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Endoplasmic Reticulum Stress Triggers an Acute Proteasome-Dependent Degradation of ATF6

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Abstract ATF6, a 670 amino acid endoplasmic reticulum (ER) transmembrane glycoprotein with the electrophoretic mobility of a 90 kDa protein, is a key transcriptional activator of the unfolded protein response (UPR) that allows mammalian cells to maintain cellular homeostasis when the cells are subjected to a variety of environmental and physiological stress. Previous studies have established that ATF6 is a short-lived protein, the activation of which involves relocation from the ER to the Golgi where it is cleaved by the S1P/S2P protease system to generate a nuclear form that acts as a transcriptional activator for ER-stress inducible target genes such as Grp78/BiP. We report here that in addition to this process, ER-stress mediated by thapsigargin triggers an acute proteasomal degradation of the pre-existing pool of p90ATF6 independent of S1P/S2P cleavage. We showed that ATF6 is a direct target of proteasome-ubiquitin pathway, and this process can be suppressed by proteasome inhibitors, ALLN and MG115. We further observed that in non-stressed cells, p90ATF6 can be stabilized by MG115 but not ALLN and that treatment of cells with MG115 results in Grp78 induction in the absence of ER stress. These studies suggest that ER-stress induced acute, transit degradation of p90ATF6 could represent a novel cellular defense mechanism to prevent premature cell death resulting from p90ATF6 activation. Further, inhibition of proteasome activity can result in chaperone protein gene induction through stabilization of p90ATF6 as well as accumulation of malfolded proteins. J. Cell. Biochem. 92: 723–732, 2004. © 2004 Wiley-Liss, Inc.

Key words: ATF6; proteasome; ER stress; Grp 78

The ubiquitin-proteosome pathway is the major mechanism utilized by eukaryotic cells for targeted degradation of protein with short half-lives [Bachmair and Varshavsky, 1989]. The covalent attachment of ubiquitin to lysine residues of the targeted protein signals the recognition and rapid degradation by the proteasome, a large multi-subunit protease. In the endoplasmic reticulum (ER), conditions that perturb folding or glycosylation of surface or secreted protein, and that causes efflux of intracellular calcium stores, result in the accumulation of abnormal proteins in the lumen of ER and triggering of the unfolded protein response (UPR) [Kaufman, 1999]. As part of the protective mechanisms elicited by the UPR, transcription of ER molecular chaperone genes are elevated to produce more chaperones to prevent protein aggregation [Lee, 2001]. Moreover, translation is arrested temporarily and a process referred to as endoplasmic reticulum associated degradation (ERAD) is activated. ERAD is an active mechanism for cells to reduce the lumenal concentration of malfolded proteins by packing the ER proteins into anterograde vesicles and transporting them out of the ER to be degraded by ubiquitin-proteasomes [Hampton, 2002]. Interestingly, inhibition of proteasome function can lead to constitutive induction of ER chaperone protein genes [Bush et al., 1997]. While it has been speculated that this could arise from failure to degrade malfolded protein in ER, as well as prevent the degradation of a key transcription factor required for ER chaperone protein gene activation, the later is not proven.

Among UPR activators, thapsigargin (Tg) which depletes ER calcium store by specific

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inhibition of the ER-calcium ATPase [Thastrup et al., 1990] is a potent inducer of the ER chaperone protein genes [Li et al., 1993]. One of the best characterized ER-stress inducible chaperone genes is the glucose-regulated protein (GRP) gene, Grp78, also referred to as BiP. Transcriptional activation of Grp78 does not occur until a few hours after addition of Tg and once transcription is triggered, it is sustained as long as the cells are exposed to the drug. A key activator for the Grp78 promoter is ATF6, which is a member of the leucine zipper protein family that acts through the endoplasmic reticulum stress response element (ERSE) [Yoshida et al., 1998; Roy and Lee, 1999]. ATF6, a 670 amino acid glycoprotein with the electrophoretic mobility of a 90 kDa protein (p90ATF6), is constitutively expressed in a variety of mammalian cells [Zhu et al., 1997]. ATF6 contains a single transmembrane domain with 272 amino acids oriented in the ER lumen. ER stress induces proteolysis of the membrane-bound p90ATF6 releasing the soluble amino-portion of ATF6 (p60ATF6), which relocates to the nucleus and activates the transcription of a wide variety of ER stress-inducible promoters. It is well established that the ER-stress induced proteolytic cleavage of p90ATF6 to p60ATF6 is mediated by the Golgi localized S1P and S2P proteases, as part of a process referred to as the regulated intramembrane proteolysis (RiP) pathway [Ye et al., 2000]. Nonetheless, authentic cleavage of endogenous or transfected ATF6 by classic ER-stress inducers such as Tg and tunicamcyin (Tuni) that exhibit a delayed response has been difficult to detect [Wang et al., 2000; Ye et al., 2000]. This is due in part to the low amount of p90ATF6 that translocates to the Golgi and rapid degradation of the newly generated p60ATF6 by proteasomes [Ye et al., 2000]. Further, we noted that p90ATF6 in NIH3T3 cells was rapidly degraded within the first 2 h of Tg treatment but quickly reverted to elevated levels with the additional appearance of a faster-migrating form [Li et al., 2000]. The nature of the faster-migrating form has recently been identified as an underglycosylation form of ATF6 induced by Tg [Hong et al., 2004]. However, the molecular basis for the acute decrease in p90ATF6 is not known. In this study, we discover that p90ATF6 is ubiquitinated and degraded by proteasomes as a general acute response to ER-stress mediated by Tg and Tuni. This process is independent of S1P/S2P cleavage and can be blocked by the proteasome inhibitors, MG115 and ALLN. Our studies further reveal that in non-stressed cells, p90ATF6 can be stabilized by the proteasome inhibitor MG115 but not ALLN, leading to induction of Grp78 transcription in the absence of ER stress, thus providing an additional mechanism for induction of the UPR by inhibition of proteasome function.

