Selective Accumulation of Aggregation-Prone Proteasome Substrates in Response to Proteotoxic Stress[†]

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Conditions causing an increase in misfolded or aberrant proteins can impair the activity of the ubiquitin/proteasome system (UPS). This observation is of particular interest, given the fact that proteotoxic stress is closely associated with a large variety of disorders. Although impairment of the UPS appears to be a general consequence of proteotoxic insults, the underlying mechanisms remain enigmatic. Here, we show that heat shock-induced proteotoxic stress resulted in conjugation of ubiquitin to detergent-insoluble protein aggregates, which coincided with reduced levels of free ubiquitin and impediment of ubiquitin-dependent proteasomal degradation. Interestingly, whereas soluble proteasome substrates returned to normal levels after a transient accumulation, the levels of an aggregation-prone substrate remained high even when the free ubiquitin levels were restored. Consistently, overexpression of ubiquitin prevented accumulation of soluble but not aggregation-prone substrates in thermally stressed cells. Notably, cells were also unable to resume degradation of aggregation-prone substrates after treatment with the translation inhibitor puromycin, indicating that selective accumulation of aggregation-prone proteins is a consistent feature of proteotoxic stress. Our data suggest that the failure of the UPS to clear aggregated proteins in the aftermath of proteotoxic stress episodes may contribute to the selective deposition of aggregation-prone proteins in conformational diseases.

The integrity of the cellular proteome is continuously threatened by nonnative protein-protein interactions (40), which can readily lead to the formation of insoluble protein aggregates (10). When left unattended, protein aggregates are inherently toxic, probably due to the presence of oligomeric species that interfere with various cellular processes (38). To counteract protein aggregation and its consequences on vital processes, cells are equipped with protective mechanisms that constantly scrutinize the intracellular environment for nonnative proteins and assist in their (re)folding or degradation (16, 41). Although even under normal conditions cells constitutively produce large amounts of aberrant proteins (37, 39), proper handling of nonnative proteins by protein quality control mechanisms becomes particularly challenging during proteotoxic insults. Such insults can be provoked by physiological and environmental factors, such as protein oxidation, aberrant transcription and translation, mutant gene products, and protein denaturation (13, 24). In fact, gradual accumulation of misfolded proteins is associated with a variety of disorders, collectively referred to as conformational diseases (4). An important unresolved question is why protein quality control mechanisms fail to keep cells

free of aberrant proteins, allowing their accumulation in intra- and extracellular protein aggregates.

Misfolded or aberrant proteins are singled out by protein quality control mechanisms and targeted for degradation through the ubiquitin/proteasome system (UPS) (40). In addition, the UPS facilitates regulated turnover of short-lived proteins involved in, for example, cell cycle progression and transcriptional regulation (17). Both aberrant and short-lived proteins are directed for proteasomal degradation through a common mechanism, namely conjugation of chains of the small protein modifier ubiquitin (32). Ubiquitylated proteins selectively bind to the proteasome, a large multisubunit complex that unfolds and translocates ubiquitylated proteins into the inner proteolytic chamber where they are hydrolyzed into short peptide fragments. At the proteasome, specific deubiquitylation enzymes disassemble ubiquitin chains into monomers that can be reused in new rounds of ubiquitylation (27). In addition, ubiquitylation is essential for a broad range of nonproteolytic processes. Various classes of polyubiquitin linkages play a role in membrane trafficking and DNA repair (33). Proteins can also be modified by a single ubiquitin, i.e., monoubiquitylation, which does not target proteins for degradation. Rather, monoubiquitylation regulates protein function in biological processes such as membrane trafficking, DNA repair, and transcriptional regulation (9).

In the present study, we demonstrate that heat shock causes dramatic changes in the intracellular ubiquitin equilibrium, which causes inhibition of proteasomal degradation. In the aftermath of heat shock-induced proteotoxic stress, a major fraction of ubiquitin was conjugated to large immobile and insoluble proteasome substrates, accompanied by a sharp de-

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cline in the levels of free ubiquitin. Ectopic expression of ubiquitin fully restored UPS function and prevented stress-induced impairment of the degradation of soluble substrates but not of aggregation-prone substrates. Also puromycin-induced proteotoxic stress caused a transient accumulation of soluble proteasome substrates, whereas increased levels of aggregation-prone substrate persisted even after UPS function had been restored. Thus, the selective accumulation of aggregation-prone proteins can be explained by the inability of the UPS to cope with these proteins once their levels have been increased as a consequence of proteotoxic stress.

MATERIALS AND METHODS

Plasmids and constructs. The human *UBC* open reading frame (NM_021009) was PCR amplified from cDNA using the primers 5'-GGCAAGCTTTGTGGA TCGCTGTGATCGT-3' and 5'-CGGGATCCGTGAACCTTAAAGGGG A-3'. The *UBC* PCR product was cloned in the HindIII and BamHI sites of mRFP-N1 (where mRFP is monomeric red fluorescent protein) (gift from J. Neefjes, Netherlands Cancer Institute, The Netherlands). The stop codon of *UBC* was replaced by a glycine-encoding codon in order to express a fusion protein consisting of a ubiquitin precursor encoding nine ubiquitin monomers in frame with mRFP (Ub^{9L}-mRFP¹¹, where Ub is ubiquitin) using a QuikChange site-directed mutagenesis kit (Stratagene). The plasmids PAGFP-Ub (where PAGFP is photoactivatable green fluorescent protein [GFP]), PAGFP fused to a ubiquitin lacking internal lysine residues and carrying the mutation G76V (PAGFP-Ub^{K0,G76V}), mRFP-Ub (8), luciferase (Luc)-GFP (gift from H.H. Kampinga, University Medical Centre Groningen, The Netherlands), EGFP-N1 (Clontech), mRFP-N1, and Ub^{9L}-mRFP¹¹ were introduced into the cell lines by electroporation.

