

Inhibition of Proteasome Activity Induces Concerted Expression of Proteasome Genes and *de Novo* Formation of Mammalian Proteasomes*

Received for publication, January 30, 2003, and in revised form, March 24, 2003
Published, JBC Papers in Press, April 3, 2003, DOI 10.1074/jbc.M301032200

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The 26 S proteasome is a high molecular mass proteinase complex that is built by at least 32 different protein subunits. Such protease complexes in bacteria and yeast are systems that undergo a highly sophisticated network of gene expression regulation. However, regulation of mammalian proteasome gene expression has been neglected so far as a possible control mechanism for the amount of proteasomes in the cell. Here, we show that treatment of cells with proteasome inhibitors and the concomitant impairment of proteasomal enzyme activity induce a transient and concerted up-regulation of all mammalian 26 S proteasome subunit mRNAs. Proteasome inhibition in combination with inhibition of transcription revealed that the observed up-regulation is mediated by coordinated transcriptional activation of the proteasome genes and not by post-transcriptional events. Our experiments also demonstrate that inhibitor-induced proteasome gene activation results in enhanced *de novo* protein synthesis of all subunits and in increased *de novo* formation of proteasomes. This phenomenon is accompanied by enhanced expression of the proteasome maturation factor POMP. Thus, our experiments present the first evidence that the amount of proteasomes in mammalia is regulated at the transcriptional level and that there exists an autoregulatory feedback mechanism that allows the compensation of reduced proteasome activity.

To ensure protein homeostasis ATP-dependent proteases play a crucial role in all organisms. The molecular architecture of such protease complexes in pro- and eukaryotes is similar in that they form multi subunit ring structures. In principle, they are composed of a proteolytic core subcomplex performing peptide hydrolysis and a regulatory subcomplex responsible for substrate recognition, unfolding, and providing accessibility to the proteolytic active sites (1, 2). Eukaryotic 20 S proteasome core complexes consist of four staggered heptameric rings of 14 nonidentical but homologous subunits. The outer rings contain α type subunits having a gating function for substrate entrance and product release (3). Only three of the seven β type subunits

in the inner rings exhibit peptide hydrolyzing activity and form the six proteolytic active threonine sites (4). The 19 S regulatory complex binds to the 20 S catalytic core unit via a substructure called the “base,” which is composed of six ATPases of the AAA family plus two non-ATPase subunits (1, 2). The ATPases exert a chaperone-like activity and are believed to unfold substrates and channel them into the 20 S core, thereby controlling access of substrates to the proteolytic chamber (5, 6). The residual non-ATPase subunits of the 19 S regulator form a “lid” structure that is thought to possess regulatory functions.

In eukaryotic cells, the 26 S proteasome is the most important ATP-dependent proteolytic system recognizing its substrates via a multi-ubiquitin tag. In bacteria, such systems are exemplified by various kinds of Clp proteases (7).

The complex processes of eukaryotic proteasome biogenesis comprise three main steps that finally lead to the formation of active proteolytic complexes (8, 9). The first step is the biosynthesis of seven different α type and the proforms of seven different β type 20 S core subunits. All of these proteins assemble into various stages of proteasome precursor complexes. Two distinct precursor intermediates can be distinguished: the 13 and 16 S precursor complexes. It is thought that two 16 S complexes interact to form a large and probably short-lived precursor intermediate, where the final maturation steps take place (8, 9).

With the exception of $\beta 3/C10$ and $\beta 4/C7$, all proteasomal β subunits are synthesized as inactive propeptides with N-terminal propeptides. These propeptides play an essential role during the biogenesis process and are processed by *cis*- or *trans*-autocatalysis in a two-step mechanism (10). The propeptides of the active β subunits are cleaved off autocatalytically, whereas the propeptides of the inactive subunits are removed by their active neighbors (10–13).

In eukaryotes, proteasome formation is assisted by accessory proteins that support the final assembly steps. One factor playing a crucial role in proteasome maturation is the short-lived protein Ump1 of yeast. Ump1 is part of proteasome precursor complexes and is degraded upon completion of proteasome maturation (14). Mammalian Ump1 homologues have been identified in 16 S precursor complexes and are referred to as POMP (proteasome maturation protein), proteasemblin, or human/mouse UMP (8, 15–17).

Gene expression of ATP-dependent proteolysis systems is strongly regulated in bacteria. Thus, genes encoding Clp proteases in Gram-positive bacteria are negatively controlled by a repressor that is degraded by the ClpCP protease under inducing conditions resulting in a positive autoregulatory feedback loop (18). In *Escherichia coli*, such a mechanism of self-control

* This work was supported by grants of the Deutsche Forschungsgemeinschaft (to P.-M. K. and E. K.) and by the Fonds of the Chemical Industry. 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.

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was shown for σ^{32} activating gene expression of Clp proteases (19, 20).

Indications for a coordinated control of proteasomal gene expression also exist in eukaryotes. In *Saccharomyces cerevisiae*, genes encoding proteasomal subunits are preceded by a common upstream activating *cis*-element, the so-called proteasome-associated control element. This proteasome-associated control element serves as a target sequence for the transcription factor Rpn4 that activates proteasomal gene expression in a concerted manner (21). Rpn4 is essentially required to control balanced levels of proteasomal subunits and thereby for balanced intracellular proteolysis. Interestingly, the extremely short-lived Rpn4 protein is not only a transcriptional regulator for the 26 S proteasome but also becomes a substrate of the 26 S proteasome. Such a regulatory mechanism yields in a negative feedback circuit in yeast. The same protein that controls and induces proteasome formation is destroyed by the fully assembled and matured proteasome (22). More recently, transcriptional profiling revealed a concerted Rpn4-dependent up-regulation of all proteasomal subunits upon treatment with proteasome inhibitors, suggesting that Rpn4 is indeed a master regulator responsible for the ability of the cell to compensate for proteasome inhibition (23).

Moreover, a probably similar system was found in *Drosophila* where proteasomal activity was inhibited by the silencing of different proteasomal genes. This approach not only resulted in reduced expression of the targeted subunit but also in increased expression of several nontargeted proteasomal subunits. However, the details of this regulation mechanism in *Drosophila* seem to be different from that in yeast (24).

Although regulated gene expression is also an essential prerequisite and the initial step in mammalian proteasome formation, the knowledge available on its regulation is still very limited. In this study, we therefore investigated the expression pattern of genes encoding proteasome subunits in response to proteasome inhibitors in mammalian cells. Moreover, we analyzed the effect of proteasome inhibitors on *de novo* formation of proteasome complexes. Our data present the first experimental evidence that mammalian cells respond to impairment of cellular proteasomal enzyme activity by a coordinated up-regulation of proteasome subunits at both the transcriptional and translational level. In consequence, mammalian cells compensate proteasome inhibition by *de novo* proteasome formation. In addition, our experiments demonstrate that mammalian cells must possess a sensor that by a so far unknown mechanism is able to tightly control the level of active cellular proteasomes.

