Transient arrest in proteasomal degradation during inhibition of translation in the unfolded protein response

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The UPR (unfolded protein response) activates transcription of genes involved in proteasomal degradation. However, we found that in its early stages the UPR leads to a transient inhibition of proteasomal disposal of cytosolic substrates (p53 and p27Kip1) and of those targeted to ER (endoplasmic reticulum)-associated degradation (uncleaved precursor of asialoglycoprotein receptor H2a). Degradation resumed soon after the protein synthesis arrest that occurs in early UPR subsided. Consistent with this, protein synthesis inhibitors blocked ubiquitin/proteasomal degradation. Ubiquitination was inhibited during the translation block, suggesting short-lived E3 ubiquitin ligases as candidate depleted proteins. This was indeed the case for p53 whose E3 ligase, Mdm2 (murine double minute 2), when overexpressed, restored the degradation, whereas a mutant Mdm2 in its acidic domain restored the ubiquitination but did not completely restore the degradation. Inhibition of proteasomal degradation early in UPR may prevent depletion of essential short-lived factors during the translation arrest. Stabilization of p27 through this mechanism may explain the cell cycle arrest in G1 when translation is blocked by inhibitors or by the UPR.

Key words: endoplasmic reticulum-associated degradation (ERAD), p27, p53, proteasomal degradation, ubiquitin, unfolded protein response (UPR).

INTRODUCTION

The UPR (unfolded protein response) is responsible for the up-regulation of the expression of certain genes that are needed for eukaryotic cells to cope with a load of misfolded proteins in the ER (endoplasmic reticulum) [1]. These genes encode proteins like chaperones and other folding factors and others involved in ubiquitin/proteasomal degradation. While the cell is preparing for this transcriptional activation there is a transient arrest in translation initiation mediated by phosphorylation of the translation factor eIF2α (eukaryotic initiation factor 2α) by the ER membrane kinase PERK [PKR (protein kinase R)-like ER kinase] [2]. This transient arrest in translation prevents the continued synthesis and accumulation of misfolded proteins. How does this inhibition of translation not result in the fast depletion of essential short-lived proteins through degradation? We show here that a block in protein synthesis by the UPR or by protein synthesis inhibitors leads to an arrest in ubiquitin/proteasomal degradation. How is this achieved? The ubiquitin/proteasomal pathway for protein degradation involves multiple factors, many of them short-lived [3]. Notably, many E3 ubiquitin ligases have a short half-life [4] and may be depleted upon inhibition of protein synthesis, abrogating ubiquitination, as we show for p53.

The ubiquitin/proteasome machinery is a major regulator of the cell cycle. An important target is the CDK (cyclin-dependent kinase) inhibitor p27Kip1; degradation of p27 relieves the inhibition of the cell cycle in G1, possibly by inhibition of the degradation of p27, which we show is also affected.

In addition, the ubiquitin/proteasome machinery is responsible for ERAD (ER-associated degradation) [6–8]. Incubation of cells with CHX (cycloheximide) blocks the degradation of proteins from the ER [9,10]. We find that also during the initial stages of the UPR, degradation of an ERAD substrate is transiently arrested, a counterintuitive finding, as this leads to a temporary inhibition of the ability of the cell to dispose of misfolded secretory proteins.

EXPERIMENTAL

Materials

Rainbow 14C-labelled methylated protein standards were obtained from Amersham Biosciences (Piscataway, NJ, U.S.A.). Promix cell labelling mixture ([35S]methionine plus [35S]cysteine, > 1000 Ci/mmol) was from PerkinElmer Life Sciences (Boston, MA, U.S.A.). Protein A–Sepharose was from RepliGen (Needham, MA, U.S.A.) and Protein G from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). N-glycosidase F was obtained from Roche (Basel, Switzerland). MG-132 (the proteasome inhibitor carboxbenzoxyl-L-leucyl-L-leucyl-L-leucinal) and CHX were from Calbiochem (La Jolla, CA, U.S.A.). Tunicamycin, thapsigargin, puromycin and other common reagents were from Sigma.