MATERIALS AND METHODS

Cell Culture Conditions

NIH3T3 and 293T cells were maintained in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, Inc., GIBCO BRL, Gaithersburg, MA) and 1% penicillin-streptomycin-neomycin antibiotics at 37°C. The CHO-7, S1P-defective SRD-12B cells, and S2P-defective M19 cells were generously provided by Dr. Joseph L. Goldstein (University of Texas, Southwestern Medical Center) and have been described [Ye et al., 2000]. Tg and tunicamycin were purchased from Sigma Co. (St. Louis, MO).

For stress induction, cells were grown to 80% confluence and treated with 300 nM Tg, or 1.5 μg/ml tunicamycin for various time intervals as indicated. The proteasome inhibitors ALLN and MG115 were purchased from Sigma Co. and MG132 from CalBiochem (La Jolla, CA). The z-DEVD-fmk peptide and calpastatin peptide were purchased from Calbiochem. To test the effect of caspase inhibitor in vivo, the cells were pretreated with 120 μM z-DEVD-fmk for 3 h prior to addition of Tg. For inhibition of proteasome activity, the cells were pretreated with either 45 μM ALLN or 50 μM proteasome inhibitor MG115 for 4 h prior to addition of Tg. For inhibition of calpain activity, the cells were pretreated with the 1 μM calpain specific inhibitor calpastatin peptide for 3 h. For stabilization of the ATF6-ubiquitin conjugates, the cells were treated with 20 μM of MG132 for 6 h.

Plasmids

pTK-HSV-ATF6 was provided by Dr. Joseph L. Goldstein and its construction has been described [Ye et al., 2000].

Western Blotting

Conditions for Western blot analysis of endogenous ATF6 using a rabbit polyclonal ATF6 antibody have been described [Li et al., 2000].

For the detection of endogenous ATF6 using a mouse monoclonal antibody against the N-terminal 273 amino acids of ATF6 (Imgenex, San Diego, CA), the primary antibody was diluted at 1:500 in 5% milk and incubated with the transferred membrane at 4°C overnight. The secondary antibody used was HRP-conjugated goat anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:3,000. For the detection of HSV-ATF6, the mouse monoclonal anti-HSV-Tag antibody (Novagen, Madison, WI) was used as the primary antibody at a dilution of 1:10,000. For detection of GRP78, the anti-KDEL mouse monoclonal antibody (StressGen, Victoria, British Columbia, Canada) was used as the primary antibody at a dilution of 1:1,500.

Co-Immunoprecipitation Assays

The conditions for co-immunoprecipitation have been described [Li et al., 2000]. For immunoprecipitation with ATF6, 500 μg of whole cell extract from each sample were incubated with Protein A-sepharose beads (Sigma) and 20 μl of mouse anti-ATF6 monoclonal antibody (Imgenex) for 4 h with rotation. After the incubation, the beads were washed four times. The immunoprecipitates were resolved by SDS–PAGE and then subjected to Western blotting. For detection of ubiquitinated ATF6, mouse monoclonal anti-ubiquitin antibody (Santa Cruz Biotechnology) was used at a dilution of 1:200.

Northern Blotting

The methods for total cellular RNA extraction and Northern blot hybridizations have been described [Cao et al., 1995]. The cDNA probes used for the detection of Grp78 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) have been described [Zhou and Lee, 1998]. The level of transcript was quantitated by phosphorimager Model 445 SI (Molecular Dynamics, Sunnyvale, CA).