EXPERIMENTAL PROCEDURES

Chemical Reagents and Antibodies—Epoxomicin, MG132, Calpain inhibitor II: Ac-Leu-Leu-methionine (ALLM),¹ and clasto-lactacystin β -lactone were purchased from Calbiochem-Novabiochem and prepared as 10 mM stock solutions in Me₂SO. α -Amanitin (1 mg/ml stock solution in water) was obtained from Roche Applied Science. The fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-aminomethyl-coumarin (AMC) was from Bachem. Antibodies against p53 (polyclonal antibody 240) and Hsp70 (K29) were purchased from Santa Cruz Biotechnology, and the anti-ubiquitin antibody was from DAKO. Antibodies against proteasomes and proteasomal subunits were purchased from Affinity (Rpt1/MSS1, and C2/ α 6), and other anti-proteasome antisera and the anti-POMP antibody are laboratory stock and have been described previously (15).

Cell Culture—Primary cultures of vascular smooth muscle cells (VSMCs) were prepared from carotids of Wistar rats and cultivated as described elsewhere (25). Purity of VSMC preparations was confirmed to be 95% by immunostaining with anti-smooth muscle α -actin antibody

(Sigma-Aldrich). VSMCs were synchronized by replacing the growth medium with medium containing 2% fetal calf serum for 24 h. For all experiments the cells were used between passages 3 and 12. Other mammalian cell lines were cultivated under standard conditions (15).

For treatment with proteasome inhibitors, the culture media were supplemented with either epoxomicin (3 μ M) or c-lactacystin, MG132, ALLM in concentrations from 0.05 to 10 μ M, or 0.1% Me₂SO as a solvent control for the times indicated. For transcriptional inhibition VSMCs were treated for 8 h with 50 μ g/ml α -amanitin to block transcription, and 1 μ M MG132 was added for the last 6 h to induce mRNA levels of exemplified proteasomal subunits. Subsequently, the cells were harvested for RNA isolation or for preparation of total protein lysates.

Real Time RT-PCR and Northern Blotting—Total RNA was extracted using Trizol reagent (Invitrogen). 500 ng of RNA was DNase-digested (Ambion) and reverse transcribed with murine Moloney leukemia virus reverse transcriptase (Invitrogen). The PCR primers to amplify rat proteasomal cDNAs and the housekeeping gene hypoxanthine phosphoribosyl transferase were purchased from TIB MOLBIOL. The SYBR Green method was applied for quantitative amplification of the cDNA of rat proteasomal subunits using real time PCR. PCR amplification was carried out in 25 μ l of TaqMan Universal PCR Master Mix (Perkin Elmer/Applied Biosystems) containing either 0.3 or 0.9 μ M primer, and 0.4 μ l of the reverse transcription reaction in a 5700 Sequence Detection System (Perkin Elmer/Applied Biosystems). Thermal cycling conditions comprised activation of uracil-N-glycosylase at 50 °C for 2 min, an initial denaturation step at 95 °C for 10 min, followed by 95 °C for 15 s and 60 °C for 1 min for 40 cycles. The C_t (threshold cycle) is defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. mRNA expression was standardized to hypoxanthine phosphoribosyl transferase gene as a housekeeping gene, the transcription level of which was not influenced under our experimental conditions. Expression of the target gene relative to the housekeeping gene was calculated as the difference between the threshold values of the two genes ($2^{-\Delta C_t}$). Expression of proteasomal genes in the presence of proteasome inhibitor was normalized to expression under control conditions (0.1% Me₂SO) by means of the comparative C_t method ($2^{-\Delta\Delta C_t}$). The values are given as the means \pm S.E. of three independent experiments.

For Northern Blots 3 μ g of total RNA isolated as described above were loaded, vacuum-blotted, and hybridized overnight with digoxigenin-labeled riboprobes of PSMA2, PSMB5, PSMC2, and POMP. The gel loading was equalized using ethidium bromide staining of 28 S rRNA. The denaturing gel electrophoresis, vacuum blotting, and chemiluminescence detection were performed as described earlier (26).

Immunoblotting and Sucrose Gradient Fractionation—Equal amounts of protein extract were separated on SDS-Laemmli gels, transferred by electroblotting onto polyvinylidene difluoride membranes, and immunodetected with specific antibodies as described (25). 4 mg of protein of total cell extracts were fractionated by size gradient ultracentrifugation and immunodetected as described (15).

Metabolic ³⁵S Labeling—VSMC cells were incubated for 8 h with 10 μ M c-lactacystin, ALLM, or 0.1% Me₂SO as controls and then pulsed with ³⁵S translabel for 6 or 16 h, respectively. Radioactivity was determined by liquid scintillation counting. Equal counts were used for immunoprecipitation processed with an anti-proteasome antibody as described (27). The radioactive protein pattern was detected by phosphorimaging (Fuji FLA3000), and the band intensities were evaluated by the AIDA software according to the manufacturer's instructions (Raytest).

Measurement of Proteasome Activity in Cellular Lysates and Cell Viability Assay—Chymotryptic activity of the proteasome was assessed in cell lysates of VSMCs using the synthetic peptide substrate Suc-Leu-Leu-Val-Tyr linked to the fluorometric reporter AMC. The cells were treated with proteasome inhibitor (0.05–10 μ M) or Me₂SO (0.1%) for different time periods, washed in phosphate-buffered saline, and lysed under hyposmotic conditions by repeated freezing and thawing in liquid nitrogen (28). After centrifugation supernatants were used for determination of protein concentration and enzymatic activity. The lysates were incubated for 30 min at 37 °C in incubation buffer containing an ATP-regenerating system (50 mM Tris-HCl, pH 8.2, 18 mM KCl, 3 mM Mg(CH₃COO)₂, 3 mM MgCl₂, 1.1 mM dithiothreitol, 6 mM ATP, 5 mM phosphokreatin, 0.2 unit of phosphokreatinkinase) and 0.2 mM Suc-Leu-Leu-Val-Tyr AMC. AMC hydrolysis was quantitated in a BioTek FLx800 plate reader using 360-nm excitation and 460-nm emission wavelengths. Enzymatic activity was normalized to protein concentration and expressed as a percentage of activity of control lysates. The values are given as the means of three independent experiments \pm S.D. Cell viability was tested after 8 h of inhibition using Cell Prolifer-

¹ The abbreviations used are: ALLM, acetyl-Leu-Leu-methionine; c-lactacystin, clasto-lactacystin- β -lactone; Suc, succinyl; AMC, aminomethyl-coumarin; VSMC, vascular smooth muscle cells; RT, reverse transcriptase.

