognition of Ub-conjugates followed by their deubiquitination (Fig. 1). We designed an affinity matrix based on recombinant, truncated tagged S5a peptide (His<sub>6</sub>-MLG) encompassing the two polyUb binding sites and covalently bound via an amide bond to the resin. We verified that the MLG affinity matrix binds both synthetic Ub chains and physiological substrates from C2C12 muscle cells (Fig. 2, A and C). By contrast, the MLG peptide showed virtually no affinity for synthetic mono-Ub. Furthermore, the binding of Ub-conjugates on the MLG was specific as no protein was detected when concentrated bacterial lysate was incubated with the MLG matrix (Fig. 2, B and C and not shown). The MLG affinity matrix was able to specifically bind native Ub-conjugates from C2C12 myotubes (Fig. 2, B and C). Ub-conjugates accumulate in cells during highly catabolic situations or when the proteasome is inactivated. We exposed C2C12 cells to MG132 and observed that the accumulation of Ub-conjugates was linear with time up to 12 h (Fig. 2D). Because inhibition of the proteasome resulted in Ub-conjugates accumulation, experiments were performed using a 12-h inhibition of the proteasome. However, following the isolation of polyUb conjugates by the MLG affinity matrix, we consistently observed that a small fraction of material reacting with the polyUb antibody was still present in the unbound fraction (not shown and Figs. 2E and 3E). As the binding capacity of our matrix could be limiting, we



