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Endoplasmic Reticulum Stress Induces the Phosphorylation of Small Heat Shock Protein, Hsp27

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Abstract There are several reports describing participation of small heat shock proteins (sHsps) in cellular protein quality control. In this study, we estimated the endoplasmic reticulum (ER) stress-induced response of Hsp27 and αB-crystallin in mammalian cells. Treatment targeting the ER with tunicamycin or thapsigargin induced the phosphorylation of Hsp27 but not of αB-crystallin in U373 MG cells, increase being observed after 2–10 h and decline at 24 h. Similar phosphorylation of Hsp27 by ER stress was also observed with U251 MG and HeLa but not in COS cells and could be blocked using SB203580, an inhibitor of p38 MAP kinase. Other protein kinase inhibitors, like Gö6983, PD98059, and SP600125, inhibitors of protein kinase C (PKC), p44/42 MAP kinase, and JNK, respectively, were without major influence. Prolonged treatment with tunicamycin but not thapsigargin for 48 h caused the second induction of the phosphorylation of Hsp27 in U251 MG cells. Under these conditions, the intense perinuclear staining of Hsp27, with some features of aggresomes, was observed in 10%–20% of the cells. J. Cell. Biochem. 95: 932–941, 2005.

Key words: endoplasmic reticulum stress; small heat shock protein; phosphorylation; aggresome

Quality control of proteins in cells is important for normal cellular function, and the endoplasmic reticulum (ER) is considered as a site where this occurs. Newly synthesized protein is folded by ER-resident chaperones such as Bip and calnexin and accurately folded forms are directed to the Golgi apparatus. On the other hand, unfolded or misfolded proteins are retrotranslocated out to the cytoplasm and degraded by proteasomes, this process being known as ER-associated degradation (ERAD) [Sommer and Wolf, 1997; Bonifacino and Weissman, 1998]. When ER functions are perturbed by stimuli, such as interference with glycosylation, disruption of calcium homeostasis, or accumulation of mutant proteins, the ER stress response is induced to counter the abnormal conditions [Kaufman, 1999; Mori, 2000]. Induction of ER-resident chaperones, such as Bip, suppression of the production of newly synthesized protein, and acceleration of the degradation of unfolded or misfolded proteins are considered as representative ER stress responses.

Small heat shock proteins (sHsps) are a family of proteins that have a molecular mass of about 15–30 kDa and share several features: possession of a homologous amino acid sequence called the "α-crystallin domain" [Caspers et al., 1995]; induction by various stimuli including heat, oxidative reagents, and heavy metals [Klemenz et al., 1991; Inaguma et al., 1993]; and phosphorylation in response to various stresses and growth factors [Arrigo, 1990; Landry et al., 1991; Ito et al., 1997]. Hsp27 is reported to be phosphorylated by several kinases downstream of p38 MAP kinase such as MAPKAP kinase-2/3 [Stokoe et al., 1992; McLaughlin et al., 1996] and PRAK [New et al., 1998]. Moreover, the delta isoform of protein kinase C (PKC-δ) [Maizels et al., 1998], cGMP dependent protein kinase [Butt et al., 2001], and Akt [Rane et al., 2003] are reported to have catalytic potential. We previously reported that

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phosphorylation of Ser-59 in αB-crystallin is catalyzed by MAPKAP kinase-2, while its Ser-45 is targeted by p44/42 MAP kinase [Kato et al., 1998]. Recently, we and other groups have provided evidence of a connection between sHSPs and the protein quality control system in mammalian cells. We demonstrated that proteasome inhibitors cause the induction, phosphorylation, and recruitment to aggresomes of Hsp27 and αB-crystallin [Ito et al., 2002]. The interaction of αB-crystallin with FBX4, a component of the SKP1/CUL1/F-box (SCF) E3 ubiquitin ligase, promotes the FBX4-dependent ubiquitination of proteins [den Engelsman et al., 2003]. Furthermore, Parcellier et al. [2003] demonstrated that Hsp27 binds to both polyubiquitinated proteins and 26S proteasomes and enhance degradation processes.