Cell culture and transfections. The human melanoma Mel JuSo cell lines were cultured in Iscove's modified Dulbecco medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma-Aldrich), 10 units/ml penicillin, and 10 µg/ml streptomycin (Sigma-Aldrich). Generation of stable cell lines expressing Ub-R-YFP (where YFP is yellow fluorescent protein), Ub^{G76V}-YFP, and YFP-CL1 has been described previously (23). The Mel JuSo ZsProSensor-1 cell line was created by transfection of Mel JuSo cells with the ZsProSensor-1 plasmid (BD Bioscience Clontech) using Lipofectamine (Invitrogen). Clones were selected in the presence of 0.5 mg/ml G418 (Gibco) and screened for ZsGreen fluorescence upon administration of the proteasome inhibitor MG132 (Biomol).

For electroporation, cells were harvested and resuspended in 2 mM HEPES, 15 mM K₂HPO₄-KH₂PO₄, pH 7.2, 250 mM mannitol, and 1 mM MgCl₂. The cell suspension was mixed with 10 µg of plasmid DNA, transferred to 4-mm electroporation cuvettes (Bio-Rad), and subsequently pulsed with the Bio-Rad GenePulser (15 pulses; 350 V; 25 µF). Directly after electroporation, cultivation medium was added, and cells were cultivated for 24 h.

Where indicated, proteasome activity was inhibited with 200 nM epoxomicin (Biomol) or 10 µM MG132 (Biomol). Proteotoxic stress was induced by a 1-h incubation at 42°C or a 1-h incubation with 5 µg/ml puromycin (BD Biosciences). Inhibition of protein translation was performed by the addition of 50 µg/ml cycloheximide for the indicated times.

Fluorescence microscopy and flow cytometry. Mel JuSo cell lines were grown on coverslips, fixed with 4% paraformaldehyde (Sigma) or 3:1 methanol-acetone for γ-tubulin detection, permeabilized with 0.2% Triton X-100, and stained with Hoechst 33258 (Molecular Probes). Immunostainings were performed using rabbit polyclonal ubiquitin and mouse monoclonal γ-tubulin antibodies (Sigma-Aldrich). Fixed cells were examined with a Zeiss LSM 510 confocal laser scanning microscope (Plan-Neofluar 40× oil objective with a 1.3 numerical aperture). Quantitative analyses of fluorescence intensities were performed using ImageJ software. Flow cytometry was performed with a FACSort flow cytometer (Becton, Dickinson, and Co.) and analyzed with Cellquest software.

Time-lapse imaging. Confocal laser scanning microscopy was performed using a Zeiss LSM 510 META equipped with a cell culture microscopy stage at a constant temperature of 37°C. Photoactivation of PAGFP-Ub in transfected cells was performed by applying a single pulse with 405-nm laser light at full intensity to a small region in the cell. Prior to and directly after the 405-nm laser pulse, images were taken. The fluorescence intensities in nonexposed regions of the cellular compartments were quantified using ImageJ software.

For fluorescent recovery after photobleaching (FRAP), the GFP fluorescence in a 0.8-µm strip of a selected cell was bleached using a single pulse of the 488-nm argon laser at 100% intensity. GFP fluorescence was monitored every 20

ms using the 488-nm argon laser excitation at 0.5% intensity. The obtained fluorescence recovery curves were normalized to the prebleach fluorescence.

Protein solubility assay. For determining protein solubility, cells were trypsinized and lysed in 1% Triton X-100 in phosphate-buffered saline supplemented with complete protease inhibitor cocktail (Roche Diagnostics). Supernatant and pellet fractions were separated by centrifugation at 10,000 × g for 10 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Immunoprecipitation. Untreated and thermally stressed cells were harvested and lysed in 1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, containing 20 mM *N*-ethylmaleimide (Sigma-Aldrich), 10 µM MG132 (Biomol), and complete protease inhibitor cocktail (Roche). Lysates were precleared by centrifugation at 10,000 × g for 10 min. The YFP-tagged proteins were immunoprecipitated using rabbit polyclonal anti-GFP antibodies (Molecular Probes).

Western blot analysis. Cells were harvested and lysed in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and transferred to Protan BA 85 nitrocellulose (Whatman) or Immobilon-P polyvinylidene difluoride (Millipore) membranes. The membranes were probed with rabbit polyclonal antibodies directed against GFP (Molecular Probes), mRFP (gift from J. Neefjes, Netherlands Cancer Institute, The Netherlands), ZsGreen (BD Biosciences) or ubiquitin (Dako) or mouse monoclonal antibodies directed against GFP (Roche), conjugated ubiquitin (FK2; Biomol), p53 (DO-1; Santa Cruz), c-Myc (9E10; Santa Cruz), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Research Diagnostics). After incubation with horseradish peroxidase-conjugated secondary antibodies, the blots were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech). Densitometric analysis was performed using ImageJ software.

