Requirement of the p38 mitogen-activated protein kinase signalling pathway for the induction of the 78 kDa glucose-regulated protein/immunoglobulin heavy-chain binding protein by azetidine stress: activating transcription factor 6 as a target for stress-induced phosphorylation

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Malfolded protein formation and perturbance of calcium homoeostasis results in the induction of the endoplasmic reticulum (ER) chaperone protein, namely the 78 kDa glucose-regulated protein (GRP78)/immunoglobulin heavy-chain binding protein. Various ER stress inducers can activate grp78, but signal transduction mechanisms are not well understood. We report in the present study that the induction of endogenous grp78 mRNA by the amino acid analogue azetidine (AzC) requires the integrity of a signal transduction pathway mediated by p38 mitogen-activated protein kinase (p38 MAPK). In contrast, induction of grp78 by thapsigargin that depletes the ER calcium storage can occur even when the p38 MAPK pathway is blocked. Treatment of cells with AzC results in the sustained activation of p38 MAPK. We identified an ER transmembrane activating transcription factor 6 (ATF6) as a target of p38 MAPK phosphorylation in AzC-treated cells. ATF6 undergoes proteolytic cleavage on AzC treatment, releasing a nuclear form that is an activator of the grp78 promoter. We show here that constitutively active mitogen-activated protein kinase kinase 6, a selective p38 MAPK activator, enhances the ability of the nuclear form of ATF6 to transactivate the grp78 promoter. Our results provide direct evidence that different ER stress inducers use diverse pathways to activate grp78 and that in addition to activation by proteolytic cleavage, ATF6 undergoes specific ER stress-induced phosphorylation. We propose that phosphorylation of ATF6 is a novel mechanism for augmenting its potential as a transcription activator.

Key words: endoplasmic reticulum, malfolded protein response, stress kinase activation, transcription activation.

INTRODUCTION

The endoplasmic reticulum (ER) serves essential functions in maintaining cellular homoeostasis. In addition to a major intracellular store for calcium and the production site for lipids and sterols, ER is the site for the synthesis, assembly and glycosylation of proteins destined for secretion or transport to the cell surface. Perturbations of ER function trigger stress signalling from the ER to the nucleus, resulting in the activation of specific sets of genes [1,2]. One of the ER stress responses, known as the 'unfolded protein response' (UPR), is conserved during evolution [3,4]. In yeast, genetic analysis revealed that the yeast UPR is mediated by a unique signalling pathway, which requires ER stress-inducible splicing of the transcript of the transcription factor Hac1 by a novel bifunctional serine kinase/endoRNase, Ire1p [5–7]. In mammalian cells, the induction of ER resident protein synthesis can be elicited by a variety of environmentally and physiologically adverse conditions [8–10], as exemplified by the stress induction of the 78 kDa glucose-regulated protein (GRP78)/immunoglobulin heavy-chain binding protein, a major ER chaperone protein with calcium-binding properties [9,11]. Aberrant proteins are synthesized when mammalian cells are exposed to amino acid analogues [12]. Azetidine (AzC) is a proline analogue that has been commonly used for the generation of malfolded proteins with substitution of proline by AzC, resulting in the induction of the UPR. Thapsigargin (Tg) stress is unique to mammalian cells [13]. Tg is a specific inhibitor of the mammalian ER calcium ATPase that pumps calcium into the ER [14]. Treatment of mammalian cells with Tg results in the depletion of ER calcium. Since calcium is actively involved in protein folding and transport in the ER [15], an attractive hypothesis is that a common stimulus of ER stress inducers is the accumulation of malfolded proteins in the ER [3]. This leads to the activation of signal transduction pathways such as that mediated by Ire1p, resulting in the induction of grp78 and other ER chaperone genes.

Other studies have suggested the existence of pathways independent of Ire1p that allow mammalian cells to mount an UPR. For example, the ER transmembrane activating transcription factor 6 (ATF6) undergoes proteolytic cleavage and other changes during ER stress and strongly activates the grp78 promoter [6,16,17]. Previously, we have shown that the stimulatory activity of ATF6 towards the ER stress-response element (ERSE) present on the grp78 promoter exhibits properties distinct from those of human Ire1p, suggesting that ATF6 and Ire1p stimulate the ERSE through diverse pathways [16]. Although most of the recent studies on ATF6 in response to ER stress focus on its

Abbreviations used: AzC, azetidine; ATF6, activating transcription factor 6; CAT, chloramphenicol acetyltransferase; ER, endoplasmic reticulum; ERSE, ER stress-response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GRP78, 78 kDa glucose-regulated protein; HA, haemagglutinin; K71R, $Lys^{71} \rightarrow Arg$; mAb, monoclonal antibody; p38 MAPK, p38 mitogen-activated protein kinase; MKK, mitogen-activated protein kinase kinase; PDGF, platelet-derived growth factor; Tg, thapsigargin; UPR, unfolded protein response.

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role in promoter activation [16–20] and proteolytic cleavage [6,21], ER stress-induced kinase signalling pathways resulting in post-translational ATF6 modification have not been described yet. Using mouse fibroblast NIH3T3 and HEK-293 cells as model systems, we report in the present study that AzC results in sustained activation of p38 mitogen-activated protein kinase (p38 MAPK) and that p38 MAPK activity is required for AzC induction of grp78 transcripts. We identify ATF6 as a target of p38 MAPK-mediated phosphorylation in AzC-treated cells, and constitutively active mitogen-activated protein kinase kinase (MKK) 6, a selective p38 MAPK activator, enhances the ability of the nuclear form of ATF6 to transactivate the grp78 promoter. In contrast, Tg induction of grp78 is independent of p38 MAPK. Our studies provide direct evidence that different ER stress stimuli utilize different mechanisms to activate grp78 and that AzC induces phosphorylation of ATF6, thereby augmenting its potential as an activator for the grp78 promoter.

MATERIALS AND METHODS

Cell culture and treatments

NIH3T3, COS-7 and HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % foetal bovine serum (Life Technologies and Gibco BRL, Gaithersburg, MD, U.S.A.). For induction of ER stress, the cells were either treated with 300 nM Tg or 5 mM AzC, in the presence or absence of p38 MAPK inhibitor SB203580 (Calbiochem, La Jolla, CA, U.S.A.). For platelet-derived growth factor (PDGF) treatment, NIH3T3 cells were serum-starved (Dulbecco's modified Eagle's medium with 0.2 % foetal calf serum) for 16 h before PDGF-BB (50 ng/ml; Cintein LLC, Union, KY, U.S.A.) was added to the culture. For inhibition of PDGF, AG1295 (Calbiochem) was used. The culture conditions for the C6 rat glioma cells and HiMut.2 cells have been described previously [22]. After the indicated time of treatment, the cells were harvested for extraction of total cell lysate or total RNA.