Cells, culture and plasmids

NIH 3T3 fibroblasts expressing human ASGPR (asialoglycoprotein receptor) H2α (2–18 cells) [11] were grown in DMEM (Dulbecco’s modified Eagle’s medium) plus 10% fetal calf serum under 5% CO2 at 37°C. HEK-293 cells (human embryonic kidney cells) were grown similarly but with 10% fetal calf serum. Plasmids carrying cDNAs for wild-type HA (haemagglutinin)-tagged mouse Mdm2 (murine double minute 2) and a double point mutant in the acidic domain, Mdm2 E246A/E248A (in pxj41 vector), were a gift from Moshe Oren (Department of Molecular Cell Biology, Weizmann Institute, Israel).

Abbreviations used: ASGPR, asialoglycoprotein receptor; CDK, cyclin-dependent kinase; CHX, cycloheximide; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK-293 cells, human embryonic kidney cells; Mdm2, murine double minute 2; MG-132, carboxbenzoxyl-L-leucyl-L-leucyl-L-leucinal; NP40, Nonidet P40; ODC, ornithine decarboxylase; PERK, PKR (protein kinase R)-like ER kinase; UPR, unfolded protein response.

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Rehovot, Israel). These plasmids and also human p35 in pcMV-neo-BAM (a gift from Sara Lavi, Cell Research and Immunology, Tel-Aviv University Tel-Aviv, Israel) and Myc-tagged human ubiquitin in pUB221 (a gift from Ron Kopito, Department of Biological Sciences, Stanford University, Stanford, CA, U.S.A.) were transfected into HEK-293 cells in 35 mm dishes using the calcium phosphate transfection method. p53 and Mdm2 were co-transfected at a 1:2 ratio.

Antibodies
Polyclonal ‘anti-H2a’ antibody against the region of the extra pentapeptide of H2a as compared with H2b was the one used in earlier studies [12]. Mouse monoclonal anti-ubiquitin was obtained from BabCO (Richmond, CA, U.S.A.). Rabbit polyclonal anti-p27 was from Santa Cruz Biotechnology. Anti-p53 was a rabbit polyclonal from Santa Cruz Biotechnology or a mouse monoclonal from Moshe Oren. Rabbit polyclonal anti-phosphorylated eIF2α was from Biosource (Camarillo, CA, U.S.A.) and anti-calnexin C-terminal from StressGen (Ann Arbor, MI, U.S.A.). Mouse monoclonal anti-Myc was from Cell Signaling Technology (Beverly, MA, U.S.A.) and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) from Chemicon (Temecula, CA, U.S.A.). Mouse monoclonals anti-eIF2α and anti-Mdm2 were gifts from Orna Elroy-Stein (Department of Cell Signaling Technology, Tel-Aviv University, Tel-Aviv, Israel) and from Moshe Oren respectively. Goat anti-mouse and goat anti-rabbit antibodies conjugated to HRP (horseradish peroxidase) were from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.).

Metabolic labelling with [35S]cysteine/methionine, immunoprecipitation and immunoblotting
For a stable NIH 3T3 cell line expressing H2α, subconfluent (90%) cell monolayers in 60 mm dishes were labelled with [35S]cysteine, lysed and immunoprecipitated as described previously with anti-H2α antibody [11,12]. For p53 and p27 cells were lysed with lysis buffer containing 50 mM Tris (pH 8.0), 5 mM EDTA, 0.15 M NaCl and 0.5% NP40 (Nonidet P40). Immunoprecipitation was done for 16 h using Protein A–Sepharose for rabbit or Protein G–Sepharose for mouse antibodies. Washes were done with 5% (w/v) sucrose, 50 mM Tris (pH 7.4), 5 mM EDTA, 0.5 M NaCl and 0.5% NP40.

For experiments involving UPR induction, labelling was done on 50–60% confluent cells to work with low basal levels of eIF2α-P.