RESULTS

Thermal proteotoxic stress impairs ubiquitin-dependent proteasomal degradation. To assess the functional status of the UPS during proteotoxic stress and the subsequent recovery phase, we subjected cells to mild heat shock conditions since this type of insult is titratable and reversible. We found that exposure of human melanoma Mel JuSo cells to 42°C for 1 h was well tolerated and did not affect cell viability (data not shown). To monitor the functionality of the UPS, we exploited three cell lines, each stably expressing a different YFP-based reporter substrate of the UPS (23). The Ub-R-YFP, Ub^{G76V}-YFP, and YFP-CL1 reporter substrates are targeted for constitutive degradation by an N-end rule (N-terminal arginine), a ubiquitin fusion degradation (N-terminal ubiquitin), or a CL1 (C-terminal hydrophobic polypeptide of 16 amino acids) degradation signal, respectively (26).

Upon exposure of YFP reporter cell lines to 42°C, the levels of the reporters rapidly accumulated during the 2 h following the temperature elevation (Fig. 1A). Whereas Ub-R-YFP and Ub^{G76V}-YFP accumulated homogeneously throughout the nuclear and cytosolic compartments, a fraction of YFP-CL1 accumulated in a single inclusion in the perinuclear region in a vast majority of stressed cells (Fig. 1B). This indicates that YFP-CL1 is an aggregation-prone protein, which is in line with earlier studies showing that this particular substrate aggregates (22), forms aggresomes (23), and cosequesters with pathological aggregation-prone proteins (44). Biochemical analysis confirmed the presence of YFP-CL1 aggregates under stress conditions since heat shock caused redistribution of YFP-CL1 from Triton X-100-soluble to -insoluble fractions, whereas Ub-R-YFP and Ub^{G76V}-GFP remained soluble (Fig. 1C). Quantitative analysis performed by flow cytometry showed that the increase in substrate levels was similar for all three reporter substrates and corresponded approximately to the levels after a 1-h inhibition of the UPS with a potent inhibitor of the proteasome (see Fig. S1 in the supplemental material). We

FIG. 1. Thermal proteotoxic stress impairs ubiquitin-dependent proteasomal degradation. (A) Fluorescence micrographs of Mel JuSo cells expressing Ub-R-YFP, Ub^{G76V}-YFP, and YFP-CL1. YFP fluorescence in untreated cells, heat-shocked cells, and cells 2 h after heat shock is shown. Scale bar, 50 μ m. (B) Magnifications of the cells as depicted in panel A at 2 h after heat shock. The arrows in the micrograph of the YFP-CL1 cells indicate aggregate-like structures. Scale bar, 20 μ m. (C) Western blot analysis of Triton X-100 solubility assay of untreated and heat-shocked Ub-R-YFP, Ub^{G76V}-YFP, and YFP-CL1 cells. The YFP reporter levels were detected in total lysates (T), insoluble pellet fraction (P), and soluble fraction (S) using GFP antibodies. GAPDH is used as a control for soluble proteins. (D) Histogram plots from flow cytometric analyses of the ZsProSensor-1 reporter cell line, which was left untreated, treated with proteasome inhibitor for 8 h, or heat shocked. One representative experiment out of three is shown. (E) Quantification of flow cytometric analyses of ZsProSensor-1 fluorescence up to 8 h after heat shock. The fluorescence intensities of ZsProSensor-1 in untreated cells are normalized to 1. Error bars are standard deviations ($n = 5$). (F) Fluorescence micrographs of Mel JuSo cells expressing ZsProSensor-1; cells were either left untreated or incubated for 1 h at 42°C (middle panel) or for 1 h with 10 μ M MG132 (right panel). Scale bar, 50 μ m. (G) Western blot analysis of Ub-R-YFP cells that were left untreated, heat shocked, and followed for 0, 2, 4, 6, and 8 h after heat shock (post-heat shock). The blots were probed with antibodies against GFP, ubiquitin, and GAPDH. Ubiquitin conjugates and free ubiquitin are indicated. A long exposure of the band corresponding to free ubiquitin is shown. Exp., exposure. (H) Western blot analysis of anti-GFP immunoprecipitations of parental Mel JuSo (par) and Ub^{G76V}-YFP Mel JuSo lysates (YFP) that

conclude that thermally induced proteotoxic stress causes a general impairment of the UPS.

Although most proteasome substrates are degraded in a ubiquitin-dependent fashion, a few substrates have been reported that do not require ubiquitylation prior to proteasomal degradation (25). We generated a stable cell line that expresses the fluorescent UPS reporter ZsProSensor-1, which is composed of ZsGreen fluorescent protein destabilized by the ornithine decarboxylase degradation signal. Treatment of this cell line with a proteasome inhibitor caused accumulation of ZsProSensor-1 (Fig. 1D and E). However, the same proteotoxic stress regimen that caused a substantial accumulation of the ubiquitin-dependent substrates did not affect degradation of ZsProSensor-1, whereas 1 h of proteasome inhibition showed an increase in fluorescence intensity (Fig. 1D to F). We monitored the steady-state levels of ZsProSensor-1 up to 8 h after the heat shock but did not detect an increase in emitted fluorescence (Fig. 1E) or protein levels (see Fig. S2 in the supplemental material). These data show that the degradation of a ubiquitin-independent substrate was not inhibited and imply that proteotoxic stress selectively impairs degradation of ubiquitin-dependent substrates.