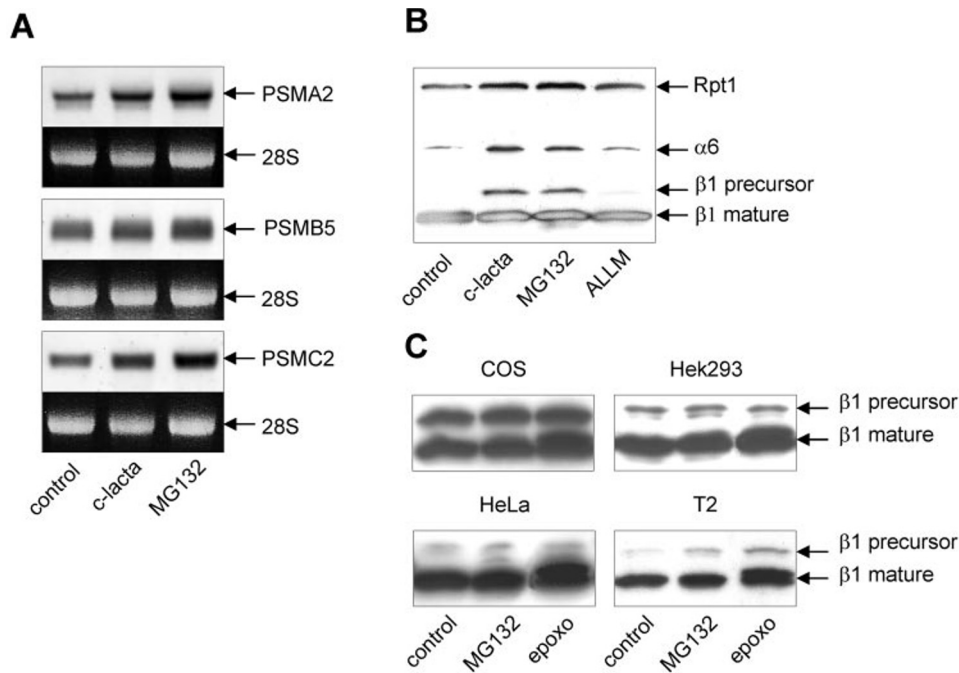


FIG. 1. **Treatment with proteasome inhibitors up-regulates RNA and protein expression of proteasomal subunits.** A, VSMCs were treated with 10 μ M c-lactacystin (*c-lacta*), 10 μ M MG132, or Me₂SO for 6 h, and RNA expression of PSMA2, PSMB5, and PSMC2 was analyzed by Northern blotting using digoxigenin-labeled riboprobes. Ethidium bromide-stained 28 S rRNA is shown as an internal standard. B, VSMCs were treated for 8 h with 10 μ M of the indicated inhibitors or Me₂SO, and protein expression of α 6, β 1, and Rpt1 was analyzed by Western blotting with the indicated antibodies. C, COS7, Hek293, HeLa, and T2 cells were treated with 10 μ M MG132 for 6 h, 3 μ M epoxomicin (*epoxo*) for 3 h, or Me₂SO and immunoblotted with a β 1-specific antibody to detect the mature and precursor forms of β 1.

ation Kit II according to the manufacturer's instructions (Roche Applied Science).

RESULTS

Proteasome Subunits Are Up-regulated in Response to Cellular Proteasome Inhibition—A first indication that proteasome inhibitor treatment of mammalian cells may affect the levels of proteasome mRNAs was obtained by gene expression profiling. Using a commercially available cDNA microarray (BD Biosciences), we observed a 2–3-fold up-regulation of several genes encoding proteasome subunits, *i.e.* α 2/PSMA2, α 4/PSMA7, α 6/PSMA1, β 1/PSMB6, β 3/PSMB3, β 4/PSMB2, and β 7/PSMB4, in response to proteasome inhibition with c-lactacystin and MG132 (data not shown).

To investigate whether such an increased expression of various subunits can also be detected by Northern or Western blot analysis primary VSMCs of the rat were treated with 10 μ M c-lactacystin, 10 μ M MG132, or Me₂SO for 6 h. Northern blot analysis of the α -subunit α 2/PSMA2, the catalytic β -subunit β 5/PSMB5, and the ATPase Rpt1/PSMC2 revealed a distinct up-regulation of these proteasome subunits in response to c-lactacystin and MG132 (Fig. 1A). Western blot analysis showed that increased proteasome mRNA levels were paralleled by increased protein levels because treatment of VSMCs with c-lactacystin and MG132 for 8 h resulted in enhanced levels of α 6/C2, Rpt1/MSS1, and β 1/ δ subunits (Fig. 1B). Interestingly, cells treated with proteasome inhibitors also showed expression of the β 1/ δ proprotein, indicating that the enhanced levels of proteasome subunits are due to *de novo* protein synthesis and not the result of protein stabilization. In contrast, treatment of VSMCs with the nonproteasomal, calpain-specific inhibitor ALLM, which is structurally similar to the peptide aldehyde inhibitor MG132, did not result in increased expression of proteasomal subunits (Fig. 1B).

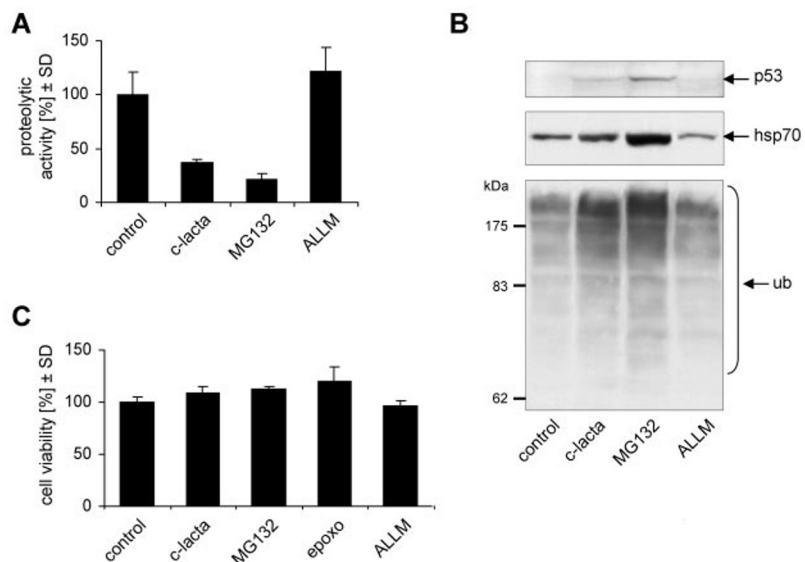
To see whether the observed effect is a cell line-specific phenomenon, we tested several mammalian cell lines for an exemplified proteasomal subunit by immunoblotting. The hu-

man cell lines HeLa, HEK293, and T2 as well as green monkey COS7 cells were exposed to epoxomicin or MG132 and immunodetected for the catalytic β 1/ δ subunit, which had shown an up-regulation in its mRNA level (see above). Independent of the cell line used, Western blot analysis revealed a moderate increase in β 1/ δ proprotein as well as in matured β 1/ δ subunit (Fig. 1C). Although the increased expression of proteasome subunits in response to proteasome inhibition seems to be less pronounced in immortal cell lines than in a primary cell line, the results suggest that the observed up-regulation is a general phenomenon in mammalian cells that is irrespective of the cell type.