Lentivirus infection

The dominant-negative p38α MAPK (AF mutant) lentivirus and the green fluorescent protein (GFP) lentivirus were kindly provided by Dr Wei Li (University of Southern California, Los Angeles, CA, U.S.A.). The conditions for infection have been described previously [23]. HEK-293 cells were cultured at 30–40 % confluence and subjected to infection with either dominant-negative p38α MAPK or GFP lentivirus. After 72 h of infection, the cells were either untreated or treated with AzC or Tg for 8 h. Total RNA was extracted from the cells and subjected to Northern-blot analysis.

Northern-blot analysis

Conditions for RNA extraction and Northern-blot hybridizations were performed as described previously [24]. The cDNA probes used for the detection of grp78 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been described previously [25]. The level of transcript was determined quantitively by the use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Western-blot analysis

The total cell extract was prepared by resuspending the cell pellet in RIPA buffer and the supernatant [16] obtained from the cell pellets was directly lysed in SDS-loading buffer (2 % SDS/50 mM Tris/HCl (pH 6.8)/100 mM dithiothreitol/10 % glycerol). Conditions for Western-blot analysis of β-actin and haemagglutinin (HA)-ATF6 have been described previously [16]. For GRP78 detection, a mouse anti-GRP78 monoclonal antibody (mAb; StressGen Biotechnologies Corp., Victoria, BC, Canada) was used (1:1000 dilution). A rabbit polyclonal antibody recognizing specifically the phosphorylated form of p38 MAPK(Thr^{180}/Tyr^{182}) (New England Biolabs, Beverly, MA, U.S.A.) was used to monitor the activation of p38 MAPK according to the manufacturer's instructions. After stripping, the same membrane was blotted with a rabbit polyclonal antibody raised against p38 MAPK (1:1000 dilution) to determine its total protein level. For detection of endogenous ATF6, a mouse mAb directed against the N-terminal 273 amino acid of ATF6 (gift from Dr Chun Wu, Imgenex, San Diego, CA, U.S.A.) was used at 1:500 dilution. All protein bands were revealed by ECL® chemiluminescence (Amersham Life Science, Piscataway, NJ, U.S.A.). The autoradiograms were determined quantitively by densitometry (Scion Corporation, Frederick, MD, U.S.A.) to obtain the relative protein levels.

Plasmids

The construction of the −457/chloramphenicol acetyltransferase (CAT) reporter gene has been described previously [13]. The −169/LUC reporter gene was constructed by subcloning the rat grp78 promoter subfragment spanning −169 to −29 [26] generated by PCR into the *Sac*I and *Xho*I sites of the basic luciferase vector PGL3 (Promega, Madison, WI, U.S.A.). The expression vector for HA-tagged full-length ATF6 (pCGN-ATF6) plasmid was provided by Dr Ron Prywes (Columbia University) and its construction has been described previously [18]. Plasmid pCGN-ATF6(373) was constructed by inserting the PCR fragment spanning the N-terminal 1–373 amino acids of ATF6 followed by a stop codon into the *Xba*I and *Bam*HI sites of the pCGN vector. The expression vectors MKK6(Glu) and MKK6 [Lys^{71} → Arg (K71R)] were gifts from Dr Roger Davis (University of Massachusetts) and have been described previously [27].

Transient transfection and assay of reporter-gene activity

HEK-293 cells were grown to 60 % confluence in 6-well plates, and transfected using polyfect reagent (Qiagen, Valencia, CA, U.S.A.). For each well, 1.2 μg of −169/LUC, 0.2 μg of pCGN-ATF6(373) and 0.6 μg of either MKK6(Glu) or MKK6(K71R) were co-transfected into the cells, and to the control groups the same amounts of empty vectors were added instead. Each transfection was performed in duplicates or triplicates. For inhibition of p38 MAPK, 2 h after transfection, SB203580 was added to a final concentration of 5 μM, and the cells were further incubated for 24 h before the preparation of cell lysate. The luciferase activity was measured according to the manufacturer's instructions (Promega). Conditions for transfection of the −457/CAT construct into NIH3T3 cells have been described previously [16]. To inhibit p38 MAPK activity, 20 μM of SB203580 was used.

Metabolic labelling with [^{32}P]P_i

COS-7 cells grown to 60 % confluence in 60 mm diameter dishes were transfected with pCGN-ATF6 (3 μg/dish) using superfect reagent (Qiagen). After 24 h transfection, the cells were labelled with [^{32}P]P_i (250 μCi/dish) in P_i-free medium (Gibco BRL) for 3 h in the presence or absence of AzC or SB203580. The cells were then lysed in Triton X-100 buffer [1 % (v/v) Triton X-100/20 mM Tris/HCl (pH 7.6)/0.3 M NaCl/20 mM NaF/500 nM