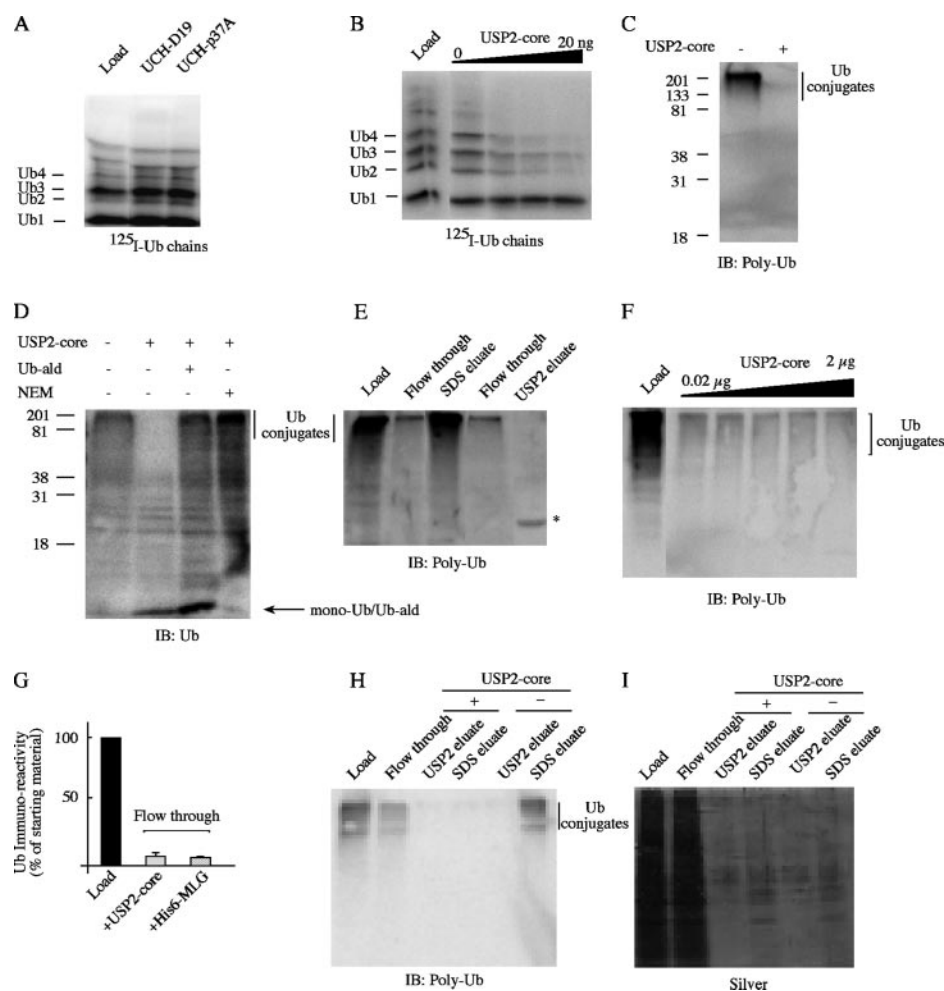
**FIGURE 2. Efficient isolation of Ub-conjugates from MG132-treated C2C12 cells.** *A*, synthetic  $^{125}\text{I}$ -labeled Ub chains (K48 linkage) were used to determine the efficiency and specificity of the His<sub>6</sub>-MLG affinity matrix. 0.45  $\mu\text{Ci}$  of labeled chains (*Load*) were incubated with the His<sub>6</sub>-MLG matrix (50  $\mu\text{l}$  of 50% slurry) for 20 min at room temperature. Beads were washed, and bound material was eluted (*Eluate*) by 2 $\times$  Laemmli. His<sub>6</sub>-MLG potentially bound synthetic Ub chains but had virtually no affinity for monoubiquitin. *B*, C2C12 myotubes and BL21(DE3) *E. coli* were lysed by sonication and French press, respectively, and 30  $\mu\text{g}$  and 1 mg of soluble proteins were, respectively, loaded onto the MLG matrix (50  $\mu\text{l}$  of 50% slurry) for 2 h at room temperature as described under "Experimental Procedures." Samples were eluted by adding Laemmli sample buffer, loaded onto SDS-PAGE, and silver-stained. The MLG matrix showed high specificity as the vast majority of the C2C12 myotube proteins were not retained by the resin. *E. coli* proteins (1 mg) also did not bind to the MLG matrix. *C*, same experiment as in *B*. Samples eluted by SDS (0.5% final) were used for Western blot analysis using an anti-polyUb antibody. Native Ub-conjugates from C2C12 lysates efficiently bound to the MLG matrix (*lane 2*), but no signal was detected when *E. coli* lysate was used (*lane 3*). *D*, upon MG132 treatment, Ub-conjugates accumulated in the soluble fraction of C2C12 cells in a time-dependent fashion. C2C12 myotubes were treated with 50  $\mu\text{M}$  MG132 for up to 12 h. Cell viability was not affected by 12 h of treatment (not shown). 30  $\mu\text{g}$  of C2C12 soluble proteins per time point were loaded onto SDS-PAGE. Anti-polyUb antibody revealed a time-dependent accumulation of Ub-conjugates (*left panel*) that was quantified by densitometry (3-fold increase, *right panel*) using the ImageJ software (NIH, v. 1.34s). *E*, purification of Ub-conjugates was efficient. A first purification of C2C12 Ub-conjugates (30  $\mu\text{g}$  of soluble proteins) was performed using the His<sub>6</sub>-MLG matrix (50  $\mu\text{l}$  of 50% slurry, 2 h at room temperature), and the unbound material (*Flow through*) was subjected to a second round of purification with fresh beads. Beads from both round of purification were treated with SDS (0.5% final) for 10 min at room temperature, and the eluates were analyzed by Western blot using the anti-polyUb antibody. The second step of purification did not appreciably increase the amount of purified Ub-conjugates and a faint smear of residual protein revealed by the anti-polyUb antibody was still present in the unbound material after round 2. The amount of residual reactive material was highly comparable throughout experiments (see Fig. 3, G).

tried to further purify the potential Ub-conjugates present in the flow through with fresh MLG. Surprisingly, the second eluate did not show significant levels of polyUb signal, while there was still some in the second flow-through (Fig. 2E). This suggests that this fraction was resistant to the capture by fresh MLG. One possibility may be that different Ub linkages were not retained by our affinity matrix. Previous *in vitro* observations with synthetic Ub chains concluded that S5a had comparable affinities for synthetic K6, K11, K29, K48, and K63 Ub linkages (6, 7, 15). K48 linkage targets proteins for degradation by the 26 S proteasome, whereas other linkages are implicated in other pathways, such as nuclear translocation of signaling

proteins (K63) (16). Our work did not identify the nature of the Ub-containing proteins not purified by S5a, but we cannot rule out that conjugates not targeted for degradation (K6, K11, K63) were protected from recognition by the proteasome machinery.