To assure that the observed effects are indeed due to proteasome inhibition and not the result of apoptosis or cell death, the degrees of both proteasome inhibition and cell viability were analyzed after treatment of cells with proteasome inhibitors. Inhibition of proteasome activity was measured in cellular extracts using a fluorogenic peptide substrate. As shown in Fig. 2A, c-lactacystin and MG132 inhibited the chymotrypsin-like activity by 63 and 74% compared with controls, respectively. In contrast, the calpain inhibitor ALLM showed no effect (Fig. 2A). In addition, we analyzed the accumulation of p53, a specific substrate of the proteasome and that of multi-ubiquitinated proteins. Increased levels of p53 and accumulation of multi-ubiquitinated proteins was observed after c-lactacystin and MG132 treatment (Fig. 2B). Proteasome inhibitors also strongly induced the expression of Hsp70, which is a well known cellular response upon inhibition of the proteasome (29–33). ALLM, on the other hand, failed to demonstrate these effects. Furthermore, cell viability tests showed that the cells were perfectly viable under the inhibitor conditions used (Fig. 2C).

Up-regulation of Proteasome Subunits Occurs in a Concerted Manner—To analyze whether up-regulation of proteasome subunits occurs in a concerted fashion and also holds true for other proteasome subunits, we performed an extensive expression

FIG. 2. Proteasome inhibition is effective and nontoxic in VSMCs. VSMCs were stimulated with 10 μ M c-lactacystin (*c-lacta*), 10 μ M MG132, 1 μ M epoxomicin (*epoxo*), 10 μ M ALLM, or Me₂SO for 8 h. **A**, measurement of the chymotrypsin-like proteolytic activity of cellular extracts. The values are given as the means of three independent experiments \pm S.D. **B**, representative Western blots of VSMCs protein extracts using p53, Hsp70, and ubiquitin antibodies. The arrows indicate the specific bands, *ub*, multi-ubiquitin conjugates. **C**, cell viability assay. The values are given as the means of three independent experiments \pm S.D.



analysis for most of the known subunits of the 26 S proteasome. Proteasome mRNA levels were quantified by real time RT-PCR analysis and normalized to the mRNA level of hypoxanthine phosphoryltransferase. Alterations in mRNA expression are expressed as the relative mRNA levels of treated *versus* control cells.

As summarized in Table I, treatment with c-lactacystin or MG132 resulted in a concerted up-regulation of the mRNA level of all constitutive 20 S and ATPase subunits by a factor of 1.5–4. In most cases, both inhibitors increased mRNA levels of individual subunits to a similar extent, *e.g.* the mRNA of the α 5 subunit (PSMA5) was up-regulated by a factor of 4 or 3.9 by c-lactacystin or MG132, respectively, whereas β 5/MB1 (PSMB5) mRNA was up-regulated by the two inhibitors by a factor of only 1.5. In a few cases, however, *i.e.* α 4 and α 1 subunits (PSMA7 and PSMA6) and the ATPase subunits Rpt2 (PSMC1) and Rpt6 (PSMC5), c-lactacystin and MG132 induced up-regulation of proteasome subunit mRNAs to different extents. The change in mRNA levels of the non-ATPase subunits of the 19 S complex appeared to be only moderate and less uniform. For example, whereas Rpn3 (PSMD3) was induced nearly by a factor of 3.5 by both inhibitors, induction of other lid subunits was less pronounced. Interestingly, mRNA levels of the two immunosubunits LMP2 (PSMB9) and LMP7 (PSMB8) were not at all or only slightly increased, and PA28 α and β subunits were not up-regulated at all (data not shown). In summary, the data shown in Table I demonstrate that treatment of cells with proteasome inhibitors results in an up-regulation of proteasome subunit mRNAs and that this up-regulation occurs in a concerted manner.

The Cellular Response to Proteasome Inhibitors Is Time- and Concentration-dependent—The data obtained thus far suggest the onset of an autoregulatory feedback mechanism in response to inhibition of proteasome activity. If so, then the increased expression of the proteasome ought to be dependent on time and on the concentration of proteasome inhibitor.

We therefore treated VSMCs with different concentrations of MG132 (0.05–1 μ M) for 4, 6, 12, and 24 h. RNA expression of three representative proteasome subunits, α 6 (PSMA1), β 1 (PSMB6), RPT2 (PSMC1), and Hsp70 was quantified by real time PCR analysis. Up-regulation of the mRNA expression of all three proteasome subunits by MG132 occurred in a time- and concentration-dependent manner (Fig. 3A). Proteasome mRNA expression started to increase with concentrations as low as 50 nM and reached its maximal (nearly 2-fold) increase