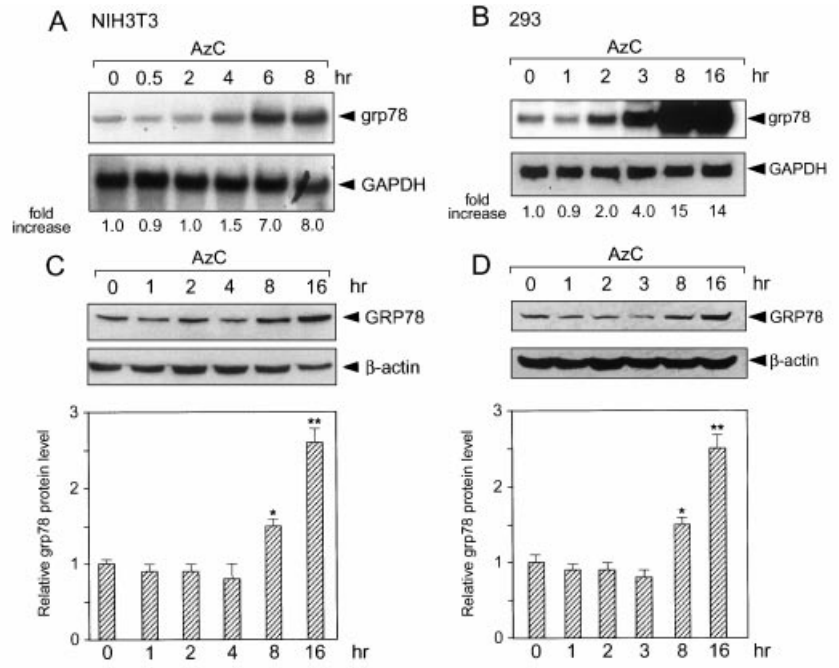

Figure 1 Kinetics of GRP78 induction by AzC

(**A**) NIH3T3 cells were treated with AzC for the time indicated on top and subjected to Northern-blot analysis with grp78 and GAPDH cDNA as probes. The relative levels were determined by quantification of Northern blots using the PhosphorImager and normalized against GADPH with the level at time 0 set as 1. The fold induction of the grp78 mRNA with relation to the time of AzC treatment is indicated below the autoradiogram. (**B**) HEK-293 cells treated with AzC were subjected to Northern-blot analysis for grp78 mRNA level as described in (**A**). (**C**) NIH3T3 cells were treated with AzC for the time indicated on top. The protein lysates were subjected to SDS/PAGE and Western-blot analysis using antibodies raised against GRP78 and β-actin. The experiment was repeated 3–4 times. The relative levels of GRP78 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. The level at time 0 was set as 1 and analysed by Student's t test ($^{*}P < 0.05$, $^{**}P < 0.01$). (**D**) HEK-293 cells treated with AzC were analysed for GRP78 protein level as described in (**C**).

Na_3VO_4/2.5 mM metabisulphite/5 mM benzamidine/1 mM EDTA/0.5 mM EGTA/10% glycerol/2 mM dithiothreitol/1 mM PMSF]. HA-ATF6 was immunoprecipitated with anti-HA mAb (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at 1:50 dilution. One-third of the immunoprecipitate from each sample was resolved by SDS/PAGE, and the dried gel was subjected to autoradiography. The remaining sample was subjected to Western-blot analysis with the anti-HA antibody at 1:500 dilution.

RESULTS

AzC induces sustained accumulation of grp78 mRNA and protein

The kinetics of induction of the transcript level of grp78 by the amino acid analogue AzC was examined in NIH3T3 cells. A gradual increase in the steady-state level of grp78 mRNA was detected after 2 h, and by 8 h there was an 8-fold increase in the grp78 mRNA level that was sustained up to 20 h (Figure 1A; results not shown). For HEK-293 cells, a 2-fold induction of the grp78 mRNA level was detected after 2 h of AzC treatment and by 8 h there was a 15-fold increase that remained at that high level for 16 h (Figure 1B). This delayed but sustained induction profile for grp78 by AzC was similar to that previously reported for Tg [13]. Thus both AzC and Tg are potential ER stress inducers. In both cell lines, the accumulation of higher levels of grp78 transcript in AzC-treated cells resulted in a gradual increase in the steady-state level of GRP78 protein in AzC-treated cells (Figures 1C and 1D). For both cell lines, quantification of the GRP78 protein levels after normalization to the β-actin loading control revealed a 1.5-fold increase in GRP78 protein level by 8 h, and by 16 h it was increased to 2.5-fold (Figures 1C and 1D). The lag in the kinetics of the increase in GRP78 protein level as compared with the mRNA could be due to transient blockage of protein translation during the UPR and the long half-life of pre-existing GRP78.

AzC induces sustained phosphorylation of p38 MAPK

The stress-activated p38 MAPK has been implicated in multiple signalling pathways initiated by cellular metabolic stress [28,29]. To test the involvement of p38 MAPK in the grp78 induction by AzC, we examined whether p38 MAPK is activated under different stress conditions in NIH3T3 cells. The activation of p38 MAPK was measured using an antibody recognizing the phosphorylated form of p38 MAPK. We observed a 4-fold increase in p38 MAPK phosphorylation in the first few hours following AzC treatment that reached a 7-fold increase after 16 h (Figure 2A). A 5-fold increase in p38 MAPK activity after 16 h of AzC treatment in NIH3T3 cells was confirmed by immunoprecipitation kinase assays, using ATF2 as a substrate (results not shown). Further, we observed that p38 MAPK can be activated by growth factors such as the PDGF (Figure 2A) and that PDGF can act as an inducer for grp78 (S. Luo and A. S. Lee, unpublished work), AzC activation of p38 MAPK is independent of the PDGF-receptor signalling pathway. This was based on two separate observations. First, treatment of NIH3T3 cells with the PDGF receptor inhibitors AG1295 and AG1296 failed to suppress AzC-induced phosphorylation of p38 MAPK (Figure 2B; results not

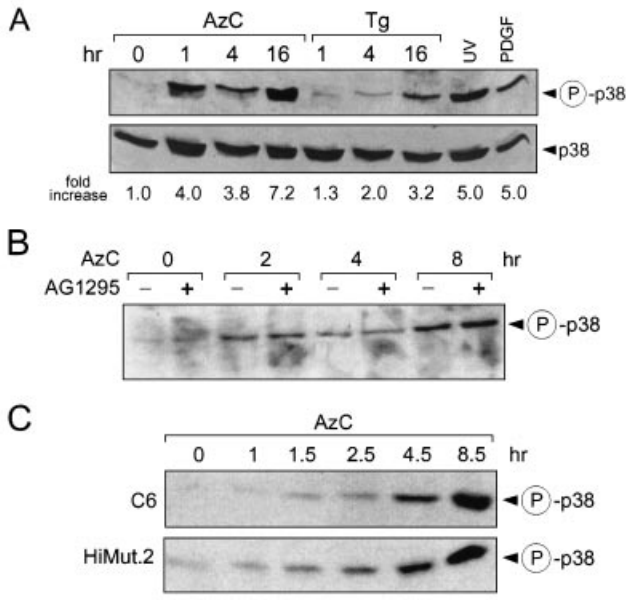

Figure 2 AzC treatment activates p38 MAPK independent of growth factor PDGF receptor

(**A**) NIH3T3 cells were treated with AzC or Tg for indicated time, UV for 5 min, or PDGF for 5 min following 16 h of serum starvation. The cell lysates were subjected to Western-blot analysis against phospho-p38 MAPK (p-p38) and p38 MAPK (p38). The relative levels of p38 MAPK activation were determined by quantification of the Western-blot bands using densitometry and were normalized against the level of total p38 MAPK. The fold increase in p38 MAPK phosphorylation for each sample is indicated below the autoradiograms. The experiment was repeated three times. (**B**) AzC-induced p38 MAPK phosphorylation is independent of the PDGF-receptor signalling pathway. NIH3T3 cells were either untreated (−) or pretreated (+) with 10 μM of AG1295 for 30 min before AzC addition. After the indicated time of AzC treatment, the cell lysates were subjected to Western-blot analysis against phospho-p38 MAPK (p-p38). (**C**) Kinetics of p38 MAPK phosphorylation in C6 cells are unaffected by defective PDGF-receptor signalling. The parental C6 cells and HiMut.2 cells overexpressing a dominant-negative mutant of PDGF receptor were treated with AzC for the time indicated and then subjected to Western-blot analysis against phospho-p38 MAPK (p-p38).

shown). This lack of inhibition was observed in cells treated with AzC from 2–8 h. Secondly, AzC-induced p38 MAPK phosphorylation was observed with similar kinetics and magnitude in both C6 rat glioma cells with functional PDGF receptor and HiMut.2 cells with a kinase-deficient PDGF receptor (Figure 2C). Similarly, AzC treatment of HEK-293 cells also resulted in the phosphorylation of p38 MAPK, with maximal phosphorylation observed at 8 h after the drug treatment (results not shown).