In the next set of experiments, we followed in parallel the accumulation of a reporter substrate and the levels of free and conjugated ubiquitin. Western blot analysis showed that heat shock caused an increase in high-molecular-weight ubiquitin conjugates mirrored by a substantial reduction in the levels of free endogenous ubiquitin (Fig. 1G). The levels of free ubiquitin gradually increased after the transient reduction observed directly after heat shock, in line with the fact that the human genome encodes two stress-inducible ubiquitin precursor genes (12). Two hours after the proteotoxic insult when the levels of free ubiquitin had been restored, the levels of Ub-R-YFP were stabilized, suggesting that degradation of soluble substrates had been resumed. After a short delay, excess of Ub-R-YFP was gradually cleared from the cells. The reduction in free ubiquitin levels was accompanied by a decrease in the amount of polyubiquitylated Ub^{G76V}-YFP (Fig. 1H). This suggests that the increase in polyubiquitylated proteins in thermally stressed cells occurs at the expense of the levels of free ubiquitin and ubiquitylated reporter substrates, leading to their stabilization.

Thermally induced proteotoxic stress causes global changes in the ubiquitin equilibrium. Our observation that degradation of ubiquitin-dependent but not ubiquitin-independent substrates is hampered during proteotoxic stress and that there is an inverse correlation between the levels of free ubiquitin and the reporter substrate suggested that limited ubiquitylation may be involved in the stress-induced UPS dysfunction. In order to study the dynamics of ubiquitin in living cells, we used a cell line stably expressing GFP-Ub. Several studies showed that GFP-Ub behaves in a way very similar to endogenous

ubiquitin (8, 34, 35). We observed the formation of GFP-Ub-positive inclusions in the perinuclear region of cells exposed to heat shock (Fig. 2A). These structures were not observed with the conjugation-deficient GFP-Ub^{K0,G76V} fusion (see below). The most predominant of the GFP-Ub inclusions were associated with γ -tubulin, a localization typical for aggresomes (Fig. 2B). Notably, aggresomes are the primary disposal sites for aggregation-prone proteins (18, 42), underscoring the notion that a large fraction of stress-induced ubiquitylated substrates are misfolded proteins (40).

Next, we photoactivated PAGFP-Ub in a small region in the cytosol and quantified the instant redistribution of PAGFP-Ub to the nucleus. Conjugation of PAGFP-Ub to a protein substrate is likely to result in a product that exceeds the size exclusion limit for passive diffusion through the nuclear pore, whereas free PAGFP-Ub based on its molecular weight can, like untagged ubiquitin, freely diffuse between the compartments (8). We therefore used the instant exchange between the cytosolic and nuclear compartments as a surrogate marker for free ubiquitin levels. Although due to its larger molecular weight the behavior of PAGFP-Ub may be slightly different from untagged ubiquitin, we anticipated that the comparison of the diffusion of this fusion protein under two different conditions might nevertheless give useful information on the pool of free ubiquitin in living cells. We found that heat shock significantly decreased the amount of PAGFP-Ub that reached the nucleus (Fig. 2C and D) but did not affect the exchange of the conjugation-deficient PAGFP-Ub^{K0,G76V} mutant (Fig. 2D). Thus, analysis of PAGFP-Ub in living cells supports the observation that heat shock causes a reduction in the pool of free ubiquitin.

Increased conjugation of ubiquitin to stress-induced immobile proteins. In order to distinguish mobile from immobile ubiquitin pools, we performed FRAP experiments (36) in control and stressed cells stably expressing GFP-Ub or the GFP-Ub^{K0,G76V} mutant. The heat shock-induced formation of γ -tubulin, GFP-Ub-positive foci was dependent on ubiquitin conjugation as this was not observed in GFP-Ub^{K0,G76V}-expressing cells (see Fig. S3 in the supplemental material). FRAP analysis revealed that heat shock caused a substantial increase in the pool of immobile ubiquitin in the nuclear and cytosolic compartments (Fig. 3A, upper panels). Proteotoxic stress did not affect the mobility of GFP-Ub^{K0,G76V}, indicating that the stress-induced immobilization is due to conjugation to ubiquitin to immobile substrates (Fig. 3A, lower panels). Immobilization of nuclear ubiquitin was not attributed to conjugation of ubiquitin to histones (a major fraction of immobile nuclear ubiquitin) since heat shock has been shown to cause a global deubiquitylation of histones (8). This suggests that the immobilization of ubiquitin is due to conjugation to ubiquitin of stress-induced immobile targets that are localized in the nucleus and cytosol.

had been left untreated (Untr) or had been subjected to a heat shock (HS). Samples were collected directly after heat shock. As a control, immunoprecipitations were performed with beads only. The blots were probed with antibodies that recognize conjugated ubiquitin (upper panel) or GFP (lower panel). Heat shock causes a reduction in the amount of polyubiquitylated Ub^{G76V}-YFP. Note that the high-molecular-weight signal in heat-shocked samples is mainly due to background (compare the third, fourth, and eighth lanes). α , anti; IP, immunoprecipitation; Mw, molecular weight in thousands.