In the present study, we investigated the response of sHsps, Hsp27, and αB-crystallin to ER stress and found Hsp27 to be phosphorylated several hours after exposure to tunicamycin and thapsigargin, mediated mainly by activation of p38 MAP kinase. Moreover, we found prolonged treatment with tunicamycin but not thapsigargin to the second induction of the phosphorylation of Hsp27 and accumulation of Hsp27 in aggresomes in U251 MG cells.

MATERIALS AND METHODS

Reagents and Antibodies

Tunicamycin thapsigargin, okadaic acid, and calyculin A were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). SB203580 and PD98059 were obtained from Tocris Cookson Ltd. (Bristol, UK), and Gö6983 and SP600125 from Calbiochem (La Jolla, CA). We used affinity purified antibodies raised in rabbits against human Hsp27 [Kato et al., 1992], phosphopeptides corresponding to the three phosphorylation sites of human Hsp27 (p15S, p78S, and p82S) [Ito et al., 2002], the carboxyl-terminal decapeptide of αB-crystallin [Kato et al., 1991], and phosphopeptides corresponding to the three phosphorylation sites of αB-crystallin (p19S, p45S, and p59S) [Ito et al., 1997]. Antibodies against phospho-JNK, JNK, phospho-p38 MAP kinase, p38 MAP kinase, phospho p44/42 MAP kinase, p44/42 MAP kinase were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal antibodies against Bip (StressGen Biotech., Victoria, BC, Canada), vimentin, β-tubulin (Sigma Chemical, Co., St Louis, MO), and ubiquitin (Medical and Biological Laboratories, Nagoya, Japan) were also employed.

Cell Culture and Preparation of Cell Extracts

U373 MG human glioma cells and U251 MG human glioma cells were grown in Eagle's minimal essential medium (EMEM; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (Equitech-Bio, Inc., Ingram, TX) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HeLa cells and COS-m6 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co.). When cells reached confluence, they were treated with various chemicals at 37°C. Cells in each dish were rinsed with phosphate-buffered saline (PBS, containing 8 g of NaCl, 0.2 g of KCl, 1.15 g of $Na_2HPO_4 \cdot 12H_2O$ and 0.2 g of KH_2PO_4 in 1,000 ml of H_2O) and frozen at −80°C. Cells were collected and suspended in a 50 mM Tris-HCl suspension buffer (buffer S), pH 7.5, containing 0.1 M NaF, 5 mM EDTA, 0.2 μM okadaic acid and 0.2 μM calyculin A, 1 mM sodium orthovanadate, and a 1/100th volume of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Each suspension was sonicated at 0°C for 1 min and centrifuged at 100,000*g* for 20 min at 4°C. The supernatants were used as soluble fractions of cells, and the pellets were washed once by sonication and centrifugation with buffer S and then solubilized with buffer S containing 2% sodium dodecyl sulfate (SDS) for SDS–polyacrylamide gel electrophoresis (PAGE). In some experiments, cells were suspended in buffer S containing 2% SDS and extracts were prepared by sonication and centrifugation as described above. The supernatants were then used as whole cell extracts.

Electrophoresis and Western Blot Analysis

SDS–PAGE was performed by the method of Laemmli [1970] in 7.5%, 10%, or 12.5% polyacrylamide slab gels. For Western blot analysis, proteins in the gels were transferred electrophoretically to nitrocellulose membranes and immunostained with primary antibodies, and then peroxidase-labeled goat antibodies against rabbit IgG or mouse IgG, as described previously [Ito et al., 1996]. Peroxidase activity in nitrocellulose membranes was visualized on X-ray films using a Western blot chemiluminescence

reagent (Western Lightning; PerkinElmer Life Sciences, Inc., Boston, MA).