Activation of p38 MAPK was not restricted to AzC and PDGF, since treatment of cells with UV and Tg also resulted in a 5-fold increase of p38 MAPK phosphorylation (Figure 2A). Nonetheless, UV is not an inducer of grp78 [29] and in Tg-treated cells, the kinetics of p38 MAPK phosphorylation was slower and the magnitude of p38 MAPK phosphorylation was less than AzC (Figure 2A).

Differential requirement for p38 MAPK activity for AzC and Tg induction of grp78

To test whether p38 MAPK is required for AzC induction of grp78, HEK-293 and NIH3T3 cells were treated with AzC in the presence or absence of SB203580, an inhibitor of p38 MAPK [30]. Total RNA was isolated and subjected to Northern-blot analysis for grp78 and GAPDH transcript. With HEK-293 cells, we observed a dosage-dependent inhibition of AzC induction of the grp78 mRNA by SB203580 (Figure 3A). Northern-blot analysis performed with NIH3T3 cells showed SB203580 suppression of grp78 induction by AzC, whereas Tg induction was not affected (Figure 3B). The relative mRNA levels for both cell lines treated with both inducers, after normalization against GAPDH as a loading control, are summarized in Figure 3(C). We observed that the addition of SB203580 did not affect the basal level of grp78 mRNA in control cells, whereas treatment of cells with 5 μM SB203580 consistently suppressed the induction of grp78 mRNA by AzC by approx. 70 % (Figure 3C). Similar results were obtained with SB202190, which also inhibits p38 MAPK (results not shown). In contrast with AzC, which requires p38 MAPK activity for grp78 mRNA induction, the induction of grp78 transcripts in response to Tg treatment was insensitive to SB203580 in both NIH3T3 and HEK-293 cells (Figure 3C).

Expression of p38 MAPK mutant containing an altered phosphorylation site (TGY at positions 180–182 to AGF), referred to as p38 MAPK(AF), has been shown to have dominant-negative effect [31]. To confirm the requirement of p38 MAPK for AzC induction of grp78, we utilized lentivirus expressing p38 MAPK(AF) to block the p38 MAPK pathway. The HEK-293 cells were either non-infected, or infected with lentivirus expressing p38 MAPK(AF) or GFP as a control. The efficiency of infection as monitored by GFP expression 72 h after infection was estimated to be approx. 85 % and expression of the dominant-negative p38 over the endogenous p38 level was confirmed by Western-blot analysis (Figure 3D). Non-infected and infected cells were treated with either AzC or Tg. The total RNA was extracted and subjected to Northern-blot analysis using grp78 and GAPDH probes. The mRNA level for grp78 was normalized against GAPDH. The level of grp78 mRNA induction by AzC and Tg was identical in the non-infected cells and cells infected with the GFP lentivirus (Figure 3E). In support of the inhibition of AzC induction of grp78 by SB203580, the dominant-negative form of p38 MAPK preferentially suppressed grp78 mRNA induction by AzC, but has no effect on Tg (Figure 3E).

Next, we investigated whether p38 MAPK is required for the AzC-mediated transcriptional activation of the grp78 promoter. In examining the effect of AzC on grp78 promoter activity, we discovered that luciferase activity is negatively affected by AzC treatment, but CAT activity is not (results not shown). In CAT assays using the grp78 promoter driving a CAT reporter gene (−457/CAT) [13], we observed the strong induction of the reporter gene by both Tg and AzC, in a manner similar to the endogenous grp78 transcript (Figure 3F). The addition of SB203580 did not affect the basal expression of the grp78 promoter. The induction of the grp78 promoter by AzC was inhibited by the p38 MAPK inhibitor, whereas the induction of Tg was not affected (Figure 3F).

AzC induces the cleavage and phosphorylation of ATF6 by p38 MAPK

In previous studies [17,21], it has been shown that the transcription factor ATF6, a 90 kDa ER transmembrane protein that is a potent activator of the grp78 promoter, undergoes proteolytic cleavage when the cells are treated with ER stress inducers such as tunicamycin. As a consequence, this generates an N-terminal fragment of about 65 kDa that enters the nucleus and activates grp78 transcription. Using an mAb directed against the N-terminal 273 amino acid of ATF6, we observed that in addition to the major 90 kDa ATF6 protein band, protein bands over the range of 50–70 kDa were detectable following AzC treatment (Figure 4A). In agreement with previous observations in