**The USP2-Core Enzyme Potently Deubiquitinates S5a-bound Ub-conjugates**—Deubiquitinase families have defined roles in the cell, such as recycling the Ub chains, proofreading conjugates or processing Ub precursors (17). The large number of DUBs present in higher eukaryotes is probably the hallmark of highly divergent functions. In our hands, *Drosophila* UCH-D19 (18) and p37A (19) enzymes did not cleave free or MLG-bound Ub chains (Fig. 3A and not shown). These DUBs have been initially characterized using small adducts (Ub-AMC or Ub-valine) but not with Ub chains or native Ub-conjugates (18, 19). These enzymes do not seem to disassemble free K48-linked Ub chains but another hypothesis is that they need cooperating factors not present in our assays. The catalytic core domain of USP2 deubiquitinates Ub-conjugates in a tissue extract (13). Interestingly, the full-length USP2 isoforms were less active in that assay (13). Thus, N- or C-terminal extensions could partly explain the lack of activity of the *Drosophila* enzymes. We show here that the USP2-core also cleaved longer K48-linked Ub chains (>3, Fig. 3B) and potently disassembled the chains present in native Ub-conjugates from the soluble fraction of C2C12 crude extracts (Fig. 3, C and D). The reaction was highly specific as both NEM and Ub-aldehyde inhibited the release of mono-Ub from Ub-conjugates by the USP2 core. An accumulation of mono-Ub was also observed upon action of the USP2-core, further indicating that unspecific proteolytic activity was not implicated in the disappearance of the polyUb signal (Fig. 3D). We also tested USP2-core activity using C2C12 MLG-bound polyUb conjugates. Interestingly, the USP2-core not only disassembled physiological Ub-conjugates but also displaced the strong interaction present between the UIM of the MLG peptide and the Ub chains (Fig. 3, E and H). Furthermore, no intermediates of chain cleavage were observed in all our assays, *i.e.* faster migrating forms of Ub-conjugates, supporting their complete disassembly by USP2-core. However, we consistently observed that a small fraction of Ub-reacting material was resistant to USP2-core activity. Increasing the amount of enzyme (up to 2  $\mu\text{g}$  per 30  $\mu\text{g}$  of crude soluble protein) and incubation time (up to 3 h) did not improve significantly the disappearance of the immunoblot signal (Fig. 3F). Thus, a fraction of the anti-Ub-reacting material was resistant to both MLG purification (Figs. 2E and 3E) and USP2-core deubiquitination (Fig. 3F). We analyzed by densitometry five independent experiments for each assay and similar amounts of resistant signal were found, *i.e.*  $6.52 \pm 0.01$  and  $6.35 \pm 0.01\%$  for MLG and USP2-core assays, respectively. Besides being unrecognized specific Ub linkages, we cannot rule out that this represents nonspecific signal. Finally, we decided to use each sample as its own control. We treated half of the sample with active USP2-core and the other half with the inactivated form (heat-denatured and NEM-treated) and observed that Ub-conjugates were eluted specifically upon action of USP2-core (Fig. 3H). Using the deubiquitinating enzyme to elute proteasome substrates decreased the amount of contaminating proteins when compared with an SDS elution (Fig. 3I and supplemental Fig. S3).