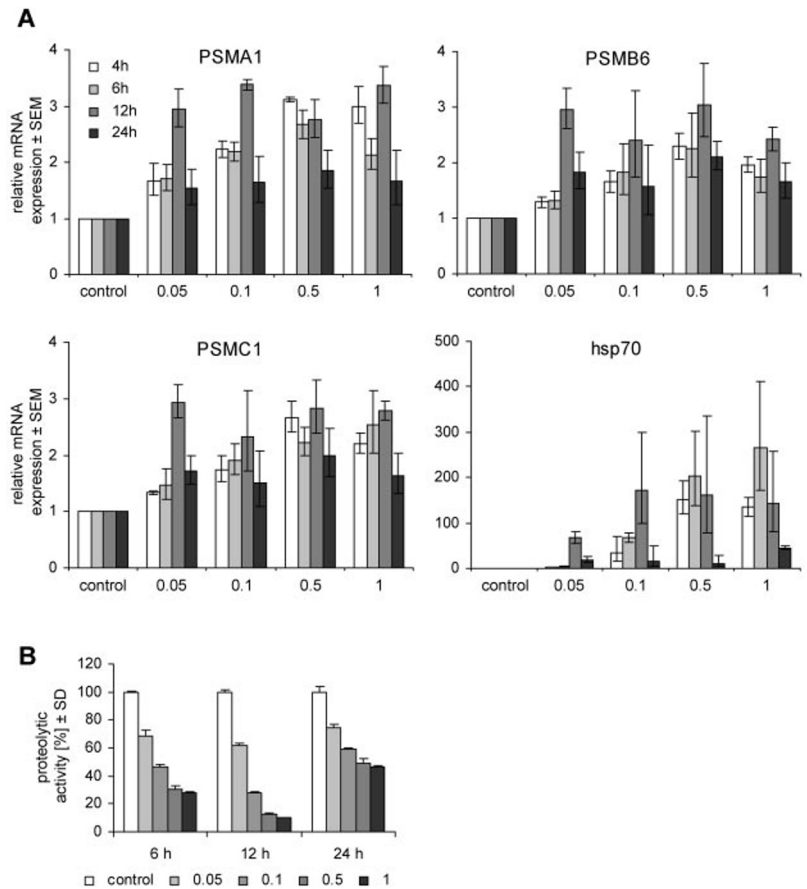
TABLE I
Real time RT-PCR expression analysis of proteasomal subunits
Rat VSMCs were stimulated with 10 μ M c-lactacystin, 10 μ M MG132, or Me₂SO (0.1%) for 6 h and analyzed for expression of proteasomal genes by real time RT-PCR. The values are given as relative RNA expression of proteasome inhibitor-treated *versus* Me₂SO-treated control cells ($2^{-\Delta\Delta Ct}$).

Gene/subunit	Relative RNA expression	
	Ratio c-lactacystin/Me ₂ SO	Ratio MG132/Me ₂ SO
PSMA1/ α 6/C2	3.8	3.4
PSMA2/ α 2/C3	1.8	1.8
PSMA3/ α 7/C8	2.4	2.5
PSMA4/ α 3/C9	2.4	1.8
PSMA5/ α 5/zeta	4.0	3.9
PSMA6/ α 1/iota	2.1	1.3
PSMA7/ α 4/XAPC7	3.6	2.5
PSMB1/ β 6/C5	1.8	1.9
PSMB2/ β 4/C7-1	2.8	2.4
PSMB3/ β 3/C10	3.1	3.4
PSMB4/ β 7/N3	2.3	2.9
PSMB5/ β 5/MB1	1.5	1.5
PSMB6/ β 1/ δ	2.5	2.6
PSMB7/ β 2/Z	2.3	2.6
PSMB8/LMP7/ β 5i	0.5	0.5
PSMB9/LMP2/ β 1i	1.2	1.2
PSMC1/Rpt2/S4	2.1	1.3
PSMC2/Rpt1/S7	2.1	1.9
PSMC3/Rpt5/S6a	2.2	2.1
PSMC4/Rpt3/S6b	2.9	2.7
PSMC5/Rpt6/S8	3.3	2.2
PSMC6/Rpt4/S10b	2.1	2.3
PSMD1/Rpn2/S1	2.4	1.6
PSMD3/Rpn3/S3	3.6	3.7
PSMD4/Rpn10/S5a	1.3	1.6
PSMD8/Rpn12/S14	2.3	1.7
PSMD9/Rpn12/S15	1.6	1.0
PSMD10/Rpn12/p28	0.4	0.3
PSMD11/Rpn6/S9	3	1.3
PSMD13/Rpn13/S11	1.5	1.5

with 0.5 and 1 μ M MG132. mRNA up-regulation started 4 h after treatment with MG132, peaked at 12 h, and started to decline after 24 h. This elevation of mRNA levels was nearly abolished after 48 h (data not shown).

To control the proteasome inhibitory effects of MG132, we monitored Hsp70 mRNA expression (Fig. 3A). Expression of Hsp70 mRNAs also increased in a time- and concentration-dependent manner starting with 0.1 μ M MG132 after 4 h and peaked with 0.5 and 1 μ M after 6 h, reaching induction levels between 200- and 400-fold. Even low concentrations of MG132

FIG. 3. Up-regulation of proteasomal gene expression is time- and concentration-dependent. **A**, VSMCs were stimulated with different doses of MG132 (0.05–1 μ M) or Me₂SO for 4, 6, 12, and 24 h under serum-reduced conditions (2% serum), and RNA expression of representative proteasomal subunits and Hsp70 was analyzed by real time RT-PCR. The values are given as relative RNA expression of proteasome inhibitor-treated *versus* Me₂SO-treated control cells ($2^{-\Delta\Delta ct}$) and are the means of three independent experiments \pm S.E. **B**, VSMCs were treated with MG132 (0.05–1 μ M) or Me₂SO for 6, 12, and 24 h, and the chymotrypsin-like activity was measured in cellular extracts. The values are normalized to controls and are given as the means of three independent experiments \pm S.D.



(50 nM) induced an Hsp70 response in the cells although to a lesser extent and at a later time point (70-fold after 12 h).

The above results were corroborated by analysis of proteasomal activity in VSMC lysates (Fig. 3B). Inhibition of the proteasome by MG132 also occurred in a time- and concentration-dependent manner, *i.e.* 50 nM MG132 inhibited the chymotrypsin-like activity of the proteasome after 6 h by up to 25% compared with controls. Maximal inhibition was achieved with 0.5 and 1 μ M MG132 after 12 h. Thus, the time- and concentration-dependent response closely mirrored the pattern of expressional up-regulation of the proteasomal subunits.

Up-regulation of Proteasome mRNAs Is Dependent on Transcriptional Activation—The observed elevated levels of proteasome mRNAs in response to inhibition of cellular proteasome activity could be the result of either mRNA stabilization or concerted activation of proteasome gene transcription. To investigate the mechanism that underlies the observed up-regulation of proteasomal gene expression, we performed expression analyses in the presence of the transcription inhibitor α -amanitin. VSMCs were treated for 8 h with α -amanitin to block transcription, and MG132 was added for the last 6 h to induce mRNA levels of exemplified proteasomal subunits. As depicted in Fig. 4, we observed a 2–3-fold proteasome inhibitor-mediated increase in RNA expression of $\alpha 6$ (PSMA1), $\beta 1$ (PSMB6), and RPT2 (PSMC1) as expected. This induction was virtually abolished by cotreatment with the transcription blocker (Fig. 4). To control efficient inhibition of RNA-polymerase II-mediated transcription by α -amanitin, we measured Hsp70 mRNA expression under the same conditions. As seen in the lower right panel, the massive induction of Hsp70 mRNA upon inhibition of the proteasome was drastically reduced by cotreatment with α -amanitin (Fig. 4), indicating that α -amanitin effectively blocks transcription of the Hsp70 gene. Because Hsp70 expression is known to be induced by transcriptional

activation, these data suggest that the monitored expressional up-regulation of proteasome genes is the result of RNA-polymerase II-mediated gene transcription.