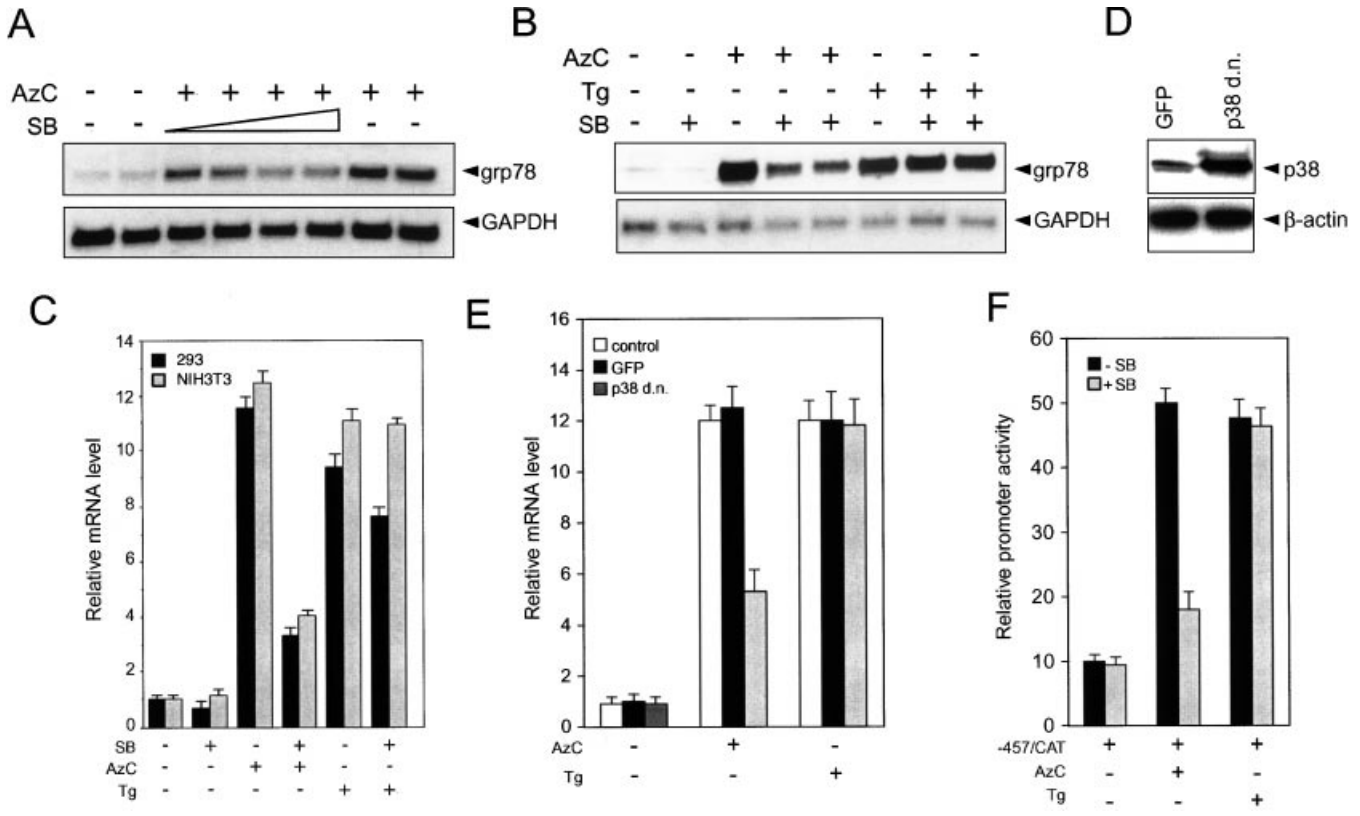

Figure 3 AzC induction of grp78 mRNA and transcription requires p38 MAPK

(**A**) HEK-293 cells were either untreated (−) or treated with AzC for 8 h (+) in the absence or presence of increasing amounts of SB (2, 3, 4 or 5 μM SB203580) as indicated on top. Analysis was performed for the untreated and AzC-treated cells in duplicates. Northern-blot analyses were performed using cDNA of grp78 and GAPDH as probes. (**B**) NIH3T3 cells were treated with AzC or Tg for 8 h in the presence or absence of 5 μM SB203580 (SB) as indicated on top. The inhibitor experiments were performed in duplicates. Northern-blot analyses were performed using cDNA of grp78 and GAPDH as probes. (**C**) The Northern-blot analyses as exemplified in (**B**) were determined quantitively by PhosphorImager. The relative grp78 mRNA level in each sample, after normalization against the GADPH level, was plotted. Identical experiments were performed with HEK-293 cells. Each experiment was repeated three times. The relative grp78 mRNA levels from the Northern-blot analyses were plotted with S.D. (**D**) Total cell lysates from HEK-293 cells infected with either the GFP lentivirus or p38 MAPK dominant-negative (p38 d.n.) lentivirus were subjected to Western-blot analysis using anti-p38 MAPK antibody or the anti-β-actin antibody. (**E**) HEK-293 cells were either non-infected (control), or infected with either GFP lentivirus (GFP) or p38 MAPK dominant-negative (p38 d.n.) lentivirus. The cells were either untreated, or treated with AzC or Tg for 8 h. Northern-blot analyses were performed using cDNA of grp78 and GAPDH as probes. The experiment was repeated twice. The relative grp78 mRNA level in each sample, after normalization against the GADPH level, was plotted with S.D. (**F**) NIH3T3 cells were transiently transfected with −457/CAT. The transfected cells were either untreated, or treated with AzC or Tg, in the presence or absence of SB203580 (SB). The experiment was repeated twice. The relative promoter activity in each sample was plotted with S.D.

Tg-treated cells [16], after AzC treatment, the level of the 90 kDa form of ATF6 decreased by one-half after 1 h and increased to twofold after 6 h. To prove that the AzC-induced band was directly derived from ATF6, full-length ATF6 with a HA-epitope tag at its N-terminus [18] was transfected into COS-7 cells. The cell extracts were prepared in a radioimmunoprecipitation assay ('RIPA') buffer. Using anti-HA antibody, we observed an increase in the intensity of the 65 kDa band in AzC-treated cells as well as Tg-treated cells (Figure 4B, upper panel). Lighter exposure of the autoradiogram revealed that the increase of the 65 kDa band was not due to a loading effect, since the increase of the 65 kDa band did not correlate with the full-length HA-ATF6 level (Figure 4B, lower panel). To improve the yield of the 65 kDa band, the cell pellets were directly lysed in SDS-loading buffer and resolved on SDS/PAGE, followed by Western-blot analysis with anti-HA antibody (Figure 4C). These experiments showed that both AzC and tunicamycin treatment yielded similar amounts of the 65 kDa band and AzC-induced cleavage of ATF6 was not affected by SB203580 treatment. For endogenous ATF6, densitometry scanning showed that the 65 kDa band represented approx. 5–10% of ATF6 in the 1 and 6 h samples respectively (Figure 4A). Therefore, in agreement with previous studies with other ER stress inducers [21], AzC treatment can also generate protein bands of 65 kDa range representing the N-terminal proteolytic product of full-length ATF6.

In search of the downstream targets of the p38 MAPK pathway that activate the grp78 promoter on AzC treatment, we localized the *cis*-elements sensitive to SB203580 on the grp78 promoter by reporter assay. We found that the AzC induction of −154grpCAT, which contains two ERSEs, was still sensitive to SB203580 (results not shown). This suggests that at least one of the transcription factors that interact with the ERSE is the downstream target of the p38 MAPK pathway and that phosphorylation by p38 MAPK is either required or potentiates its *trans*-acting activity. Among the known transcription factors activating the grp78 gene by the ERSEs, ATF6 has been shown to be phosphorylated by p38 MAPK *in vitro* [32]. To determine whether ATF6 was phosphorylated on AzC treatment, COS-7 cells were transfected with HA-tagged ATF6 and labelled with [^{32}P]P_i. The cells were either untreated or subjected to AzC