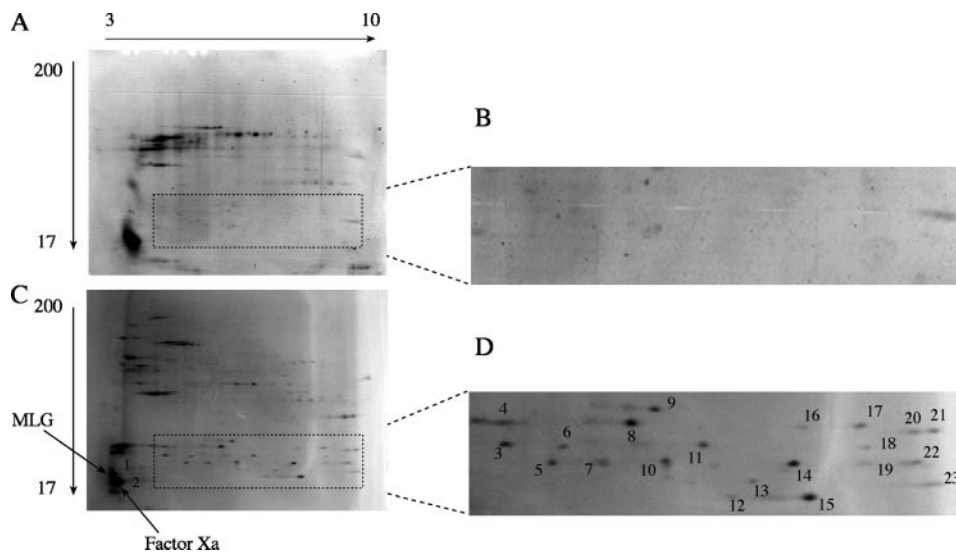
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**FIGURE 3. The testis USP2-core is a potent deubiquitinating enzyme for both synthetic and native Ub chains.** Three deubiquitinases were tested for their ability to trim synthetic Ub chains. *A*,  $^{125}\text{I}$ -Ub chains (*Load*) were incubated with either 2  $\mu\text{g}$  of UCH-D19 or 2  $\mu\text{g}$  of UCH-p37A. The reaction was stopped by addition of Laemmli buffer (1 $\times$  final), and samples were separated by SDS-PAGE and analyzed by autoradiography. Ub chains were not cleaved by these enzymes. *B*, USP2-core depolymerizes polyUb chains. K48-linked  $^{125}\text{I}$ -Ub chains were incubated with increasing amounts of USP2-core (0–20 ng) in deubiquitination buffer for 30 min at 37  $^{\circ}\text{C}$ . The reaction was stopped by addition of Laemmli buffer (1 $\times$  final). Autoradiography revealed that the Ub chains were efficiently disassembled with a corresponding accumulation of mono-Ub. *C*, C2C12 soluble proteins (30  $\mu\text{g}$ ) were treated with either 20 ng of native USP2-core or buffer and incubated for 2 h at 37  $^{\circ}\text{C}$ . Immunoblot analyses of the products with anti-polyUb antibody revealed an almost complete disappearance of Ub-conjugates upon treatment with USP2-core. *D*, USP2-core enzyme specifically trimmed C2C12 Ub-conjugates and accumulated mono-Ub. Soluble proteins (30  $\mu\text{g}$ ) of the soluble fraction of C2C12 myotubes were exposed to USP2-core as described above with or without the inhibitors Ub-aldehyde or NEM. A Western blot was performed using an antibody that recognizes both mono and polyUb to visualize the accumulation of mono-Ub following USP2-core treatment. Note, Ub-aldehyde is also recognized by the antibody. Treatment of the soluble fraction with either Ub-aldehyde (0.5  $\mu\text{M}$ ) or NEM (10 mM) inhibited the deubiquitination of the Ub-conjugates. *E*, Ub-conjugates purified with the His<sub>6</sub>-MLG affinity matrix were efficiently deubiquitinated *in situ*. Ub-conjugates were purified from 30  $\mu\text{g}$  of C2C12 soluble proteins using 50  $\mu\text{l}$  of the MLG affinity matrix as described above. The unbound proteins were saved for Western blot analysis (*Flow through*). The polyUb proteins bound on beads were divided and eluted with either 0.5% SDS (*SDS eluate*) or USP2-core (*USP2 eluate*). Eluates were collected, and 2 $\times$  Laemmli buffer was also added to the beads and boiled. All samples were analyzed by immunoblot with anti-Ub antibodies. PolyUb detection revealed that both elution techniques were powerful as virtually no Ub-conjugate was present on the beads after elution (not shown). *Load*, starting material. *Flow through*, after pelleting the beads, the supernatant was concentrated and loaded onto the gel. The asterisk denotes a cross-reacting band. *F*, increased amounts of USP2-core did not improve deubiquitination. 30  $\mu\text{g}$  of soluble proteins from C2C12 myotubes (*Load*) were incubated with varying amounts of USP2-core for 3 h at 37  $^{\circ}\text{C}$ . Western blot with anti-polyUb antibodies revealed that 20 ng of USP2-core were sufficient to reach maximal deubiquitination although a faint smear of immunoreactive material was still present. *G*, immunoreactive material resistant to purification by the His<sub>6</sub>-M6-LG affinity matrix or to deubiquitination by the USP2-core enzyme was analyzed by densitometry (ImageJ v.1.34s). Thirty micrograms of C2C12 soluble proteins were used for each assay, and five independent experiments were pooled. The unbound (+*His6-MLG*) or not deubiquitinated (+*USP2-core*) fractions were expressed as percent of starting material (*Load*). We observed indistinguishable levels of remaining material revealed by the anti-polyUb antibody after purification or deubiquitination. *H*, Ub-conjugates were purified from 60  $\mu\text{g}$  of C2C12 soluble proteins using the MLG matrix. The beads were divided in 2 equal fractions, and elution was performed using native (+) or heat plus NEM-inactivated (–) USP2-core (20 ng). Following the elutions, the beads were further treated using 0.5% SDS. Fractions were analyzed by anti-polyUb antibody. The immunoblot revealed that the USP2-core efficiently disassembled the Ub chains thus eluting proteasome substrates. The inactivated USP2-core is a good control as there was no detectable loss of Ub-conjugates. *I*, an experiment identical to the one shown in *G* was performed, and the gel was silver-stained. Our purification procedure eliminated most of the cellular proteins present in the sample (*Flow through*). The amount of starting material per assay (30  $\mu\text{g}$  soluble proteins) was too low to detect Ub-conjugates by silver staining.