RESULTS

ER-Stress Mediated by Tg and Tuni Results in Transient Degradation of p90ATF6

Upon treatment of NIH3T3 cells with Tg, the level of Grp78 mRNA remained low during the first 2 h and gradually increased to a high level that was sustained up to 16 h (Fig. 1A). Correlating with a gradual increase in the Grp78

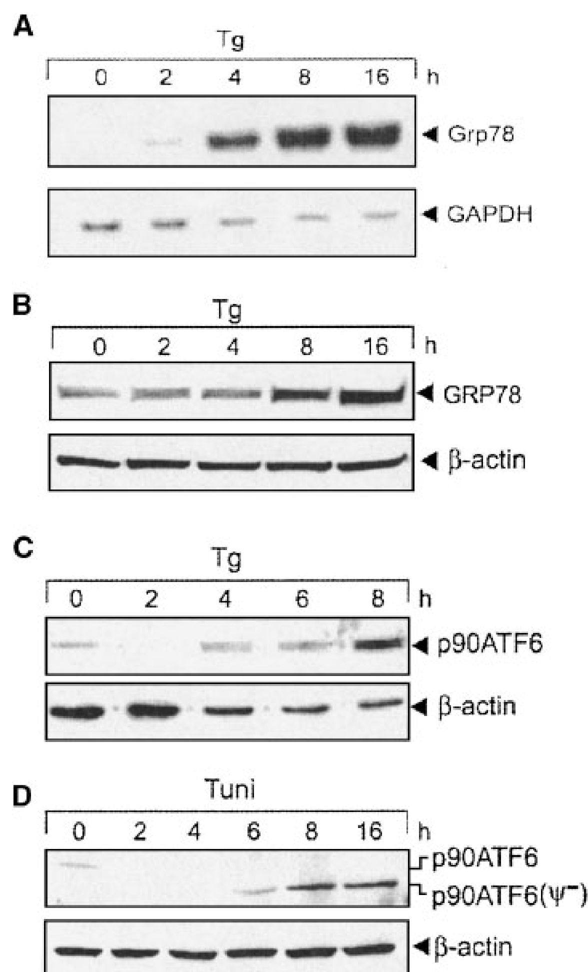

Fig. 1. ER-stress induced transient degradation of endogenous ATF6. **A:** Total RNA was prepared from NIH3T3 cells treated with Tg for the time indicated and then subjected to Northern blot analysis with radiolabeled Grp78 and GAPDH cDNA probes. The autoradiograms are shown. **B:** Total cell lysates were prepared from NIH3T3 cells treated with thapsigargin (Tg) for the indicated time in hours (h) and analyzed by immunoblotting using anti-GRP78 antibody and an antibody against β-actin as loading control. **C:** The cells were treated with Tg for the indicated time and immunoblots were performed using an anti-ATF6 antibody and an antibody against β-actin as loading control. **D**: It was same as (C) except that the cells were treated with tunicamycin (Tuni). The positions of GRP78, p90ATF6, the non-glycosylated form [p90ATF6(ψ^-)] and β-actin are indicated.

mRNA level, the level of GRP78 protein also increased gradually reaching a plateau at about 16 h (Fig. 1B). This delayed kinetics of Grp78 induction was also observed for cells treated with tunicamycin [Kim et al., 1987]. In non-stressed NIH3T3 cells, p90ATF6 was present at a low basal level. Surprisingly, after 2 h of Tg treatment, the level of p90ATF6 was much diminished. This decrease was transient and occurred during the first 2 h of Tg treatment; by

4 h, the level of p90ATF6 was already replenished (Fig. 1C). By 8 h of Tg treatment, the amount of p90ATF6 was elevated instead, correlating with sustained high level induction of Grp78 mRNA.

The transient degradation of endogenous p90ATF6 is not limited to Tg treatment. NIH3T3 cells treated with the protein N-linked glycosylation-blocking agent tunicamycin (Tuni) also resulted in the near complete disappearance of p90ATF6 between 2 and 4 h of the drug treatment (Fig. 1D). As in the case of Tg treatment, the level of p90ATF6 was later replenished and even increased in amount. The single faster-migrating band that reappears after 6 h of tunicamycin treatment represents the completely non-glycosylated form of ATF6, referred to as [ATF6(Ψ^-)] with a faster electrophoretic mobility than p90ATF6.

Acute ATF6 Degradation Following Tg Stress Is Independent of S1P/S2P Cleavage

To address directly whether the acute disappearance of p90ATF6 is entirely due to S1P/S2P cleavage upon Tg stress, we compared the level of p90ATF6 in Tg-treated CHO-7 cells as well as its two derivatives, SRD-12B and M19 that are defective in S1P and S2P, respectively [Ye et al., 2000]. In all three types of cells, irrespective of the functional status of S1P and S2P, the level of p90ATF6 decreased within 1 h of Tg treatment and recovered by 3 h (Fig. 2A–C). Next we tested whether the acute degradation of ATF6 could be observed with ectopic ATF6. To mimic the physiologic level of endogenous ATF6, we utilized the pTK-HSV-ATF6 expression vector encoding the full-length human ATF6 tagged with two copies of the HSV-glycoprotein epitope [Ye et al., 2000]. The epitope-tagged ATF6 is expressed under the control of the HSV thymidine kinase (TK) promoter, which gives a low but detectable level of expression. Cell lysates were prepared from the transfected cells following Tg treatment. Using amounts of cell lysate below the saturation limit for the p90ATF6 band in the immunoblots performed with the anti-HSV antibody, acute decrease and replenishment of the ectopic ATF6 were observed with kinetics similar to endogenous p90ATF6 (Fig. 2D). Collectively, these experiments demonstrate that both endogenous and ectopic ATF6 are subjected to acute proteolysis immediately following Tg stress. While the acute disappearance of p90ATF6 could be in part due