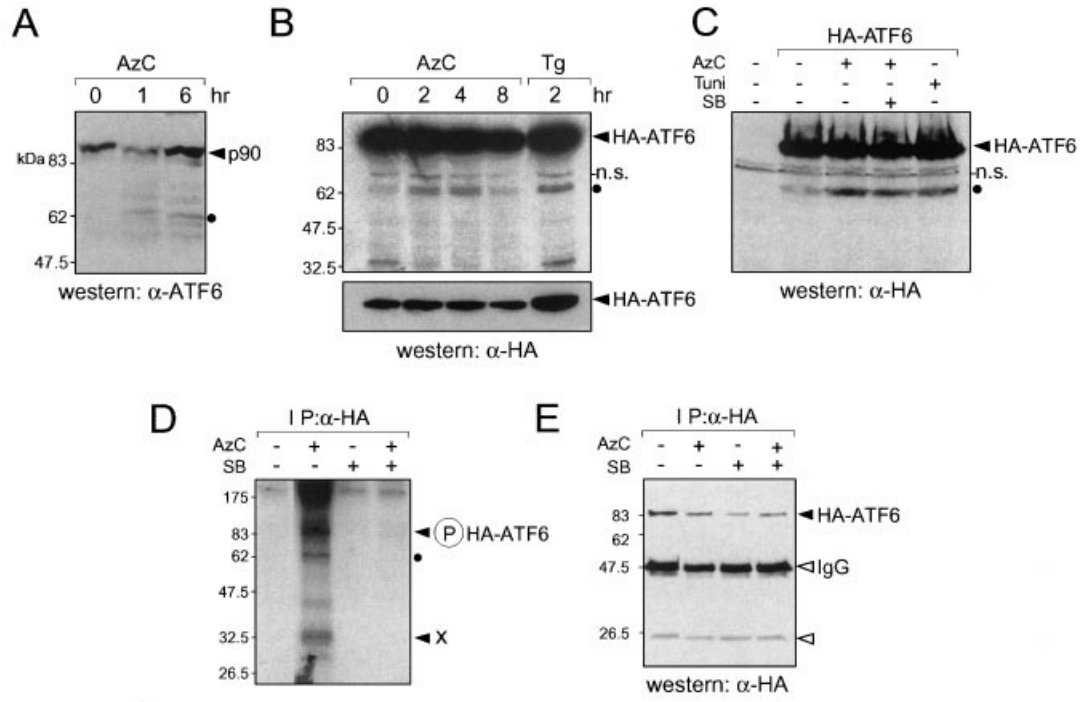

Figure 4 Cleavage and phosphorylation of ATF6 in AzC-treated cells

(**A**) Proteolytic cleavage of endogenous ATF6 following AzC treatment. Total cell lysate was prepared from NIH3T3 cells treated with AzC for the times indicated. The cells were lysed in SDS-loading buffer and subjected to Western-blot analysis using anti-ATF6 antibody. The positions of the full-length 90 kDa ATF6 (p90) and a major 65 kDa protein band (●) with increased intensity after AzC treatment are indicated. (**B**) Proteolytic cleavage of transfected HA-tagged ATF6 following AzC treatment. Upper panel, COS-7 cells were transiently transfected with pCGN-ATF6. The cells were either untreated or treated with AzC for the indicated time. As a control for ATF6 cleavage, one set of cells was treated with Tg for 2 h. The cells were lysed in RIPA buffer and subjected to Western-blot analysis against the HA tag. The positions of the full-length HA-ATF6, a 65 kDa protein band (●) with increased intensity after AzC and Tg treatment and a non-specific band (n.s.) as revealed in (**C**) are indicated. Lower panel, a lighter exposure of the autoradiogram showing the protein level of full-length HA-ATF6. (**C**) SB203580 (SB) does not block the ATF6 cleavage induced by AzC treatment. HEK-293 cells transiently transfected with pCGN-ATF6 were treated with AzC for 3 h in the presence (+) or absence (−) of 5 μM SB203580. Another set of cells was treated with tunicamycin (Tuni) (1.5 μg/ml) for 3 h as a control for stress-induced ATF6 cleavage. The cells were lysed in SDS-loading buffer and subjected to Western-blot analysis against the HA tag, in parallel with cell lysates prepared from non-transfected HEK-293 cells (lane 1). The positions of the full-length HA-ATF6, a 65 kDa protein band (●) with increased intensity after AzC and Tuni treatment and a non-specific band (n.s.) are indicated. (**D**) ATF6 phosphorylation in AzC-treated cells. COS-7 cells transiently transfected with pCGN-ATF6 were labelled with [^{32}P]P_i *in vivo*. Cell lysates prepared from control cells or cells treated with AzC in the presence (+) or absence (−) of 5 μM SB203580 (SB) were immunoprecipitated with anti-HA antibody. The immunoprecipitate was applied on to SDS/PAGE (10% gel). The autoradiogram is shown. The positions of the phosphorylated full-length HA-ATF6 (ⓟHA-ATF6), a 65 kDa protein with electrophoretic mobility similar to its cleaved products (●), and a 35 kDa band of unknown identity (X) are indicated. (**E**) Western-blot analysis with anti-HA antibody to determine the amount of HA-tagged ATF6 immunoprecipitated in (**D**). The positions of the 90 kDa HA-ATF6 and IgG are indicated. The experiments were repeated three times.

treatment. Following immunoprecipitation with anti-HA antibody and SDS/PAGE, phosphorylated ATF6 was detected by autoradiography (Figure 4D) and the level of immunoprecipitated HA-ATF6 in each sample was determined by immunoblot analysis (Figure 4E). In control cells, the level of ATF6 phosphorylation was below the detection limit (Figure 4D). On AzC treatment, major phosphorylated protein bands at 90, 65 and 35 kDa were detected. The increase in phosphorylation was blocked by treatment of cells with 5 μM SB203580, indicating its dependence on p38 MAPK (Figure 4D). Immunoblot analysis with anti-HA antibody confirmed the ability of the anti-HA antibody to immunoprecipitate HA-ATF6 in all the samples; thus the lack of phospholabelling in the control and SB203580-treated samples was not due to a failure to immunoprecipitate the protein (Figure 4E). Electrophoretic mobilities of the phosphorylated 90 and 65 kDa bands were the same as the full-length HA-ATF6 and its cleavage product respectively; however, the level of the 65 kDa protein might be too low to be detected by the anti-HA antibody. The identity of the 35 kDa band (X) is unknown. Although it is not certain that the 35 kDa protein is derived from the N-terminus of HA-ATF6, a faint protein band around 35 kDa was detectable using anti-HA antibody by Western-blot analysis, but its appearance varied among the stress-treated cells (Figure 4B).