For transiently transfected HEK-293 cells, similar conditions were used except that labelling was done in 60 mm dishes 2 days after transfection with 100 μg/ml [35S]cysteine plus [35S]methionine mix. Tunicamycin (10 μg/ml), thapsigargin (2 μg/ml), CHX (300 μM) or the proteasome inhibitor MG-132 (20 μM) was added to the chase medium. Treatment with N-glycosidase F was performed after immunoprecipitation as described previously [11].

Protein transfer and immunoblotting were performed as described previously [13].

Gel electrophoresis, fluorography and quantification
Reducing SDS/PAGE was performed on 10% Laemmli gels except if indicated otherwise. The gels were analysed by fluorography using 20% 2,5-diphenyloxazole and were exposed to a Kodak BioMax MR film. Quantification was performed in a Fuji BAS 2000 phosphorimaging device.

RESULTS
The UPR leads to transient arrest in the degradation of p53 and p27
It was shown that the UPR leads to an up-regulation of the ubiquitin/proteasomal machineries to expedite the ERAD of misfolded proteins [14,15]. The UPR also increases the degradation of cytosolic proteasomal substrates like p53 [16]. We wondered whether there is any change in ubiquitin/proteasomal degradation at the early stages of the UPR, before transcriptional up-regulation occurs. Surprisingly, we observed that the degradation of p53 is inhibited upon short incubations (1.5 h) of cells with UPR-inducing agents (Figure 1A). The activation of an early stage of the UPR was evidenced by a large transient increase in the
Transient proteasomal degradation arrest in UPR

Figure 2  UPR induction causes a transient block in the degradation of p53 and p27, which correlates with an arrest in protein synthesis

(A) Similar to Figure 1(A) except for the inclusion of a longer chase in the absence or presence of 2 µg/ml thapsigargin (thap). (B) Similar to (A) but in this case p27 was immunoprecipitated from cell lysates after pulse-labelling or chase in the absence or presence of 10 µg/ml tunicamycin for the indicated times and run on SDS/12 % PAGE. (C) Phosphorimage quantification of the gel in (B) and plot of percentage of pulse-label remaining as a function of chase time. (D) Relative rates of protein synthesis were measured by pre-incubating NIH 3T3 cells with 10 µg/ml tunicamycin (tun) for the indicated times and then labelling with 20 µCi/ml [35S]cysteine plus [35S]methionine mix for 30 min. Total trichloroacetic acid-precipitable c.p.m. were measured as detailed in the Experimental section. The graph is an average of three independent experiments; error bars represent standard deviations.

Phosphorylation of eIF2α (Figure 1B). After longer incubations p53 was degraded (Figure 2A). A similar effect was observed with the proteasomal substrate p27 (Figures 2B and 2C). The degradation of p27 is slower than that of p53 and there was an even more dramatic block after short incubations with a UPR-inducing agent, tunicamycin, followed by recovery of the degradation at longer times.

Phosphorylation of eIF2α leads to transient inhibition of protein synthesis and recovery at later stages of UPR when eIF2α is dephosphorylated [17]. Thus, we investigated whether a decrease in translational activity correlated with the inhibition in proteasomal degradation by incubation with tunicamycin. Indeed, this was the case. There was a close correlation between a reduction in the levels of overall protein synthesis and inhibition of the degradation, and later of a recovery of degradation with a recovery in translational activity (Figures 2C and 2D). This was also consistent with a reduction after 3 h of treatment in the levels of phosphorylated eIF2α (Figure 1B).

Inhibition of protein synthesis blocks proteasomal degradation of p53 and p27

If the arrest in proteasomal degradation is due to the block in protein synthesis during the UPR and not to another aspect of the response, incubation of cells with an inhibitor of protein synthesis should cause a similar effect. In fact, addition of CHX immediately after pulse-labelling had a strong inhibitory effect on the degradation of p53 (Figures 3A and 3B). In contrast with the...
ERAD is blocked by inhibition of protein synthesis or activation of the UPR