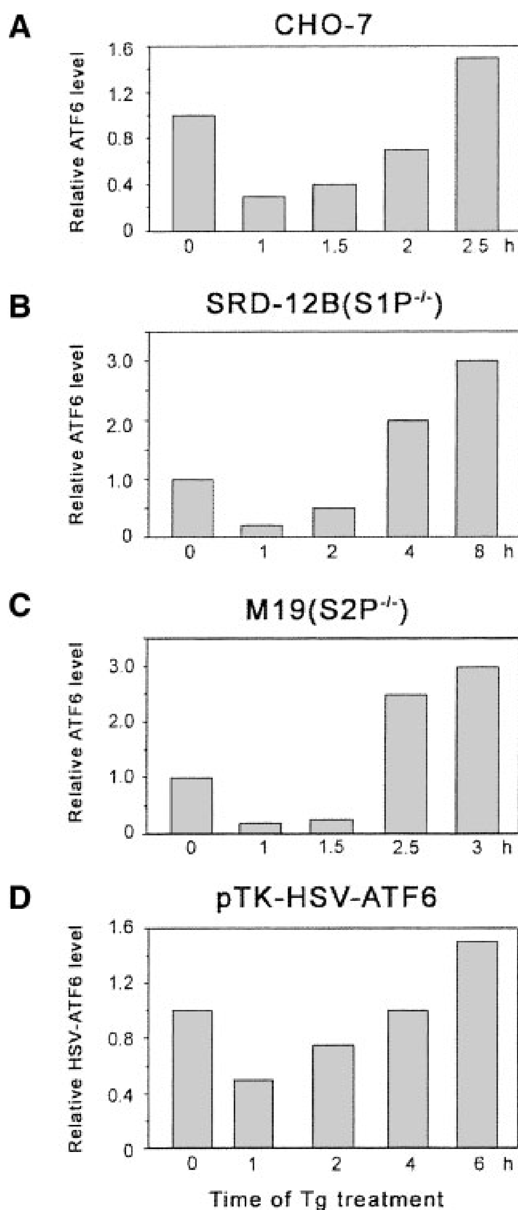

Fig. 2. Tg-induced acute degradation of endogenous and ectopic ATF6. **A:** CHO-7 cells were treated with Tg for the time indicated and the whole cell extracts were subject to immunoblotting using a monoclonal antibody against the N-273 amino acids of ATF6 and an antibody against β-actin as loading control. The relative levels of ATF6 proteins were determined by quantification of the Western-blot bands using densitometry and were normalized against the level of β-actin and plotted against the time of the drug treatment. **B**: It was same as (A) except that the cells were SRD-12B(S1P$^{-/-}$). **C:** It was same as (A) except that the cells were M19(S2P$^{-/-}$). **D:** 293T cells transfected with pTK-HSV-ATF6 were treated with Tg for the time indicated. Whole cell extracts were subject to immunoblotting using an antibody against the HSV-Tag and an antibody against β-actin. The relative levels of HSV-ATF6 proteins were determined after normalization against β-actin.

to S1P/S2P cleavage triggered by ER stress, our results reveal that additional proteolytic pathways are involved.

Suppression of Tg-Induced ATF6 Degradation by Proteasome Inhibitors

Since Tg and other ER stress inducers are known to induce apoptosis, they could trigger the activation of proteasomes and caspases. To test whether the acute disappearance of p90ATF6 after Tg treatment is due to degradation by these proteases, NIH3T3 cells were pretreated with inhibitors for proteasomes (N-acetyl-leucine-leucine-norleucinal, ALLN) and (Z-Leu-Leu-Nva-H, MG115) and caspase-3 (z-DEVD-fmk) prior to addition of Tg. After various periods of Tg treatment, whole cell lysates were prepared and the level of endogenous p90ATF6 was determined by immunoblotting. In the absence of the protease inhibitors, p90ATF6 was degraded to near completion in 2 h and accumulated to high levels at 16 h (Fig. 3A). Pre-incubation with ALLN or MG115 partially rescued Tg-induced ATF6 degradation at 2 h, and in combination, the level of ATF6 was about 80% restored. In contrast, pre-incubation with z-DEVD-fmk was without effect on the basal level of ATF6 and it did not prevent degradation of ATF6 upon Tg treatment. The same inhibitors (ALLN, MG115, and z-DEVD-fmk) exhibited no effect on the level of GRP78 or β-actin (Fig. 3A). Thus, inhibition of proteasome activity prevented the bulk of p90ATF6 degradation 2 h following Tg treatment.

Since Tg treatment results in the efflux of intracellular calcium from the ER store, the rise in cytoplasmic calcium can lead to the activation of the proteolytic properties of calpain. Calpain belongs to a family of protein cysteine proteinases that are activated at the cellular membranes in a calcium-dependent manner [Carafoli and Molinari, 1998]. Furthermore, while MG115 primarily inhibits the ubiquitin-proteasome proteolytic pathway [Rock et al., 1994], ALLN is a dual inhibitor of proteasome and calpain [Milligan et al., 1996]. To test whether calpain contributes to the acute degradation of ATF6 following Tg treatment, NIH3T3 cells were pretreated with calpastatin peptide that specifically inhibits calpain [Shiraishi et al., 2001]. Our results show that inhibition of calpain activity could not prevent the degradation of ATF6 after 2 h of Tg treatment (Fig. 3B). By 6 h, the level of ATF6 was elevated irrespective of calpastatin peptide pretreatment.