Activation of p38 MAPK enhances the transactivation activity of the nuclear form of ATF6 towards the grp78 promoter

Next, we tested the effect of ATF6 phosphorylation on transcription mediated by the grp78 promoter. A schematic drawing of an expression vector containing the nuclear form of ATF6 [ATF6(373)] is shown in Figure 5(A). The nuclear form of ATF6 is derived from the N-terminal 373 amino acids of ATF6 and contains a basic and leucine zipper domain [17]. It is a strong stimulator of the rat grp78 promoter containing multiple ERSEs (Figure 5A). To test whether the nuclear form of ATF6 is an *in vivo* target of p38 MAPK, the expression vector for ATF6(373) was co-transfected into HEK-293 cells with either MKK6(Glu), a constitutively active kinase that phosphorylates and activates p38 MAPK, or MKK6(K71R), a kinase-inactive form of MKK6 [27]. The reporter gene −169/LUC contains a subfragment of the rat grp78 promoter driving the expression

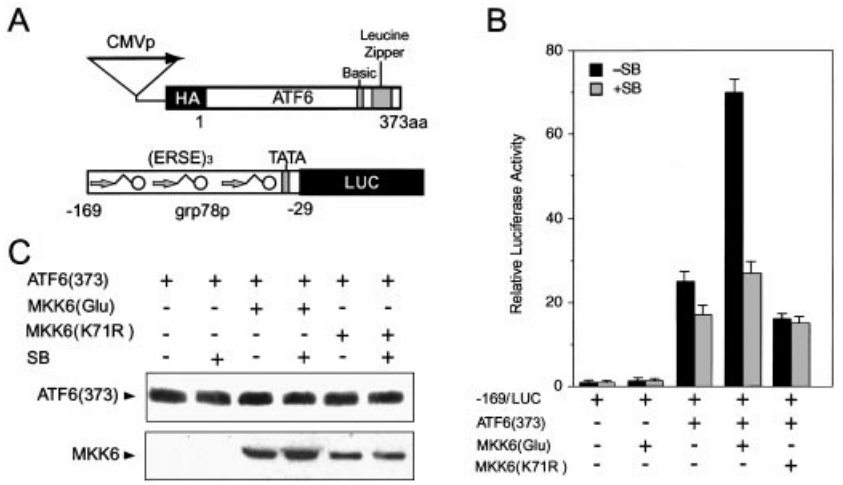

Figure 5 Activation of p38 MAPK by MKK6 enhances the ability of the nuclear form of ATF6 to transactivate the grp78 promoter

(**A**) Schematic drawing of the HA-tagged ATF6(373) expression vector and the −169/LUC. The HA-ATF6(373) is driven by the cytomegalovirus (CMV) promoter. The −169/LUC plasmid contains three ERSEs and the TATA element of the rat grp78 promoter (grp78p) fused to the luciferase gene. (**B**) The −169/LUC was used as a reporter gene and was co-transfected into HEK-293 cells with expression vectors for ATF6(373), MKK6(Glu) or MKK6(K71R) as indicated below. Plasmid containing the LacZ gene driven by the β-actin promoter was also co-transfected to normalize the transfection efficiency. The transfected cells were either untreated or treated with 5 μM SB203580 (SB). The cells were lysed and assayed for luciferase activity 24 h after transfection. The luciferase activity of the −169/LUC alone was set as 1. The experiments were repeated three times. Relative luciferase activities after normalization with β-galactosidase activities are shown with S.D. (**C**) The expression levels of the transfected ATF6(373) and MKK6 in the samples in (**B**) were determined by Western-blot analysis against the HA and the FLAG tag respectively. The loading was normalized against the β-galactosidase activity in each sample.

of the luciferase gene. This subfragment contains all three ERSEs and is fully inducible by ER stress [33]. As expected, overexpression of ATF6(373) resulted in a 25-fold induction of grp78 promoter activity (Figure 5B). Overexpression of MKK6(Glu) alone showed minimal stimulation of the grp78 promoter, suggesting that activation of p38 MAPK alone in non-stressed cells is not sufficient to induce grp78 and it needs a critical substrate for its action. One such substrate is ATF6(373) which is generated on AzC stress (Figure 4). We observed that on co-transfection of ATF6(373) with FLAG-tagged MKK6(Glu), the stimulatory activity of ATF6(373) was enhanced from 25- to 70-fold. This increase was dependent on the kinase activity of MKK6 since the kinase-inactive mutant MKK6(K71R) did not exhibit such an effect.

To inhibit p38 MAPK activity, the cells were treated with 5 μM SB203580. Addition of SB203580 alone did not affect the basal activity of the −169/LUC reporter gene; however, it slightly reduced the stimulatory activity of ATF6(373) from 25- to 17-fold (Figure 5B). In previous studies, the stimulatory activity of exogenous ATF6 in non-stressed cells was also partially reduced by SB203580 [32], suggesting that there was basal phosphorylation of exogenous ATF6 by p38 MAPK. SB203580 completely suppressed the additional induction of ATF6 activity mediated by MKK6(Glu), reducing it from 70- to 27-fold, whereas it had no effect on MKK6(K71R) (Figure 5B). To exclude the possibility of suppression of the ATF6(373) expression level by SB203580, we performed Western-blot analysis against the HA tag to detect the ATF6(373) expression level in each sample (Figure 5C). The amount of protein loaded was after normalizing against the β-galactosidase activity. The expression levels of ATF(373) were similar in all samples. Further, Western-blot analysis with the anti-FLAG antibody showed that the levels of the transfected MKK6 were not affected by SB203580 (Figure 5C). These results confirmed that MKK6 stimulation of ATF6(373) is acting via p38 MAPK. Similar results were observed when the transfections were performed with NIH3T3 cells (results not shown).

DISCUSSION

Evidence is emerging that mammalian cells may have evolved multiple mechanisms to transmit ER stress signals arising from different environmental or physiological conditions that require the up-regulation of the ER chaperone gene expression. Support of an Ire1p-dependent pathway for the mammalian UPR is based on the observation that in transient transfection assays, overexpression of Ire1p activated the grp78 promoter in a manner dependent on its endoribonuclease activity [34]. Recently [35], it was discovered that Ire1p-mediated splicing of XBP1 mRNA produces an active transcription factor capable of activating the grp78 promoter. However, since genetic knockout of both isoforms of Ire1p in mice showed no defect in the UPR including the activation of grp78 [7], there could be multiple ER stress signalling pathways. For example, genistein, an inhibitor of tyrosine kinases, suppresses stress induction of grp78, suggesting that grp78 activation is regulated by tyrosine kinase signalling pathways [25]. In support of the idea that different ER stress stimuli may elicit distinct or shared pathways to activate grp gene expression, WEIHI7.2 lymphoma cells were able to increase grp78 transcript level in response to a block in protein glycosylation but not depletion of the ER calcium storage [36]. Further investigation implied the involvement of c-*fos* in the activation of the grp78 promoter following ER calcium release, although the signalling components and the mechanism by which grp78 transcription is enhanced are not known [37].