Up-regulation of Proteasomal mRNAs Results in *de Novo* Formation of Proteasome Complexes—Induction of proteasomal gene expression would only be of physiological relevance to the cell if higher mRNA levels resulted in enhanced biosynthesis of the subunits and finally in *de novo* complex formation. In fact, proteasome inactivation has been shown to delay the autocatalytic processing step necessary for the final maturation step of the 20 S core complex (10, 11, 34, 35). Therefore, the observed increase in the amount of proteasome subunits may in part be explained by the accumulation of proteasome precursor complexes (Fig. 1).

To dissect whether the observed subunit up-regulation is due to proprotein accumulation or *de novo* protein biosynthesis, we performed pulse labeling and immunoprecipitation experiments using an anti-proteasome antiserum raised against the 20 S core complex. Under native conditions this antiserum recognizes complex structures of fully assembled 20 S proteasomes and also partially assembled proteasome precursor intermediates. The cells were treated with the inhibitors c-lactacystin or MG132 to up-regulate proteasome mRNA levels and then pulse-labeled for 16 h to immunoprecipitate mature proteasome complexes (Fig. 5A) or for 6 h to precipitate proteasome precursor complexes (Fig. 5B). As shown in Fig. 5A, inhibitor treatment resulted in an \sim 3-fold increase in precipitable pulse-labeled 20 S proteasomes when compared with the precipitates of control cells. Concomitantly, the amount of precipitated proteasome precursor complexes was increased \sim 5-fold and was paralleled by increased coimmunoprecipitation of the maturation protein POMP (Fig. 5B).

In agreement with the data shown above the enhanced gene expression of proteasome subunits in response to low inhibitor

FIG. 4. Up-regulation of proteasomal gene expression is dependent on transcriptional activation. VSMCs were treated with 50 $\mu\text{g/ml}$ α -amanitin for 8 h to block transcription and co-treated with 1 μM MG132 or 0.1% Me_2SO (control) for the last 6 h. RNA expression of exemplified proteasomal genes (PSMA1, PSMB6, and PSMC1) was analyzed by real time RT-PCR. Expression of Hsp70 was monitored under the same conditions as a positive control for efficient inhibition of transcription.

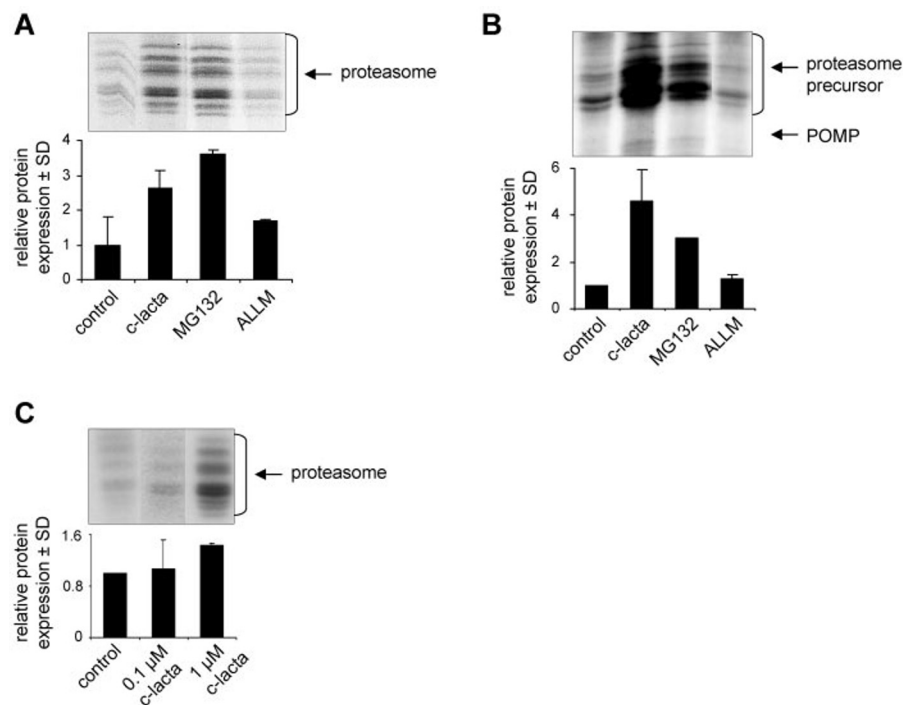
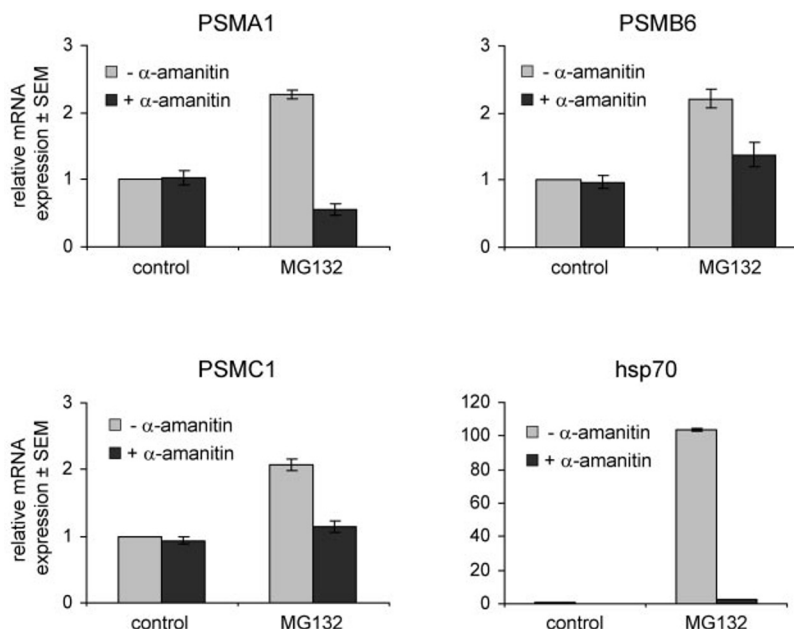


FIG. 5. Induction of proteasomal gene expression results in higher *de novo* protein synthesis of proteasome subunits. VSMCs were treated for 8 h with 10 μM of the inhibitors c-lactacystin (*c-lacta*), MG132, and ALLM or with 0.1% Me_2SO (*control*). For immunoprecipitation of proteasome complexes, the cells were then pulse-labeled with [^{35}S]methionine for 16 h (A) or for 6 h (B) to precipitate proteasome precursor complexes. Equal counts were used for immunoprecipitation processed with an anti-proteasome antibody. For determination of a concentration dependence, VSMCs were treated with 0.1 or 1 μM c-lactacystin (*c-lacta*) and Me_2SO (*control*) for 8 h, labeled for 16 h, and processed as described above (C). Representative immunoprecipitations are shown, and densitometric quantitation was performed in at least two independent experiments \pm S.D.

concentrations resulted in enhanced biosynthesis and biogenesis of proteasomes as measured by immunoprecipitation experiments (Fig. 5C). Even at concentrations of 1 μM c-lactacystin, a clear increase in the signal intensity corresponding to 20 S proteasome complexes was observed in comparison with the control. From these results we can therefore conclude that increased proteasome mRNA levels result in enhanced subunit synthesis and concomitantly in the assembly of new proteasomes.