Altogether, the inactivated form of USP2-core applied to half of the sample was a good control and was used in two-dimensional gel assays to localize co-purifying contaminants.

*Two-dimensional Gels Revealed Specific Spots Appearing upon Deubiquitination*—To increase the amount of Ub-conjugates, C2C12 cells were grown in presence of the proteasome



**FIGURE 4. Proteasome substrates were detected in C2C12 extracts.** Soluble C2C12 proteins (5.1 mg) were used to purify Ub-conjugates with the His<sub>6</sub>-MLG affinity matrix as described under "Experimental Procedures." Bound Ub-conjugates were divided in equal amounts and then eluted with native or heat plus NEM-denatured USP2-core (3.4 μg). Equal amounts of eluates were loaded on pH 3–10 strips and subjected to isoelectric focusing. Strips were loaded onto 8–16% acrylamide SDS-PAGE, and the gels analyzed by silver staining. A series of four experimental (active USP2-core) and four control (inactive USP2-core) gels was performed in parallel. A, control gel. C, experimental gel. To establish our method, we focused on an area where spots specifically appearing upon USP2-core activity were easily distinguished (B and D). Twenty-three spots were excised using pipette tips, treated as described (14), and analyzed by MALDI-TOF mass spectrometry.

**TABLE 1**

**Identification of target proteins**

Ub-conjugates were purified from C2C12 cells using the MLG affinity matrix and deubiquitinated by the recombinant USP2-core enzyme. Identification was performed by MALDI-TOF mass spectrometry.

Spot no.	Protein name	GenBank <sup>TM</sup> accession number	No. of matching peptides
14	Proteasome subunit α2	X70303	7
11	Proteasome subunit α6	NM_011968	6
20, 21	Proteasome subunit α7 <sup>a</sup>	BC008222	4, 7
13	Proteasome subunit β2	BC008265	3
10	Proteasome subunit β3	NM_011971	4
7	Proteasome subunit β4	BC008241	5
23	Proteasome subunit β5	AF060091	8
3, 6	HSP27 <sup>a</sup>	U03561	5, 5

<sup>a</sup> Two different spots were identified as the same protein suggesting post-translational modifications (phosphorylation).

inhibitor MG132. Large amounts of sample (50 mg) were purified with the MLG/USP2-core system and used to perform series of two-dimensional gels. A first series of 8 gels was performed, half of them being loaded with samples treated with the denatured USP2-core (control gels), the other half with the native enzyme (Fig. 4 and supplemental Fig. S1). Pilot experiments identified factor Xa (spot 2) and USP2-core as major contaminants in control gels, a small fraction of the MLG peptide (spot 1) being also present in experimental ones (Fig. 4, A and C). Other contaminating proteins were only present at low levels in our preparations (Fig. 4 and supplemental Figs. S1 and S2). They may include cellular proteins with Ub binding domains (15, 20) that could co-purify with the polyUb proteins.