FIG. 2. Thermally induced proteotoxic stress causes global changes in the ubiquitin equilibrium. (A) Fluorescent micrographs of GFP-Ub untreated cells, cells subjected to heat shock, and cells 2 h post-heat shock. The lower panels are higher-magnification micrographs of representative cells, depicted in the upper panel by squares. Aggregate-like structures are indicated by arrows. Scale bars, 20 μ m (top) and 10 μ m (bottom). (B) Fluorescent micrographs of the γ -tubulin immunostaining of untreated GFP-Ub cells and GFP-Ub cells 2 h after heat shock. The fluorescent signals of GFP-Ub (right), γ -tubulin (middle), and merged images (right) are shown. GFP-Ub inclusions colocalized with the centrosomal marker γ -tubulin, indicated by arrows. Scale bar, 10 μ m. (C) Fluorescent micrographs of untreated (upper panels) and heat-shocked (lower panels) cells expressing PAGFP-Ub before photoactivation in a selected region in the cytosol (prepulse) and directly after photoactivation (postpulse). The contours of the cells and the photoactivated regions (indicated by red-lined squares) are depicted. The images are shown in false colors to illustrate differences in fluorescence intensities. The look-up table bar is provided on the right. (D) Quantification of the increase in fluorescence intensities in the nucleus after photoactivation of cytosolic PAGFP-Ub and PAGFP-Ub^{K0,G76V} in untreated and heat-shocked cells. The overall intensity is averaged from the intensities in three nuclear regions of interest (ROI), indicated by the filled gray squares in the diagrams of panel C. Error bars are standard deviations ($n = 5$), and the Student's t test values are shown.

The major sources of proteasome substrates that arise during heat shock are thermolabile proteins (31). Therefore, we tested whether the increased immobilization of ubiquitin could be explained by conjugation of ubiquitin to unfolded proteasome substrates. Heat shock also immobilized a large fraction of GFP-tagged firefly luciferase (Luc-GFP), a thermolabile protein (28), in the nucleus and cytosol (Fig. 3B, upper panels), while GFP remained highly mobile (Fig. 3B, lower panels). Furthermore, Luc-GFP and mRFP-Ub cosequestered in aggregate-like structures in the perinuclear region of cells exposed to heat shock (Fig. 3C). Heat shock caused a clear increase in the amount of Triton X-100-insoluble ubiquitin conjugates (Fig. 3D) and GFP-Ub conjugates (Fig. 3E). Together, these data indicate that the stress-induced disturbance of the ubiquitin equilibrium is accompanied by increased conjugation of ubiquitin to immobile and detergent insoluble substrates.

Restoration of ubiquitin levels is followed by clearance of soluble but not aggregation-prone proteasome substrates. To gain better insight into the behavior of different substrates

after heat shock, we followed the levels of our reporter substrates for several hours after the insult. A striking difference was observed between the levels of the soluble (Ub-R-YFP and Ub^{G76V}-YFP) and the aggregation-prone (YFP-CL1) reporter substrates after heat shock. The initial accumulation of Ub-R-YFP and Ub^{G76V}-YFP during the first 2 to 4 h after heat shock was followed by clearance of the reporters to low steady-state levels, suggesting full restoration of the UPS (Fig. 4A). In contrast, the increased levels of YFP-CL1 persisted (Fig. 4A) and remained high up to 16 h after heat shock, the latest time point assessed (data not shown). Similar patterns were found when steady-state levels of all reporters were analyzed by Western blotting (Fig. 4B).

The inverse correlation between free ubiquitin and proteasome substrates was also observed for the endogenous proteasome substrates p53 and c-Myc (Fig. 4C). Short-lived proteins in general are involved in signaling pathways, and elevation in their steady-state levels can reflect transcription upregulation. To determine whether increased p53 and c-Myc levels are caused by either UPS impairment or stress-induced transcrip-

FIG. 3. Increased conjugation of ubiquitin to stress-induced immobile proteins. (A) FRAP curves of GFP-Ub and GFP-Ub^{K0,G76V} in the nucleus and cytoplasm in untreated and heat-shocked cells. (B) FRAP curves of Luc-GFP and GFP in the nucleus and cytoplasm in untreated and heat-shocked cells. The curves in panels A and B represent the average of 10 cells. (C) Fluorescent micrographs from untreated and heat-shocked cells coexpressing mRFP-Ub and Luc-GFP. The merged images are shown in the lower panels. Scale bar, 10 μm. (D) Western blot analysis of Triton X-100 solubility assays of untreated and heat-shocked parental Mel JuSo cells. Ubiquitin conjugates are detected in total cell lysate (T), detergent-insoluble pellet fraction (P), and soluble fraction (S). (E) Western blot analysis of Triton X-100 solubility assays of untreated and heat-shocked Mel JuSo cells expressing GFP-Ub. GFP-Ub conjugates are detected in total cell lysate (T), detergent-insoluble pellet fraction (P), and soluble fraction (S). α, anti; Mw, molecular weight in thousands.

tion, we inhibited protein translation following heat shock by treating with cycloheximide. Upon inhibition of protein synthesis and in the absence of proteotoxic stress, cellular levels of p53 and c-Myc drastically dropped, showing that these proteins are short-lived in Mel JuSo cells (Fig. 4D, lanes 1 to 3). Exposure of the cells to heat shock caused a striking stabilization of p53 and c-Myc, suggesting that proteasomal degradation of these proteins was blocked (Fig. 4D, lanes 4 to 6). In the absence of translation inhibition, p53 and c-Myc levels increased after proteotoxic stress in line with stress-induced stabilization (Fig. 4D, lane 7 to 9). These data demonstrate that both soluble and aggregation-prone proteasome substrates are stabilized following heat shock but that only soluble proteasome substrates are cleared once UPS function is restored.