The Onset of the Entire Biogenesis Program Is Triggered by Proteasome Inhibition—Efficient eukaryotic proteasome biogenesis is essentially assisted by accessory proteins such as Ump1p/POMP-like factors (8). Therefore, we investigated whether proteasome inhibition also affects POMP mRNA and protein levels. As shown in Fig. 6A, treatment of cells with MG132 or c-lactacystin resulted in an \sim 2-fold induction of

POMP mRNA and protein levels that correspond to the observed increase of POMP associated with precursor complexes (Fig. 5B).

To separate proteasomes and their precursor complexes for analysis of proteasome biogenesis, cellular lysates of c-lactacystin- and Me_2SO -treated cells were applied to fractionation by sucrose gradient ultracentrifugation. The following immunodetection of the ATPase subunit Rpt1/MSS1, the 20 S proteasome, the β 1/ δ catalytic subunit, and the maturation protein POMP revealed a pronounced up-regulation of the proteasome subunits and of POMP in response to treatment of cells with proteasome inhibitors (Fig. 6B). Importantly, not only was the amount of proteasome precursor complexes significantly increased upon proteasome inhibition as evidenced by the β 1 proprotein and POMP, but also the levels of matured proteasomes were strongly augmented. Furthermore, a shift toward

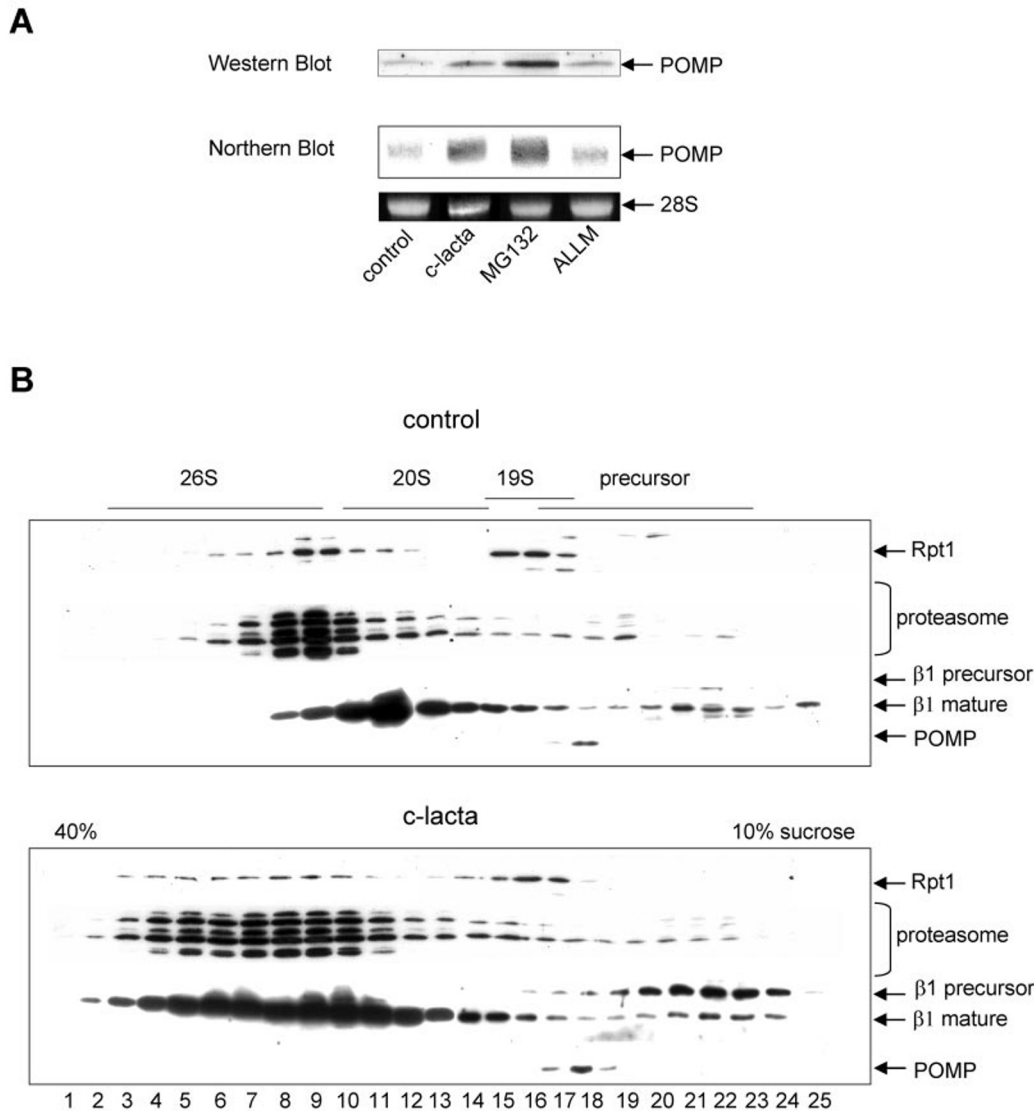


FIG. 6. Induction of *de novo* protein synthesis is accompanied by enhanced proteasome biogenesis. A, induced POMP levels were investigated by treatment of the cells with 10 μ M of the inhibitors c-lactacystin (*c-lacta*), MG132, and ALLM or with 0.1% Me₂SO (*control*) for 6 h in Northern blot or 8 h in Western blot analyses. The blots were processed with a digoxigenin-labeled riboprobe of POMP or a POMP-specific antibody, respectively. Ethidium bromide-stained 28 S rRNA is shown as an internal standard for Northern blotting. The respective bands of the chemiluminograms are indicated by arrows. B, sucrose gradient fractionation of cellular extracts from inhibitor-treated (10 μ M c-lactacystin) and control cells (0.1% Me₂SO). Gradient fractions were analyzed by Western blotting for the indicated subunits, POMP, or an anti-20 S antiserum (proteasome), which recognizes more than one 20 S subunit. The sedimentation positions of different proteasome complexes are shown on the top.

higher molecular weight fractions corresponding to 26 S complexes was observed in inhibitor-treated cells. From these experiments we can conclude that upon proteasome inhibition cells respond with a concerted up-regulation of the complete *de novo* onset of the proteasome biogenesis program.