We wondered if ERAD substrates would be affected by the same block in degradation upon UPR. We analysed an established ERAD substrate, uncleaved precursor of ASGPR H2a [11,13,18,19]. The normal half-life of H2a is approximately an hour, after an initial lag of 1 h [12]. H2a membrane-bound precursor can be cleaved to a 35 kDa C-terminal fragment, corresponding to its ectodomain (by signal peptidase [20]), although this cleavage is inefficient in cells other than hepatocytes. The fragment matures to a cleaved soluble secreted form of the receptor in hepatocytes but is mostly degraded in other cell types [12]. Degradation can also proceed directly from the uncleaved precursor [20]. Proteasomal inhibition blocks the degradation of H2a precursor and leads to cleavage, accumulation and slow secretion of the ectodomain fragment [13].

After pulse-labelling, we incubated cells expressing H2a with tunicamycin or thapsigargin for a short or for a longer chase period. Similar to what we had observed with the cytosolic proteasomal substrates, the degradation of H2a was significantly inhibited after incubation with tunicamycin or thapsigargin for 2.5 h but was back to normal after longer periods of 8 h of chase (Figure 4A). Degradation of both H2a precursor and its cleaved ectodomain [12] was inhibited after short incubations with the drugs.

Incubation of cells expressing H2a with CHX after pulse-labelling also caused a block in the degradation, although this was observed mainly as an accumulation of its cleaved fragment (Figures 4B and 4D), probably by proteasome-independent cleavage of accumulated precursor, as we had seen before when proteasomes were inhibited [13]. The levels of a stable ER protein, calnexin, remained unchanged following a similar treatment (Figure 4B, lower panel and Figure 4D). In ERAD, CHX treatment could theoretically lead to a block of proteins on ribosomes during translocation, and subsequently inhibit protein retrotranslocation to the cytosol for degradation. Therefore we incubated cells with puromycin, a protein synthesis inhibitor that does not have the same effect. Puromycin treatment leads to premature release of translating proteins from ribosomes and to release from translocating channels. As with CHX, the incubation with puromycin after metabolic labelling led to a strong inhibition of the degradation of H2a (Figures 4C and 4D). Altogether the results

 transient effect of UPR inducers, CHX still strongly inhibited the degradation after 6 h of chase, consistent with the prolonged inhibition of protein synthesis (compare Figures 2A and 3A). The effect of CHX was additive with that of the proteasomal inhibitor MG-132 (Figures 3A and 3B). Again, the same effect was observed on p27, with a stronger protection by CHX or MG-132 due to its slower degradation (Figures 3C and 3D).
suggest a general arrest in ubiquitin/proteasomal degradation, including that of ERAD substrates, upon inhibition of protein synthesis.

**Inhibition of protein synthesis leads to a block in ubiquitination**

Because degradation of ubiquitin conjugates does not seem to require protein synthesis [21], we looked at ubiquitination as a possible earlier step affected in the ubiquitin/proteasome pathway by inhibition of translation. HEK-293 cells were transfected with Myc-tagged ubiquitin, pulse-labelled and chased for 90 min in the absence or presence of CHX or MG-132, or both combined as indicated. Cell lysates were immunoprecipitated with anti-Myc antibodies. On the right the migrations of molecular-mass markers are indicated in kDa. (A) Phosphoimager quantification of the gel in (A) and plot of fold increase in label remaining (ubiquitinated species) in the presence of MG-132, with and without CHX, relative to untreated cells. (C) HEK-293 cells were incubated for the indicated times with 300 µM CHX. Cell lysates were run on SDS/15% PAGE and immunoblotted with antibodies specific for ubiquitin (upper panel) or for GAPDH as control (lower panel). The values at the bottom of the upper panel represent relative levels of ubiquitin calculated by densitometry.