To test whether ATF6 is a direct target of the ubiquitin-proteasome proteolytic pathway following Tg treatment, NIH3T3 were pretreated with MG132 that inhibits proteasome activity and stabilizes protein-ubiquitin conjugates. Our results show that in non-stressed cells, ubiquitinated p90ATF6 could be detected in the presence of MG132. Within 1–2 h of Tg treatment, the level of p90ATF6 ubiquitination increased substantially (Fig. 3C). Thus, in non-stress cells, p90ATF6 itself is subjected to proteasome-ubiquitin degradation constitutively. Furthermore, Tg triggers additional ubiquitination of p90ATF6, correlating with its acute degradation.

Stabilization of ATF6 by Proteasome Inhibitor in Non-stressed Cells Results in Grp78 Induction

While investigating the effect of ALLN and MG115 on the basal level of p90ATF6 in non-stressed cells, we observed that treatment of non-stressed NIH3T3 cells with 6 h of MG115 but not ALLN resulted in a slight increase of the steady state level of p90ATF6 (Fig. 4A). For comparison, 2-h treatment with Tg resulted in complete disappearance p90ATF6 whereas 16-h treatment with Tg resulted in elevated amount of p90ATF6. To confirm the stabilizing effect of MG115, NIH3T3 were treated with MG115 for 6, 8, and 16 h, and the increase in the level of p90ATF6 was confirmed by Western blots of total cell lysates with β-actin level as loading control (Fig. 4B). As expected, after 16 h of Tg treatment, the level of GRP78 was elevated (Fig. 4B). Collectively, these studies reveal that the normal turnover of p90ATF6 in non-stressed cells is not affected by proteasomes targeted by ALLN but is sensitive to proteasomes inhibited by MG115. Thus, prevention of p90ATF6 degradation by inhibiting the specific branches of the ubiquitin-proteasome proteolytic pathway may contribute to induction of Grp78 in the absence of ER stress.

To test this, NIH3T3 cells were treated with Tg in the presence or absence of MG115. Total RNA was prepared from the cells treated for varying periods with Tg and the level of Grp78 transcript was determined by RNA blot hybridization, and GADPH transcript served as a loading control (Fig. 4C). Quantitation of the RNA bands after normalization with the