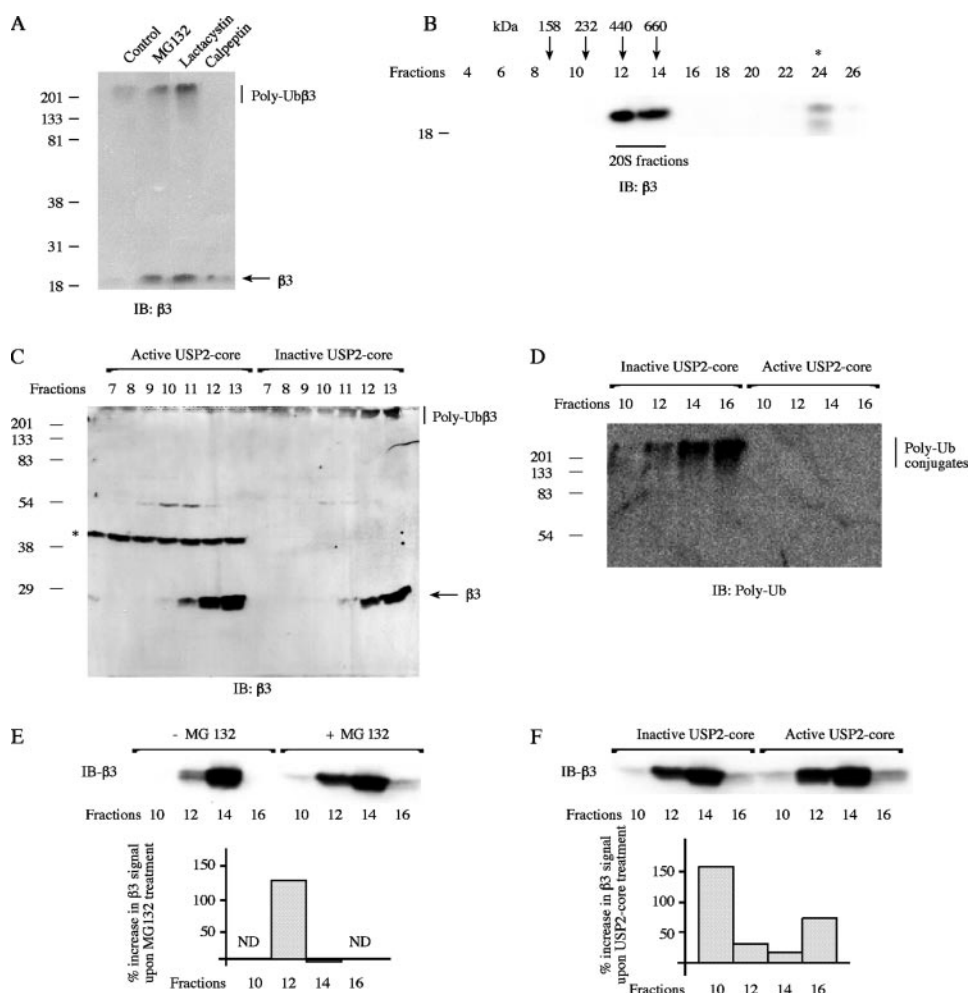
By contrast, many spots appeared specifically only upon deubiquitination and were considered as previously polyubiquitinated proteins. In this work, spots were picked on a presence/absence basis and only spots appearing in the four experimental gels and not in the control ones were considered as proteasome substrates (Fig. 4 and supplemental Figs. S1 and S2). While

spots specifically appeared throughout the gel, we focused on an area that was almost clear of contaminants to establish our method (Fig. 4, B and D). In this area, all spots detected were specific to the experimental gels. A similar experiment was performed except that Ub-conjugates were eluted by SDS and then dialyzed before deubiquitination (supplemental Fig. S3). More protein contaminants were present in the SDS-eluted two-dimensional gels, but the same spots appeared specifically upon deubiquitination. Similar results were obtained with a second set of gels performed with a different batch of Ub-conjugates. Mass spectrometry analysis was successful for 10 of 21 specific spots. Most identified spots were unambiguously 20 S proteasome subunits (Table 1). The α7 proteasome subunit and HSP27 were identified in two different spots, thus supporting

post-translational modifications (phosphorylation) previously described for these proteins (21, 22). Our 2-step purification technique followed by two-dimensional analysis and mass spectrometry should provide a good strategy to identify proteasome substrates in any kind of biological sample, even when good antibodies are not available. Narrow pH gradient two-dimensional electrophoresis could increase resolution and allow more proteins to be identified using mass spectrometry. In addition, analyses done on cell or tissue samples from various physiological or pathological conditions would provide insights into regulation of substrate targeting to the proteasome.