Many E3 ubiquitin ligases could be good candidate short-lived proteins that participate in ubiquitination and would be rapidly depleted upon inhibition of protein synthesis, before depletion of their substrates. We tested this hypothesis in the case of p53. The half-life of p53 is approx. 30–60 min, depending on cell type, whereas that of Mdm2, its specific E3 ligase, is approx. 15 min [23]. Therefore inhibition of protein synthesis would cause rapid depletion of Mdm2, the levels of which could fall under a minimum threshold while substantial amounts of p53 are still present. We first analysed degradation of p53 by pulse–chase analysis. Incubation with CHX during the chase time stabilized p53 (Figure 6A) as we had already seen above. We then analysed the total steady-state levels of Mdm2 in the same samples by immunoblot. The short incubation with CHX caused a dramatic depletion of Mdm2 (Figure 6B, lane 4). This was only partially compensated for by the simultaneous presence of MG-132 (Figure 6B, lane 5), because the inhibition of protein synthesis is immediate, whereas the proteasomal inhibitor must take some time to penetrate the cells and act. A stable protein, GAPDH, remained unchanged (Figure 6C).

We tried to compensate for the depletion of Mdm2 by overexpression, which was done in HEK-293 cells to achieve high efficiency of transfection. p53 is degraded very slowly in HEK-293 cells. It was reported that in *Saccharomyces cerevisiae* the levels of free ubiquitin are rapidly depleted upon inhibition of protein synthesis [22]. We analysed the levels of free ubiquitin in HEK-293 cells and they did not appear to be affected by CHX treatment for periods of time similar to those used above (Figure 5C). This suggests that the inhibition of the ubiquitination step is not a result of depletion of free ubiquitin.

**Figure 5 Ubiquitination is inhibited but ubiquitin is not depleted upon inhibition of protein synthesis**

(A) HEK-293 cells were transiently transfected with a plasmid carrying cDNA for Myc–ubiquitin. After 48 h they were labelled as in Figure 1(A), followed by chase in the absence or presence of 300 µM CHX or 20 µM MG-132 or both combined as indicated. Cell lysates were immunoprecipitated with anti-Myc antibodies. On the right the migrations of molecular-mass markers are indicated in kDa. (B) Phosphoimager quantification of the gel in (A) and plot of fold increase in label remaining (ubiquitinated species) in the presence of MG-132, with and without CHX, relative to untreated cells. (C) HEK-293 cells were incubated for the indicated times with 300 µM CHX. Cell lysates were run on SDS/15% PAGE and immunoblotted with antibodies specific for ubiquitin (upper panel) or for GAPDH as control (lower panel). The values at the bottom of the upper panel represent relative levels of ubiquitin calculated by densitometry.

**Figure 6 CHX causes rapid depletion of the E3 ligase Mdm2, leading to stabilization of p53**

(A) Pulse–chase analysis and immunoprecipitation (IP) of p53, similar to Figure 3(A). (B, C) Ten percent of the cell lysates from (A) were run on SDS/PAGE and immunoblotted (BLOT) for Mdm2 (B) or GAPDH (C). Note a small difference between the levels of Mdm2 between lanes 1 and 2 in (B); this result was reproducible and is probably due to a small effect of cysteine and methionine starvation 30 min before and during the pulse-labelling in NIH 3T3 cells.

Inhibition of protein synthesis causes depletion of Mdm2, leading to stabilization of p53

Inhibition of protein synthesis leads to a block in ubiquitination

Because degradation of ubiquitin conjugates does not seem to require protein synthesis [21], we looked at ubiquitination as a possible earlier step affected in the ubiquitin/proteasome pathway by inhibition of translation. HEK-293 cells were transfected with Myc-tagged ubiquitin, pulse-labelled and chased for 90 min in the absence or presence of CHX or MG-132. Immunoprecipitation with anti-Myc antibodies showed many ubiquitinated proteins that accumulated in the presence of MG-132 compared with untreated cells (Figure 5A, compare lanes 2 and 3, and Figure 5B). This accumulation of ubiquitinated proteins was almost completely abrogated when cells were incubated during the chase with CHX in addition to MG-132 (Figure 5A, compare lanes 4 and 5, and Figure 5B), leading to the conclusion that ubiquitination is indeed inhibited.