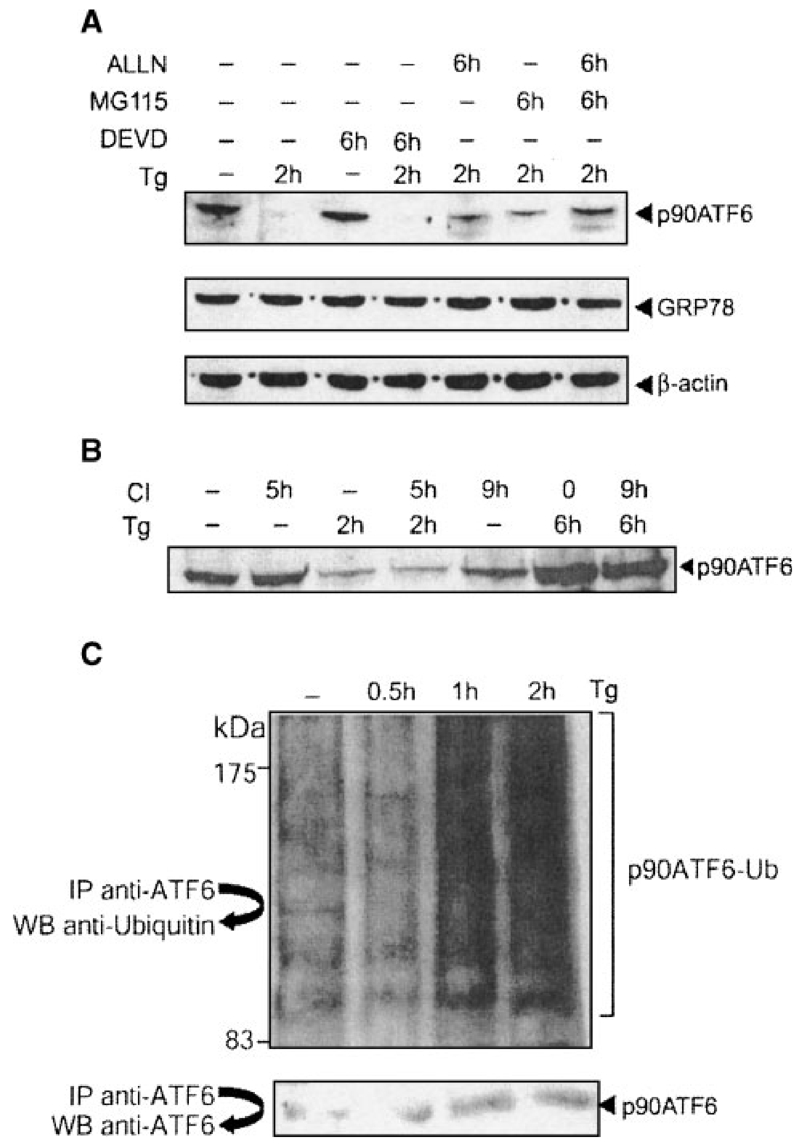

Fig. 3. Effect of inhibitors of proteolytic activities on Tg-induced acute degradation of ATF6. **A:** NIH3T3 cells were either non-treated, treated alone with Tg for 2 h, treated alone with DEVD for 6 h, or pretreated with either ALLN (45 μM), MG115 (50 μM), or z-DEVD-fmk (120 μM) for 4 h prior to combination treatment with Tg for 2 h. Whole cell extracts were immunoblotted to detect the level of ATF6, GRP78, and β-actin, which served as loading control. **B:** NIH3T3 cells were either non-treated, treated with calpastatin peptide CI (1 μM) alone for 5 or 9 h, treated with Tg alone for 2 or 6 h, or pretreated with CI for 3 h prior to 2 or 6 h of combination treatment with Tg. Whole cell extracts were immunoblotted to detect ATF6 level. **C:** Total cell lysates were prepared from NIH3T3 cells treated with thapsigargin (Tg) for the indicated time in hours in the presence of proteasome inhibitor MG132 (20 μM) for 6 h. Following immunoprecipitation (IP) with the anti-ATF6 antibody, one set of the immunoprecipitates was subjected to Western blot using anti-ubiquitin antibody, another set was subjected to anti-ATF6 antibody. The positions of p90ATF6-ubiqutin conjugates and p90ATF6 are indicated.

GADPH transcript level show that as expected, Tg treatment increased the level of Grp78 mRNA by about 12-fold by 4 h (Fig. 4D). In agreement with the ability of MG115 to stabilize p90ATF6 in non-stressed cells, MG115 treatment alone resulted in a 2.5-fold increase in Grp78 transcript (Fig. 4C,D). Correlating with the lack of increase of p90ATF6 steady state levels by ALLN treatment in non-stressed cells, the increase in Grp78 transcript level was not detected in cells treated with ALLN (data not shown).

DISCUSSION

The discovery that ATF6, a key transcriptional activator of ER-resident molecular cha-