Overexpression of ubiquitin prevents accumulation of soluble but not aggregation-prone proteasome substrates. The inverse correlation between the levels of free ubiquitin and soluble substrates provides indirect evidence suggesting that the

reduction in free ubiquitin may be responsible for UPS dysfunction in cells exposed to heat shock. An important prediction based on this model is that increasing the ubiquitin pool prior to the insult should prevent UPS dysfunction. Since total cellular ubiquitin levels are already high, overexpression of ubiquitin is not trivial. Accordingly, we found previously that expression of a single ubiquitin-encoding open reading frame from a cytomegalovirus promoter only marginally increased ubiquitin levels (8). To allow robust overexpression of ubiquitin, we cloned the open reading frame of a ubiquitin precursor encoding nine ubiquitin monomers in frame with mRFP. Since ubiquitin precursors are rapidly processed by ubiquitin C-terminal hydrolases (45), this chimeric Ub^[9]-mRFP^[1] precursor is expected to be rapidly converted into nine wild-type ubiquitin monomers and one mRFP protein (Fig. 5A).

Ubiquitin immunostaining of cells transiently transfected with the Ub^[9]-mRFP^[1] construct showed that mRFP-positive cells had increased levels of ubiquitin (Fig. 5B). Quantitative

FIG. 4. Restoration of ubiquitin levels is followed by clearance of soluble but not aggregation-prone proteasome substrates. (A) Quantification of the YFP fluorescence from flow cytometric analyses of Ub-R-YFP, Ub^{G76V}-YFP, and YFP-CL1 cell lines up to 8 h after heat shock. The fluorescence intensities of the YFP reporters are normalized to 0% in untreated cells and 100% for cells treated with proteasome inhibitor for 8 h. Error bars are standard errors of the means ($n = 5$). (B) Western blot analyses of the Ub-R-YFP, Ub^{G76V}-YFP, and YFP-CL1 cell lines before and after heat shock for 0, 2, 4, 6, and 8 h. The reporter levels are detected with GFP antibodies. GAPDH levels are shown as a loading control. (C) Western blot analysis of the endogenous short-lived proteins p53 and c-Myc. (D) Turnover of p53 and c-Myc in control cells (lanes 1 to 3) and heat-shocked cells (lanes 4 to 6). Cycloheximide (CHX) was added to block protein synthesis. In the absence of CHX, cells accumulated p53 after heat shock (lanes 7 to 9). The blots were probed with p53 and c-Myc antibodies. GAPDH loading controls are shown in panels C and D. Untr., untreated; HS, heat shock; arrow, c-Myc band; asterisk, nonspecific band.

analysis revealed a linear correlation between mRFP and ubiquitin levels in transfected cells (Fig. 5C) and showed that ubiquitin levels from Ub^[9]-mRFP^[1]-expressing cells exceeded the levels that were obtained by stress-induced expression (Fig. 5D). Western blot analysis on total lysates of transiently transfected Mel JuSo cells confirmed that expression of Ub^[9]-mRFP^[1] caused an increase in the density of the free ubiquitin band (Fig. 5E and F).

We next used expression of the Ub^[9]-mRFP^[1] precursor to increase the ubiquitin levels before the proteotoxic insult. Microscopic analysis showed that heat-shocked Ub-R-YFP (Fig. 5G; see also Fig. S4A in the supplemental material) and Ub^{G76V}-YFP (Fig. 5H) cells expressing Ub^[9]-mRFP^[1] did not accumulate the reporter substrate, unlike untransfected cells or cells expressing mRFP. Ubiquitin overexpression did not prevent accumulation of reporters in response to proteasome inhibitor, which was anticipated since the proteasome is acting downstream of ubiquitylation (Fig. 5G and H). Strikingly, we consistently found that overexpression of ubiquitin failed to prevent the accumulation of the aggregation-prone YFP-CL1 substrate (Fig. 5I; see also Fig. S4B in the supplemental material). We conclude that changes in the ubiquitin equilibrium are responsible for UPS impairment during thermal proteotoxic stress. The failure to sustain degradation of aggregation-prone substrates in stressed cells even when the ubiquitin levels

had been experimentally increased suggests that these substrates are intrinsically different from soluble proteasome substrates.

Puromycin-induced proteotoxic stress also causes UPS dysfunction and persistent accumulation of aggregation-prone proteasome substrates. Puromycin causes proteotoxic stress by inducing premature release of polypeptide chains from ribosomes (3). We noticed that puromycin treatment, unlike heat shock, did not cause a reduction in the levels of free ubiquitin (Fig. 6A). Accordingly, diffusion of PAGFP-Ub between the cytosolic and nuclear compartments was not affected in living cells exposed to puromycin (Fig. 6B). The fact that puromycin causes proteotoxic stress in the absence of a reduction in free ubiquitin levels allowed us to assess whether the selective accumulation in aggregation-prone substrates is confined to conditions that cause ubiquitin stress or whether it is a more general feature of proteotoxic stress-induced UPS impairment, regardless of the nature of the molecular mechanism responsible for the dysfunction. Exposure of cells to puromycin caused an increase in soluble and aggregation-prone substrates similar to that observed in cells subjected to heat shock (Fig. 6C and D). Notably, also in the aftermath of puromycin-induced proteotoxic stress, cells were able to clear the accumulated soluble proteasome substrates, whereas the levels of the aggregation-prone substrate remained high up to 8 h after