It was reported that in *Saccharomyces cerevisiae* the levels of free ubiquitin are rapidly depleted upon inhibition of protein synthesis [22]. We analysed the levels of free ubiquitin in HEK-293 cells and they did not appear to be affected by CHX treatment for periods of time similar to those used above (Figure 5C). This suggests that the inhibition of the ubiquitination step is not a result of depletion of free ubiquitin.
and Figure 7F). No changes can be seen in the same samples for a stable protein, calnexin (Figure 7D). The results suggest that the defect in ubiquitination upon inhibition of protein synthesis can indeed be ascribed to the depletion of the E3 ligase.

**DISCUSSION**

Our results show that inhibition of translation, which takes place during the early stages of the UPR, a condition frequently encountered in the normal physiology of eukaryotic cells, causes a transient arrest in ubiquitin/proteasomal degradation. This arrest would have important consequences as discussed below. We showed that what is affected in ubiquitin/proteasomal degradation during a block in protein synthesis is the ubiquitination step. This may be the only step affected, as it has been reported that CHX does not inhibit degradation of ubiquitin conjugates [21].

A protein that does not require ubiquitination for its proteasomal degradation, ODC (ornithine decarboxylase), still degrades in the presence of CHX as seen with an ODC–GFP (green fluorescent protein) fusion protein [25]. The short life of Mdm2 [26] and other E3s [3,4] is likely to be responsible for the effect of protein synthesis inhibition on the ubiquitination machinery (Figure 6). Consistent with this, overexpression of Mdm2 compensates for this effect and allows p53 degradation in the presence of CHX (Figure 7). Overexpression of an Mdm2 mutant in its acidic domain still leads to the ubiquitination (but defective degradation) of p53 in the absence of protein synthesis. Normally, the very fast disappearance of Mdm2 and other E3 ligases during inhibition of protein synthesis would take place before a substantial reduction in the levels of their substrates, which have a longer half-life. Upon depletion of the E3 ligases their substrates are stabilized. This must be true for a large number of substrates in the cell (Figure 5), although we cannot predict that this will apply to all substrates, as some must have longer lived E3 ligases. UPR induction would have a similar effect through its inhibition of protein synthesis. Stress conditions cause a higher instability of Mdm2 [27] and possibly of other E3 ligases, and therefore UPR could lead to an even shorter half-life of the enzymes, accelerating their disappearance and stabilizing the substrates. In early UPR, ubiquitination is probably the only step affected as it was recently reported that in human melanoma cells ER stress inhibits degradation of proteasomal substrates without decreasing the levels and activity of proteasomes [28].

In the case of the ERAD substrate ASGPR H2a, a possibility existed that CHX treatment of cells could eventually lead to a block of proteins on ribosomes during translocation, which could in turn inhibit retrotranslocation to the cytosol for degradation. However, as the same block in degradation was obtained with puromycin (that releases the translocating proteins from the ribosomes) and upon activation of the UPR, the mechanism of the inhibition of degradation by blocking protein synthesis in ERAD is likely to be the same as for cytosolic substrates, by inhibition of E3 ligases. One of these enzymes, gp78, a RING (really interesting new gene) finger-dependent ubiquitin ligase implicated in ERAD, was shown to undergo autoubiquitination and has a short half-life [29]. Another ligase implicated in ERAD, HRD1 (3-hydroxy-3-methylglutaryl-CoA reductase degradation 1) also undergoes autoubiquitination and this activity is regulated by an associated protein, HRD3 [30]. Inhibition of degradation after short incubations with CHX has been observed for H2a [10] and for other ERAD substrates [31] as well as for HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase for which it was shown that CHX inhibited its ubiquitination [9]. On the other hand, inhibition of ERAD by UPR induction had been seen (although not explained) for another substrate, truncated ribophorin [32].

The transient arrest in proteasomal degradation could help explain several physiological processes. For example, the cell cycle is arrested in G1 both by inhibitors of protein synthesis [33] and by the UPR. Our results suggest that this effect is possibly due to the inhibition of the proteasomal degradation of p27kip (Figure 2). The arrest in the cell cycle by the UPR has been attributed to a PERK-mediated block in the synthesis of cyclin
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