Towards identifying cellular kinases that may regulate grp78 induction, we discovered that AzC, an agent that causes the formation of abnormal proteins, stimulates the stress-activated kinase p38 MAPK, which phosphorylates ATF6, and that the

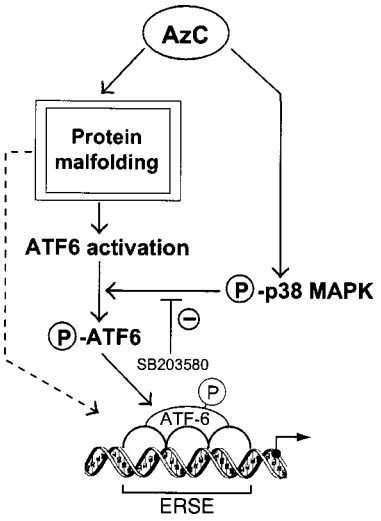

Figure 6 A model of grp78 induction by AzC through p38 MAPK pathway

AzC treatment results in the substitution of the proline analogue into newly synthesized proteins, leading to protein malfolding in the cells. The accumulation of abnormal proteins in the ER triggers the activation of ATF6 resulting in proteolytic cleavage. AzC treatment also induces sustained activation of p38 MAPK. The activated p38 MAPK phosphorylates ATF6 and possibly other targets. This process can be blocked by SB203580. We propose that phosphorylated ATF6, acting in unison with other transcription factors such as nuclear factor-Y, TFII-1 and Yin and Yang 1, induces grp78 transcription via the ERSEs. There are other signalling pathways (dashed line) such as Ire1p initiated from the ER that can contribute to AzC induction of grp78 mRNA.

AzC response is sensitive to SB203580, a specific inhibitor of p38 MAPK (Figure 6). ATF6 can physically and functionally interact with Yin and Yang 1, nuclear factor-Y and TFII-I [16,17,38], all of which bind and activate the ERSE [13,33]. Interestingly, the tyrosine phosphorylation site of TFII-I is required for its stimulatory activity and may provide the molecular link connecting the tyrosine kinase transduction pathway to transcription complexes binding the grp78 promoter [38]. Previous studies *in vitro* have established that ATF6 is a substrate of p38 MAPK in kinase assays and p38 MAPK-induced phosphorylation of ATF6 fused to a Gal4 DNA-binding domain stimulates its transactivation activity on a synthetic promoter [32]. Our investigations uncover several new observations concerning the regulation and function of phosphorylated ATF6 on ER stress. First, unlike other stimuli related to growth signalling which result in transient activation of p38 MAPK, AzC treatment elicits a sustained, long-term activation of p38 MAPK. Secondly, by multiple criteria, we showed that the activation by AzC is independent of the PDGF-signalling pathway although PDGF can stimulate p38 MAPK activity and is an inducer of grp78 (S. Luo and A. S. Lee, unpublished work). Thirdly, we showed that AzC stress induces ATF6 phosphorylation. Finally, using a natural cellular promoter, which is a physiological target for ATF6 on ER stress, we show that activation of p38 MAPK enhances the transactivation of the nuclear form of ATF6. These results lead us to propose that under specific ER stress conditions as exemplified by the treatment of cells with the amino acid analogue AzC, ATF6 not only undergoes proteolytic cleavage but is also phosphorylated by p38 MAPK. Phosphorylation enhances the transactivation activity of the nuclear form of ATF6 and contributes to the induction of grp78 by AzC (Figure 6). In support of this hypothesis, CHOP, a member of the CCAAT-enhancer-binding protein family of transcription factors that is inducible by ER stress, is also a target of p38 MAPK [29]. The stress-induced phosphorylation of CHOP by p38 MAPK enhances its ability to function as a transcription factor. Other phosphorylation targets for p38 MAPK include ATF2, a transcription factor that is implicated in the rapid induction of grp78 mediated by okadaic acid treatment followed by heat shock in a rat brain tumour cell line [28]. Nonetheless, the CRE-like site located in the upstream sequence of the grp78 promoter postulated for ATF2 action is distinct from the ERSEs and is not required for induction of grp78 by the UPR [39,40]. The target for p38 MAPK during the induction of grp78 by okadaic acid and calyculin A is not known [41].

Although p38 MAPK is required for AzC induction of grp78, its activation alone is apparently not sufficient to induce grp78. This is based on two observations. First, many external stimuli, including UV can activate p38 MAPK but are not inducers of grp78 [29]. Secondly, transfection of constitutively active MKK6 in non-stressed cells is not sufficient to activate the grp78 promoter. This is likely due to the additional requirement of proper processing of ATF6, e.g. its proteolytic cleavage specifically induced by ER stress. Thus UV can activate p38 MAPK, but it does not induce ATF6 cleavage and therefore does not result in significant grp78 activation. On the other hand, Tg treatment induces ATF6 cleavage as well as p38 MAPK activation. However, the kinetics of Tg-induced p38MAPK activation lagged behind the induction of grp78 mRNA, and the magnitude was much less. This is consistent with the idea that Tg utilizes other compensating pathways to induce grp78 when p38 MAPK is blocked by drug inhibitors or dominant-negative mutant of p38 MAPK. Further, we note that in co-transfection assays, overexpression of p38 MAPK did not affect the basal level of the grp78CAT reporter gene or its induction by Tg and it only increased by 25% the induction by AzC (results not shown). Collectively, these results show that the p38 MAPK pathway is unique for AzC induction but not Tg, and suggest that the activation of p38 MAPK rather than the expression level of p38 MAPK is the limiting factor for grp78 induction by AzC. It is also possible that there are additional targets of p38 MAPK inducible by AzC that act in concert with ATF6(373) to activate the ERSE. Future studies aimed at identifying additional p38 MAPK targets that act on the ERSE and identifying the p38 MAPK targeting site(s) in ATF6 will resolve these issues.

Our results point to the existence of diverse pathways for grp78 induction by ER stress and provide an explanation why certain cells can respond to some ER stress inducers, but may lose response to other ER stress stimuli. There may also be cell-type-specific responses to ER stress. Our results linking p38 MAPK involvement in AzC induction of grp78 are observed in at least two different cell types from two different mammalian species, suggesting that it is a common pathway in different cell types. Finally, our studies do not exclude the involvement of other stress-activated kinases as important components of the mammalian UPR. Further dissections of the signalling pathways of ER stress initiated by AzC and other inducers of chaperone genes will address these important questions. The elucidation of the mammalian UPR signalling pathways may lead to novel therapeutic approaches towards diseases associated with ER storage etiology.

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