FIG. 5. Overexpression of ubiquitin prevents accumulation of soluble proteasome substrates. (A) Schematic representation of the strategy for quantitative overexpression of ubiquitin with the Ub⁹-mRFP¹ construct. (B) Fluorescence micrograph of cells expressing Ub⁹-mRFP¹. Fluorescence of mRFP (upper panel) and immunostaining for ubiquitin (lower panel) are shown. The contours of untransfected cells are outlined. Scale bar, 20 μ m. (C) The mRFP fluorescence intensities of Ub⁹-mRFP¹-transfected cells are plotted against the corresponding ubiquitin immunostaining intensities. Each spot represents one cell. The correlation coefficient (R^2) is shown. (D) Quantitative analysis of ubiquitin immunostaining intensities in untreated parental cells after thermal stress induction (8 h after heat shock) and expressing Ub⁹-mRFP¹. Error bars are standard deviations ($n = 50$). (E) Western blot analysis of ubiquitin and mRFP levels in parental Mel JuSo cells and in mRFP-N1- or Ub⁹-mRFP¹-transfected cells. The band corresponding to free ubiquitin is indicated. GAPDH levels are shown as a loading control. (F) Analysis of the band densities corresponding to free ubiquitin on Western blots of untransfected (–) and mRFP-N1- or Ub⁹-mRFP¹-transfected cell extracts. Error bars are standard error of the means ($n = 10$), and a Student's t test value between untransfected and Ub⁹-mRFP¹-transfected cells is shown. (G to I) Quantitative analysis of the YFP reporter accumulation of mRFP- and Ub⁹-mRFP¹-expressing Ub-R-YFP (G), Ub^{G76V}-YFP (H), and YFP-CL1 (I) cells; untreated (Untr) cells, cells 2 h after heat shock (post-HS), and cells incubated with proteasome inhibitor (Inh) were analyzed. YFP intensities from RFP-negative and -positive cell populations are shown. Error bars represent standard errors of the means ($n = 50$ to 100). There is a significant differences between Ub⁹-mRFP¹-expressing cells, control, and mRFP-positive cells (unpaired Student's t test, $P < 0.001$). AU, arbitrary units; α , anti; CMV, cytomegalovirus; Mw, molecular weight in thousands.

FIG. 6. Puromycin-induced proteotoxic stress causes UPS dysfunction and persistent accumulation of aggregation-prone proteasome substrates. (A) Western blot analysis of parental Mel JuSo cells that were left untreated, heat shocked, and followed for 0, 2, 4, 6, and 8 h after puromycin treatment (postpuromycin). The blot was probed with antibodies against ubiquitin and GAPDH. Ubiquitin conjugates and free ubiquitin are indicated. (B) Quantification of the increase in fluorescence intensities in the nucleus after photoactivation of cytosolic PAGFP-Ub and PAGFP-Ub^{K0,G76V} in untreated cells and cells treated for 1 h with puromycin. The overall intensity is averaged from the intensities in three nuclear regions of interest. Error bars are standard errors of the means ($n = 5$). (C) Western blot analyses of the Ub-R-YFP, Ub^{G76V}-YFP, and YFP-CL1 cell lines before and after puromycin treatment for 0, 2, 4, 6, and 8 h. The reporter levels are detected with GFP antibodies. GAPDH levels are shown as a loading control. (D) Quantification of the YFP fluorescence from flow cytometric analyses of Ub-R-YFP, Ub^{G76V}-YFP, and YFP-CL1 cell lines up to 8 h after puromycin treatment. The fluorescence intensities of the YFP reporters are normalized to 0% in untreated cells and to 100% for cells treated with proteasome inhibitor for 8 h. Error bars are standard errors of the means ($n = 3$). Mw, molecular weight in thousands.

the puromycin exposure (Fig. 6C and D). We conclude that the selective accumulation of aggregation-prone substrates is a general feature of proteotoxic stress-induced impairment of the UPS and is not limited to ubiquitin stress.

DISCUSSION

While it seems plausible that UPS impairment reflects a lack of timely adaptation to the increased needs for protein degradation during proteotoxic stress, potential rate-limiting steps in the UPS that may be saturated have only been identified for a few stress conditions (11, 20). An attractive possibility is that an event shared by various ubiquitin-dependent substrates is responsible for heat shock-induced UPS impairment since ubiquitin expression is induced by thermal stress (12). Our observation that overexpression of ubiquitin can prevent UPS dysfunction in thermally stressed cells supports this hypothesis and strongly suggests that ubiquitin deficiency can be directly

responsible for stress-inflicted UPS impairment. It is noteworthy that puromycin, while also causing UPS impairment, does not induce any of the typical signs of ubiquitin stress such as a reduction in the levels of free ubiquitin and an increase in polyubiquitin conjugates. We have recently also reported that UPS dysfunction caused by disease-associated prion protein may be attributed to its ability to directly inhibit the proteolytic activity of the proteasome (20). Furthermore, it has been shown that UPS dysfunction caused by protein aggregation is a direct effect of the sequestration of VCP/Ufd1/Npl4, a complex essential for the degradation of some proteasome substrates, in protein inclusions (11). It is probably intrinsic to the complex nature of the UPS that malfunctioning of this system can be caused by interfering at various crucial steps that may be differentially affected depending on the stress condition.