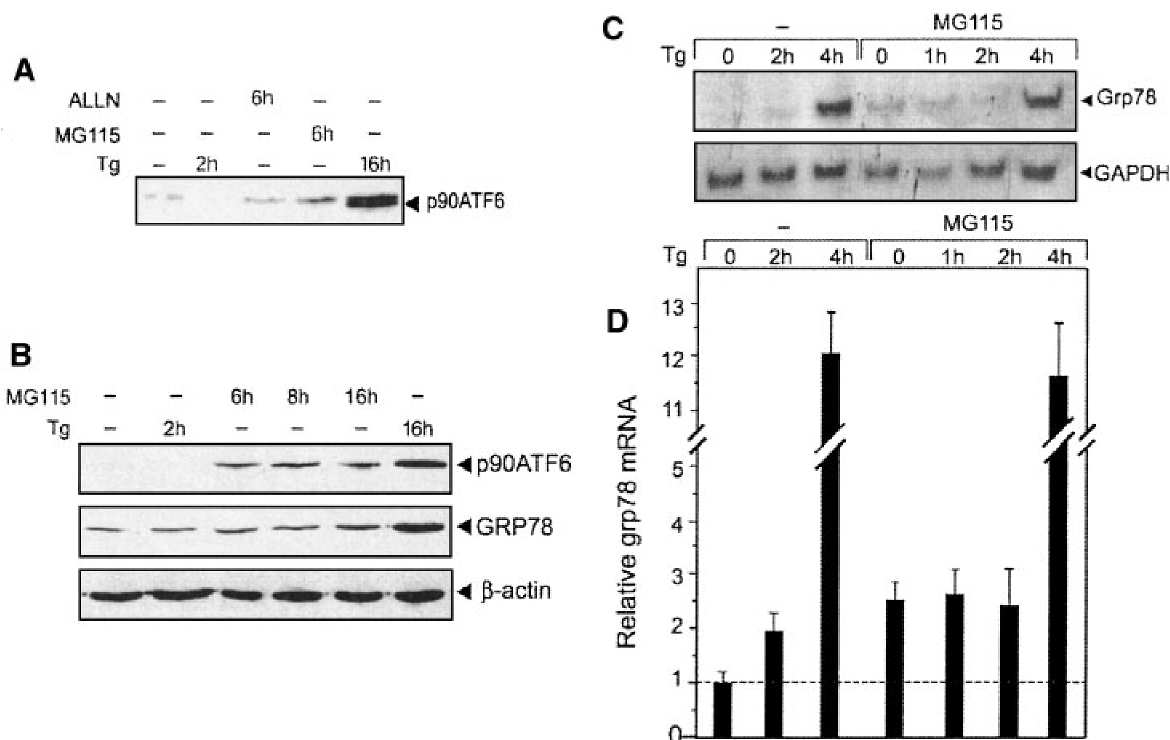

Fig. 4. Inhibition of proteasome function results in Grp78 induction in the absence of ER stress. **A:** NIH3T3 cells were either non-treated, treated with Tg for 2 or 16 h, or treated with MG115 or ALLN for 6 h as indicated on top. Whole cell extracts were immunoblotted for ATF6. **B:** NIH3T3 cells were either non-treated, treated with Tg for 2 or 16 h, or treated with MG115 for 6, 8, or 16 h as indicated on top. Whole cell extracts were immunoblotted for ATF6, GRP78, and β-actin, which served as loading control. The positions of p90ATF6, GRP78, and β-actin are indicated. **C:** Stabilization of basal ATF6 level by MG115 correlates with Grp78 induction. NIH3T3 cells were either non-treated or treated with MG115 for 4 h prior to combination treatment with Tg for the time as indicated on top. Ten micrograms of each RNA sample was subjected to Northern blot analysis. Radiolabeled Grp78 and GAPDH cDNAs were used as probes. The autoradiograms are shown. **D:** The relative levels of grp78 mRNA in (C) were determined after normalization against GAPDH mRNA. The standard deviations are shown.

perones and folding enzymes in the UPR, contributes significantly to the understanding of the molecular mechanisms employed by mammalian cells in response to stress that perturbs ER homeostasis. Most studies on ATF6 thus far focus on its role in promoter activation [Zhu et al., 1997; Li et al., 2000; Wang et al., 2000; Yoshida et al., 2000, 2001; Thuerauf et al., 2001; Okada et al., 2002] and RiP-mediated proteolytic cleavage [Ye et al., 2000; Chen et al., 2002; Shen et al., 2002]. Recently, evidence has emerged suggesting that other post-translational modifications of ATF6 also occur and they may play important roles in ATF6 activation. In mammalian cells treated with azetidine, ATF6 not only undergoes S1P/S2P proteolytic cleavage but is also phosphorylated by p38 MAPK in a sustained manner [Luo and Lee, 2002]. Phosphorylation enhances the transactivating activity of the nuclear form of ATF6 and contributes to the induction of Grp78 by AzC. In contrast, Tg induction of Grp78 is independent of p38 MAPK. Here we discover that ER stress also affects the stability of ATF6 independent of S1P/S2P proteolytic cleavage in the Golgi. Our results, as summarized in Figure 5, reveal that ATF6 is subjected to ubiquitin-proteasome degradation prior to restoration to high level and processing through the RIP pathway.

Pulse chase experiments showed that ATF6 has a half life of about 2 h [Haze et al., 2001]. Our studies show that in non-stressed cells, p90ATF6 is subjected to constitutive protein degradation, which can be inhibited by MG115 but not ALLN. The stabilization of p90ATF6 results in a 2.5-fold activation of Grp78 transcription in the absence of ER stress. Our finding that in non-stressed cells, p90ATF6 is a target for the ubiquitin-proteasome degradation pathway sensitive to MG115 provides

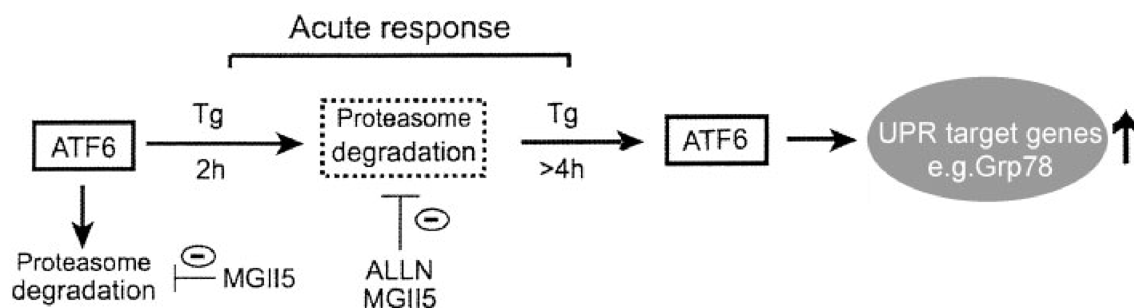

Fig. 5. ATF6 as a target of proteasome degradation. In non-stressed cells, ATF6 exists primary as a full-length ATF6 (p90). As a short-lived protein, it is constantly being turnover and its degradation by proteasomes can blocked by proteasome inhibitor MG115. Within the first 2 h of Tg treatment, the level of p90ATF6 rapidly decreased. This is in part due to proteasomal degradation which can be suppressed by proteasome inhibitors ALLN and MG115. Previous work also shows that p90ATF6 level can decrease due to Tg-induced transient block in protein translation and specific proteolytic cleavage of ATF6 by the intramembrane proteolytic process (RiP). After 4 h of Tg treatment, the steady state level of ATF6 gradually increases. While the majority of p90ATF6 still reside in the ER, a fraction of it translocates to the Golgi where it is cleaved by the S1P/S2P to yield the nuclear form, which will enter nucleus and activate Unfolded Protein Response gene such as Grp78, Grp94, and Chop.

direct support for the hypothesis that proteasome inhibitor leads to constitutive induction of chaperone protein genes in part due to stabilization of key transcription factors required for chaperone protein gene induction. Interestingly, heat shock factor 1 (HSF1), which activates the transcription of the heat shock-inducible Hsp70 gene, is also sensitive to proteasome inhibitors. Thus, proteasome inhibitors, MG132 and lactacystin, induce hypophosphorylation and trimerization of HSF1, resulting in transactivation of heat shock genes at 37°C [Kim and Li, 1999].