*The 20 S Proteasome Subunits Are Polyubiquitinated*—The β3 proteasome subunit was present in the soluble fraction from C2C12 cells both as a 23-kDa band and as a high molecular weight smear (Fig. 5, A and C), suggesting polyUb modification. The amount of both forms of the β3 subunit substantially and specifically increased in the presence of the MG132 and lactacystin proteasome inhibitors when compared with control or to cells treated with the unrelated calpeptin calpain inhibitor (Fig. 5A). On sucrose gradients the β3 subunit was mainly present in assembled 20 S proteasome or in polyUb β3 but not as free subunit (Fig. 5, B and C and not shown). The conditions used here to isolate Ub-conjugates did not preserve the 26 S structure, so that most if not all proteasomes were present as 20 S particles in the soluble fraction (Fig. 5B). To further characterize the high molecular weight form of β3, we deubiquitinated the Ub-conjugates present in the gradient fractions. We systematically found an increase in total β3 in the 300–800 kDa fractions (Fig. 5, C and F) that upon USP2-core treatment paralleled the disappearance of polyUb conjugates (Fig. 5, C and D). These observations confirm that the high molecular weight forms of β3 were Ub-conjugates (Fig. 5, C and F). Sucrose gradients analysis confirmed that MG132 induced an increase in

## Polyubiquitination of 20 S Proteasome Subunits



**FIGURE 5. 20 S proteasome subunit  $\beta 3$  is polyubiquitinated in C2C12 cells.** *A*, C2C12 myotubes were treated with proteasome (MG132, 50  $\mu\text{M}$ ; lactacystin, 25  $\mu\text{M}$ ) or calpain (calpeptin 50  $\mu\text{M}$ ) inhibitors for 4 h. Cells were then scraped and disrupted in lysis buffer. The soluble proteins were separated in SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Western blot analysis was performed using the human anti- $\beta 3$  proteasome subunit antibody. Proteasome inhibitors (but not the calpain inhibitor) increased the 20 S  $\beta 3$  subunit content. High molecular weight  $\beta 3$  was present in cell extracts incubated with proteasome inhibitors, suggesting polyubiquitination of this subunit. *B–E*, sucrose gradients confirmed the presence of polyUb  $\beta 3$ . Cells were treated or not by the proteasome inhibitor MG132, and equal amounts of soluble proteins (5 mg) were separated on 10–40% sucrose gradients (see “Experimental Procedures”) and aliquoted in 27 fractions. Equal amounts of each fraction were loaded onto a 12% SDS-PAGE, and anti- $\beta 3$  or anti-polyUb Western blot analyses were performed. Protein standards were used systematically to calibrate the gradients (see *B*). The asterisks (*B* and *C*) denote nonspecific signals. *B*,  $\beta 3$  subunit sedimented in the area expected for the 20 S proteasome without any signal in the heavier fractions where the 26 S should sediment (fractions 20–22). *C*, equal amounts of each fraction (130  $\mu\text{l}$ ) from MG132-treated C2C12 cells were subjected to deubiquitination with either the native or the heat-denatured USP2-core. Fractions 7–13 are shown. PolyUb  $\beta 3$  was detectable in fractions 12–13. Deubiquitination (active USP2-core) abrogated high molecular weight  $\beta 3$  signal in the upper part of the gel and specifically increased the  $\beta 3$  signal confirming that the high molecular weight forms were  $\beta 3$  Ub-conjugates. *D*, same experiment as in *C* but the Western blot was performed using the anti-polyUb antibody to demonstrate that the USP2-core was active in sucrose gradient samples. PolyUb conjugates disappeared upon action of the active USP2-core in fractions 10–16. *E*, MG132 induced the accumulation of the  $\beta 3$  subunit present in 20 S fractions. C2C12 cells were treated with or without 50  $\mu\text{M}$  MG132, and soluble proteins were loaded onto sucrose gradients as described above. Equal amounts of fractions (130  $\mu\text{l}$ ) were loaded onto SDS-PAGE, and anti- $\beta 3$  Western blots were performed. Only the fractions exhibiting signals are shown. Densitometry was performed (ImageJ, v.1.34s), and results were expressed as percent increase in the  $\beta 3$  signal in cells treated with MG132 compared with control. Because of the lack of detectable signal in the control cells (-MG132), even in overexposed films, no calculation could be made for the fractions 10 and 16 (ND). *F*, deubiquitination by the USP2-core enzyme increased the  $\beta 3$  signal in proteasome fractions from MG132-treated C2C12 myotubes. Aliquots (130  $\mu\text{l}$ ) were incubated with the active or inactive USP2-core as in *C*. The  $\beta 3$  signal systematically increased upon treatment with the active USP2-core (upper panel). Densitometry was performed, and data expressed as percent increase in  $\beta 3$  signal relative to inactive USP2-core-treated cells (lower panel).