Stress-induced ubiquitin disequilibrium can cause UPS impairment. The data presented in this study show that ubiquitin is implicated in heat shock-inflicted impairment of the UPS in

FIG. 7. Schematic representation of the UPS impairment induced by thermal proteotoxic stress. Model for the functionality of the UPS in the absence of proteotoxic stress (A), in cells experiencing proteotoxic stress (B), and during the recovery phase after the proteotoxic insult (C).

mammalian cells and that overexpression of ubiquitin alone is sufficient to prevent UPS dysfunction, conclusively showing that ubiquitin is a limiting factor during thermal proteotoxic stress. Although this may seem surprising given the high intracellular levels of ubiquitin in metazoans (7), it is well established that two ubiquitin precursor encoding genes are stress inducible (12) and that the yeast *Saccharomyces cerevisiae* has specific mechanisms to increase disassembly of polyubiquitin chains during proteotoxic stress (15), suggesting an intimate link between cellular stress and the ubiquitin homeostasis.

Despite the fact that cells express large quantities of ubiquitin (7), it appears that the levels of ubiquitin available for ubiquitylation can nevertheless be limiting under certain conditions (Fig. 7A). Ubiquitin modifications are reversible due to the counteracting activity of deubiquitylation enzymes, which serve to replenish the pool of free ubiquitin (45). This dynamic process of ubiquitylation and deubiquitylation appears to be essential for cellular homeostasis since a large number of ubiquitin-dependent processes compete for the limited amount of free ubiquitin (8). We have proposed that this delicate ubiquitin equilibrium may be of functional significance as it links various ubiquitin-dependent cellular processes in an intricate multilayered network with ubiquitin as a common regulator (14). The present study implies that the bulk of the proteasome substrates degraded under normal conditions face a shortage of ubiquitin during heat shock, resulting in stabilization of soluble and aggregation-prone short-lived proteins (Fig. 7B).

Several observations in this study suggest that a large fraction of the stress-induced ubiquitylated proteins may not be targeted for proteasomal degradation. First, the increase in polyubiquitin adducts is primarily due to an increase in large, insoluble, and immobile polyubiquitylated proteins, characteristics that do not match the features of typical proteasome substrates, which are generally soluble and mobile entities (32). Second, whereas protein aggregates resist proteasomal degradation (43) and can obstruct proteasome activity (1), the proteasomes in thermally stressed cells are operative since increasing ubiquitin levels restore degradation, making it unlikely that these proteasomes are actively engaged in the attempted degradation of these insoluble and immobile polyubiquitylated proteins. Although we cannot exclude at this point that ubiquitylation of these proteins reflects a misplaced and failed attempt to target them for proteasomal degradation, it seems plausible that a fraction of the stress-induced ubiquitylation may serve another function in the stress response. In this respect, it is noteworthy that a number of recent studies suggest that formation of stress granules (21) and aggresomes (19, 29), as well as the autophagy-mediated clearance of aggregated proteins (2, 30), relies on polyubiquitylation. Thus, the enhanced ubiquitylation of insoluble, immobile substrates may reflect the cell's attempt to redirect these acute substrates from proteasomal degradation to storage in inclusion bodies or clearance by the autophagosomal/lysosomal pathway. Unfortunately, our data do not allow us to draw conclusions on when aggregation-prone substrates are targeted for ubiquitylation. In the light of a potential function in handling aggregation-prone proteins, determining whether polyubiquitylation takes place before or after their incorporation in inclusions would be informative.

Persistent accumulation of aggregation-prone substrates.

An intriguing observation is that whereas cells rapidly removed accumulated soluble proteasome substrates once the free ubiquitin levels were restored, they failed to do so with the accumulated aggregation-prone substrates (Fig. 7C). This condition is not specific for proteotoxic stress that is accompanied by ubiquitin stress since a similar phenomenon was observed in puromycin-treated cells. This could reflect the intrinsic problems that the proteasome encounters in degradation of these aggregation-prone proteins in stressed cells (43). Thus, it is feasible that during proteotoxic stress, as a consequence of improper folding of proteins in combination with molecular crowding, these proteins form stable aggregates that resist proteasomal degradation. This finding may also provide an explanation for the selective accumulation of aggregated proteins in conformational diseases (6) since episodes of proteotoxic stress, which some cells may encounter as part of their normal physiology, may result in the selective precipitation of aggregation-prone proteins that have been redirected from proteasomal degradation to inclusion bodies.

Evolution and functional aspects of ubiquitin expression.

We demonstrated that overexpression of ubiquitin is sufficient to prevent accumulation of soluble proteasome substrates during thermal proteotoxic stress. Since experimentally increasing the ubiquitin levels seems to be beneficial, this brings up the question of why cells do not produce higher amounts of ubiquitin to anticipate stress conditions and avoid UPS dysfunction in the first place. Cells do generate the required quantities of ubiquitin to accommodate clearance of the substrates in the aftermath of the heat shock (12), so the UPS impairment is apparently avoidable. Although our data predict that higher ubiquitin levels probably will not prevent accumulation of aggregation-prone proteins, which in the long term may form the greatest threat for cell viability (40), it seems nevertheless beneficial for cells to keep proteasomal degradation of soluble proteins operative during stress. It may simply be a matter of a trade-off, and the costs of constitutively synthesizing excessive amounts of ubiquitin to cope with occasional or even rare stress conditions may be too high. Alternatively, the global increase in ubiquitin-dependent proteasome substrates as a consequence of the limited amounts of ubiquitin may assist the cell in coordinating the cellular response to proteotoxic stress. Persistent proteotoxic stress can induce cell death (5), and the accumulation of soluble substrates, including many regulators of the cell cycle, as a direct consequence of UPS impairment may be instrumental in transiently stalling the cell cycle or, in the case that the stress conditions persist, in inducing apoptosis.

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