The rapid turnover of p90ATF6 in non-stressed cells by proteasomes will ensure that its level be stringently regulated so that it does not accidentally trigger the UPR. However, since ATF6 is a major transcription activator for ER stress inducible genes, it is surprising that its level dropped instead of increased within the first 2 h of Tg treatment. One simple explanation is that this drop is entirely due to the ER-stress induced triggering of S1P/S2P processing of p90ATF6. While RiP processing is likely to occur, several lines of evidence argue that the acute decrease in ATF6 level when cells are treated with ER stress inducers with delayed kinetics (Tg and Tuni) is largely due to proteasome degradation. First, the majority of p90ATF6 after treatment of such stress inducers remain in the ER compartment, so the elimination of the bulk of the protein has to occur in the ER rather than in the Golgi where S1P/S2P cleavage takes place. Second, pretreatment of cells with the proteasome inhibitor ALLN, which has no effect on the basal level of ATF6, can suppress the Tg-induced degradation process. Third, depletion of intracellular calcium by Tg can activate ERAD. Since ATF6 contains specific proteasomal domains in its N-terminus [Thuerauf et al., 2002], it is a logical target for degradation by proteasomes. Fourth, previous studies showed that when HeLa or Cos cells were pretreated with an inhibitor of protein synthesis before Tg treatment, the nuclear form of ATF6 was not produced while the level of p90ATF6 was still substantially reduced within 2 h [Haze et al., 1999]. This is consistent with proteasome degradation in the absence of RiP processing. Lastly, through the use of cell lines specifically deficient in S1P or S2P function, we showed directly here that the proteasomal degradation of p90ATF6 induced by ER stress is independent of S1P or S2P function. However, we noted that this process is transient and the extent of proteasome degradation can vary among different cell lines and their growth conditions, thus accounting for some variability in the level of p90ATF6 degradation reported for various cell lines under different stress conditions. We noticed that the ER-stress induced acute proteasome degradation of p90ATF6 is most apparent in NIH3T3 cells and is less evident in HeLa cells. Also, if a saturating amount of p90ATF6 is used in immunoblots, this transient, acute decrease in p90ATF6 level may also not be readily detected. For ectopically expressed p90ATF6,

overexpression with strong promoter could overwhelm the degradation system thereby attenuating the level of proteolysis.

Why do cells establish a mechanism to destroy the pre-existing pool of ATF6 upon ER stress and onset of the UPR? We offer two explanations. First, it has been reported that ectopotic expression of p90ATF6 or p60ATF6 results in programmed cell death, presumably through induction of pro-apoptotic UPR targets such as CHOP [Oyadomari et al., 2002]. Thus, immediately following ER stress, the cells may avoid triggering the cell death pathway by eliminating the bulk of p90ATF6. However, when the stress persists, the cells need to trigger the full UPR, and to achieve this, the level of p90ATF6 is restored and actually elevated to high levels along prolonged stress. Second, we recently observed that upon Tg treatment, the newly synthesized p90ATF6 is partially underglycosylation and exhibits a faster transport rate to the Golgi [Hong et al., 2004]. This results in a new form of p90ATF6 with higher transactivating activity. By purging the pre-existing pool of p90ATF6 following ER stress, mammalian cells can utilize post-translational modification of the newly synthesized ATF6 such as its glycosylation status as a monitoring mechanism for ER homeostasis. The rationale is that when cells are starved of glucose or blocked in N-linked glycosylation that requires the induction of the GRPs that are major target genes of ATF6 [Shiu et al., 1977; Lee et al., 1986; Lee, 1987], ATF6 itself is also underglycosylated, triggering the activation mechanism. This provides a plausible explanation as to why p90ATF6 contains three N-linked glycosylation sites within its ER lumenal domain, and this could extend to other forms of modification such as phosphorylation that are induced by ER-stress.

Lastly, the ubiquitin-proteasome system targets a wide range of cellular proteins for degradation. In addition to regulating a broad array of basic cellular processes, aberrations in the proteasome function have been implicated in the pathogenesis of both inherited and acquired neurodegenerative diseases such as Parkinson's, Alzheimer's, Huntington's, and Prion's diseases [Ma et al., 2002; Ciechanover and Brundin, 2003]. It will be interesting to determine in future studies whether the stabilization of ATF6 when the proteasome system malfunctions represents a cellular defensive mechanism against pathological stress.

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