DISCUSSION

The availability of proteasome-specific inhibitors has made a great impact on our understanding of the catalytic mechanism and the physiological role of the proteasome. Over the past years, the cellular response to proteasome inhibition in particular has been studied extensively with respect to the induction of apoptosis and stress response (29–33, 36). Also the therapeutic potential of proteasome inhibitors as drugs in inflammatory and hyperproliferative diseases is increasingly realized (25, 37–39). Nevertheless, there still exists little knowledge on the effects that are exerted by proteasome inhibitors on the homeostasis of the proteasome system in mammalian cells.

Here, we have analyzed the effect of proteasome inhibition on proteasome gene expression and proteasome biogenesis in

mammalian cells. The experiments show for the first time that proteasome inhibition results in a concerted and enhanced expression of mammalian proteasome genes accompanied by increased *de novo* proteasome biogenesis. Our data imply that mammalian proteasome homeostasis is regulated by a positive autoregulatory feedback mechanism.

Proteasome mRNA expression analysis revealed that mammalian cells respond to inhibition of proteasome activity with a transient up-regulation of most of the 20 and 19 S proteasome subunits, which is time- and concentration-dependent (Table I and Fig. 3). Because proteasome inhibition had almost no effect on the expression of immunosubunits or the PA28 regulator, the data suggest that the observed concerted up-regulation of subunit expression is restricted to components of standard proteasome. With regard to the observed increase in mRNA levels, the question arose regarding whether this is a result of increased mRNA stabilization or of transcriptional activation of proteasome genes. This question was resolved by the use of a transcription inhibitor showing that the observed proteasome

inhibitor-induced up-regulation of proteasomal mRNAs is the product of a coordinated transcriptional activation of the proteasome subunit genes. A 2–4-fold up-regulation of proteasome mRNAs is in good agreement with data that describe alterations of proteasome gene expression for various diseases such as muscle atrophy, acidosis, uremia, diabetes, and cancer (40–44). Similarly, recent cDNA expression array analyses also revealed moderate changes in proteasome gene expression during treatment with drugs, circadian rhythms, and aging processes (45–48). Thus, mammalian cells respond to various stimuli by moderately regulating proteasomal gene expression, which allows the cells to cope with the increased or reduced demands for protein degradation.

Recently, proteasomal activity was inhibited in *Drosophila* S2 cells by gene silencing of different proteasomal genes using RNA interference (24). This approach resulted not only in reduced expression of the targeted subunit but also in increased expression of several nontargeted proteasomal subunits pointing to a mechanism similar to that observed for mammalian cells and yeast. However, in contrast to our data, treatment of S2 cells with lactacystin did not result in increased proteasome gene expression. The most striking difference between RNA interference and proteasome inhibitor-treated S2 cells was the significantly higher percentage of cell death after treatment with proteasome inhibitors (24), whereas the cells were perfectly viable under the conditions used here for proteasome inhibition (Fig. 2C).

Transcriptional activation of proteasome genes in response to inhibition of proteolytic activity seems to be of physiological significance only if this also leads to an increase in cellular proteasome levels. Indeed, transcriptional activation of proteasome genes as a result of proteasome inhibition induces the onset of the complete proteasome biogenesis program and finally the *de novo* formation of matured proteasomes. Eukaryotic proteasome formation requires accessory factors such as Ump1/POMP-like proteins that assist in orchestrating the correct positioning of two sets of seven different α and β subunits and the correct maturation of the β subunits (8). Interestingly, gene expression of the yeast *UMP1* gene is regulated in a Rpn4-dependent manner at the level of transcription as described for all genes encoding proteasomal subunits in yeast (23, 49). In this context it is important to note that gene expression of mammalian Ump1/POMP-like factors appears to be differentially regulated as well. The mRNA levels of the human and mouse homologues are induced after treatment with interferon γ , suggesting that these factors are somehow involved in immunoproteasome biogenesis (15–17). Here, we show that expression of mammalian POMP is also induced in response to proteasome inhibition, thereby facilitating the biogenesis pathway (Figs. 5B and 6). This supports the idea that balanced Ump1/POMP levels are necessary for balanced proteasome levels and thereby for balanced intracellular ATP-dependent proteolysis.

The time- and concentration-dependent kinetics of inhibitor induced proteasome gene expression demonstrate that the time course of gene induction closely matches the degree of inhibition of proteasomal activity *in vivo* (Fig. 3B). Less than 50% inhibition of proteasome activity seems to be sufficient to induce imbalances in protein homeostasis that are registered by the cell and result in induction of proteasome formation. This might suggest that the intracellular accumulation of proteasomal substrates that cannot be efficiently eliminated by the inhibited proteasome induces the coordinated activation of proteasome genes. In yeast, transcriptional regulation of proteasome subunits is dependent on the transcription factor Rpn4, which is itself a proteasomal substrate. Upon proteasome in-

hibition Rpn4 accumulates and activates increased proteasome production (22). In analogy, one would therefore postulate a factor of similar function but so far unknown identity in mammalian cells that senses cellular proteasome-dependent proteolytic activity and controls proteasome homeostasis by an autoregulatory feedback mechanism. Interestingly, the protein sequence of the yeast Rpn4 protein shares no significant similarities with proteins contained in the human genome data base. Furthermore, no similarities to the proteasome-associated control element, the Rpn4-binding site (21), were found in front of mammalian proteasomal subunits, indicating that the principle autoregulatory loop might be similar, but single components of this mechanism may vary. Gene expression of ATP-dependent proteolysis systems in bacteria and yeast undergo mechanisms of self-control. This was shown for σ^{32} in *E. coli*, the CtsR repressor in Gram-positive bacteria both regulating gene expression of Clp proteases (18–20) or Rpn4 of yeast controlling proteasome levels (21–23). Thus, in a remarkably parallel manner, ATP-dependent proteolysis in prokaryotes and eukaryotes is autoregulated by degradation of the same regulators of gene expression that control the production of the protease systems.

For a long time regulation of proteasomal gene expression has been neglected as a possible control mechanism for the amount of proteasomes in the cell. Its elucidation and the dissection of its components will be a future challenge. Our results point toward a more complex understanding of the cellular effects of proteasome inhibition. Because inhibition of the proteasome results in *de novo* synthesis of the proteasome, this newly expressed proteasome might not only compensate inhibited protein breakdown but might even enhance proteasomal degradation for a short time period in a compensatory response. Therefore, inhibition of the proteasome might lead to increased proteasomal activity in case the cell survives inhibitor treatment. In light of these results, the use of proteasome inhibitors as therapeutic agents has to be viewed more carefully.

Acknowledgments—We thank Carola Beier for excellent technical assistance and the members of the Klotzel and Stangl labs for valuable discussions.

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