20 S proteasome content (Fig. 5*E*). The 20 S proteasome peaked in the same fraction (around 600 kDa, see Fig. 5*B*) in cells treated or not with MG132. Additionally, the  $\beta 3$  signal after

action of the USP2-core was systematically increased in all of the analyzed fractions in both control and MG132-treated cells (not shown and Fig. 5*F*) indicating that polyUb  $\beta 3$  was also present in control cells.

Few data on proteasome turnover, if any, is available. Proteasome inhibitors increase the 20 S proteasome content caused by *de novo* synthesis of its subunits in various human and monkey cell lines (23). Proteasome inhibitors also induced polyubiquitination of proteasome subunits (Fig. 5). This may reflect either stabilization of polyubiquitinated subunits or an increase in the breakdown of proteolytically inactive particles. Altogether, these observations suggest that there is an autoregulation of active proteasome levels in living cells. Similarly, others have suggested such a mechanism in cell lines overexpressing  $\beta$  subunits (24).

A recent study based on antibody affinity suggested that proteasome subunits could be ubiquitinated. The authors hypothesized that several subunits from the 19 S regulatory particle but also from the 20 S catalytic core ( $\alpha 4$ ,  $\beta 3$ , and  $\beta 4$ ) were ubiquitinated (25). However, adequate controls were not included in this study. Indeed, only S4 was found by mass spectrometry to be at least mono-ubiquitinated on K237 in HEK293T cells previously treated with the proteasome inhibitor LLnL. In related experiments, we identified the S7, S8, and S10b 19 S subunits as potential polyubiquitinated substrates (not shown). Although we did not identify all 19 S and 20 S subunits here, it is tempting to hypothesize that all proteasome subunits may be polyubiquitinated. Additionally, polyubiquitinated  $\beta 3$  was found in  $\geq 300$  kDa fractions under both native (gradient, Fig. 5*C*) and denaturing conditions (Fig. 5, *A* and *C*), suggesting that the 20 S proteasome was at least

partially disassembled before subunits were polyubiquitinated. The 26 S proteasome may exhibit through the 19 S regulatory complex a very high affinity for Ub-conjugates



and therefore could simply be co-isolated in assays that bind polyUb chains. However, this is very unlikely. The conditions used in the present work do not favor the stability of the 26 S proteasome: (i) we did not provide ATP and  $Mg^{2+}$  to stabilize the complex, and we were unable to detect 26 S particles on sucrose gradients; (ii) intense sonication before isolation of polyUb conjugates also would contribute to 26 S proteasome disassembly; (iii) high molecular weight  $\beta 3$  conjugates were detected in cells treated with proteasome inhibitors; (iv) spots corresponding to proteasome subunits were absent in two-dimensional gels from SDS-eluted Ub-conjugates; and (v) polyUb  $\beta 3$  was specifically disassembled by the USP2-core enzyme. Altogether, it is very unlikely that 20 S proteasome subunits were contaminants in our experimental preparations. It is tempting to hypothesize that proteasome subunits were tagged by K48 chains to degrade non-functional 20 S proteasome blocked by proteasome inhibitors. However, we cannot rule out the use of other chain linkages.

In conclusion, we describe a technique that enables large scale identification of 26 S proteasome substrates and thus should contribute to a better understanding of this proteolytic machinery in any organ/tissue. In addition, our observations and the current literature suggest that proteasome levels are adjusted in mammalian cells by autoregulatory mechanisms at both the synthesis and degradation levels. Further studies are clearly needed to identify (i) the E2/E3(s) responsible for the ubiquitination of the 20 S subunits, (ii) the chain linkage involved, and (iii) the signaling pathways that control proteasome homeostasis in mammalian cells.

*Acknowledgments*—We thank Prof. Martin Rechsteiner and Dr. Patrick Young for the generous gift of the MLG clone, Prof. Cecile Pickart for supplying us with Ub chains, and Dr. Marie-Noëlle Pélissier for USP2-core subcloning.

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