Immunofluorescence

Cells were seeded on glass coverslips and when they reached 80%–90% confluence, were treated with various chemicals at 37°C. After rinsing twice with PBS, they were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature, washed three times with PBS, and blocked with 10% normal goat serum in PBS containing 0.1% sodium azide for 30 min. Incubation was then with primary and secondary antibodies, diluted in 10% normal goat serum in PBS, at room temperature for 2 and 1 h, respectively. Fluorescent images were obtained using an Axiovert 200M fluorescent microscope with an attached AxioCam HRc digital camera (Carl Zeiss, Oberkochen, Germany).

Flow Cytometric Analysis

After the various treatments, cells were collected by trypsination from culture dishes, resuspended in 10 mM HEPES-NaOH, pH 7.4 containing 100 mM NaCl and 2.5 mM $CaCl_2$, and incubated with 1/50 volume of annexin-V-FITC (Wako) and 5 μg/ml propidium iodide. After 10 min incubation at 0°C, cell fluorescence was recorded by flow cytometric analysis using a Cyto ACE-300 (JASCO, Tokyo, Japan).

Other Methods

Concentrations of protein were estimated with a micro BCA protein assay reagent kit (Pierce, Rockford, IL) or a Bio-Rad protein assay kit (Hercules, CA) using bovine serum albumin as the standard.

RESULTS

ER Stress Induces Phosphorylation of Hsp27

We estimated the response of representative sHsps, Hsp27 and αB-crystallin, to the ER-stress inducers, tunicamycin and thapsigargin, in U373 MG human glioma cells, which normally contain high levels of the two sHsps [Ito et al., 2002]. Since the induction, translocation and phosphorylation of sHsps are representative response against extracellular stimuli [Arrigo, 1990; Landry et al., 1991; Kato et al., 1993; Ito et al., 1997], we estimated these responses in this study. We could not observe the significant increase or translocation of Hsp27 after treatment with tunicamycin or thapsigargin (Fig. 1A). In contrast, increased phosphorylation of three serine residues, Ser-15, Ser-78, and Ser-82, in Hsp27 was observed at 2–10 h after treatment with the ER stress inducers, with a tendency for decrease at 16 h (Fig. 1A). The ER-resident chaperone Bip is known to be induced by ER stress and participate in the quality control proteins in cells [Gething, 1999]. We estimated the expression of Bip after treatment with tunicamycin or thapsigargin and found that the induction of Bip was observed at 5–10 h and this was sustained for 16 h (Fig. 1B). We also examined the effects of tunicamycin and thapsigargin on the levels, distribution and phosphorylation of αB-crystallin, another representative small heat shock protein, and found no significant induction, translocation or phosphorylation (Fig. 2).

Using another cell lines containing high level of Hsp27, we estimated the ER-stress induced phosphorylation of this protein and found that the agents also induced phosphorylation of Hsp27 in U251 MG and HeLa but not in COS-m6 cells (data not shown).

Phosphorylation of Hsp27 by ER Stress Is Mainly Mediated by Activation of the p38 MAP Kinase Pathway

To identify the protein kinase responsible for the phosphorylation of Hsp27 by ER stress, we examined the effects of various protein kinase inhibitors. As shown in Figure 3A, an inhibitor of p38 MAP kinase, SB203580, dramatically suppressed the phosphorylation in U373 MG cells. The delta isoform of PKC is known to catalyze phosphorylation of Hsp27, but an inhibitor of PKC, Gö6983, did not exert any suppressive effect (Fig. 3A). PD98059 and SP600125, inhibitors of p44/42 MAP kinase and JNK, respectively, tended to decrease the phosphorylation of Hsp27 but the extent was much less than with SB203580 (Fig. 3A). The similar effect of these protein kinase inhibitors on the ER stress-induced phosphorylation of Hsp27 were also observed in U251 MG cells (Fig. 3C).

We next estimated the activation (phosphorylation) of p38 MAP kinase in U373 MG and U251 MG cells. In both glioma cell lines, the level of phsophorylated (activated) form of p38 MAP kinase was increased at 2–4 h after addition of tunicamycin and thapsigargin, although the amount of the enzyme did not change (Fig. 3B,D).

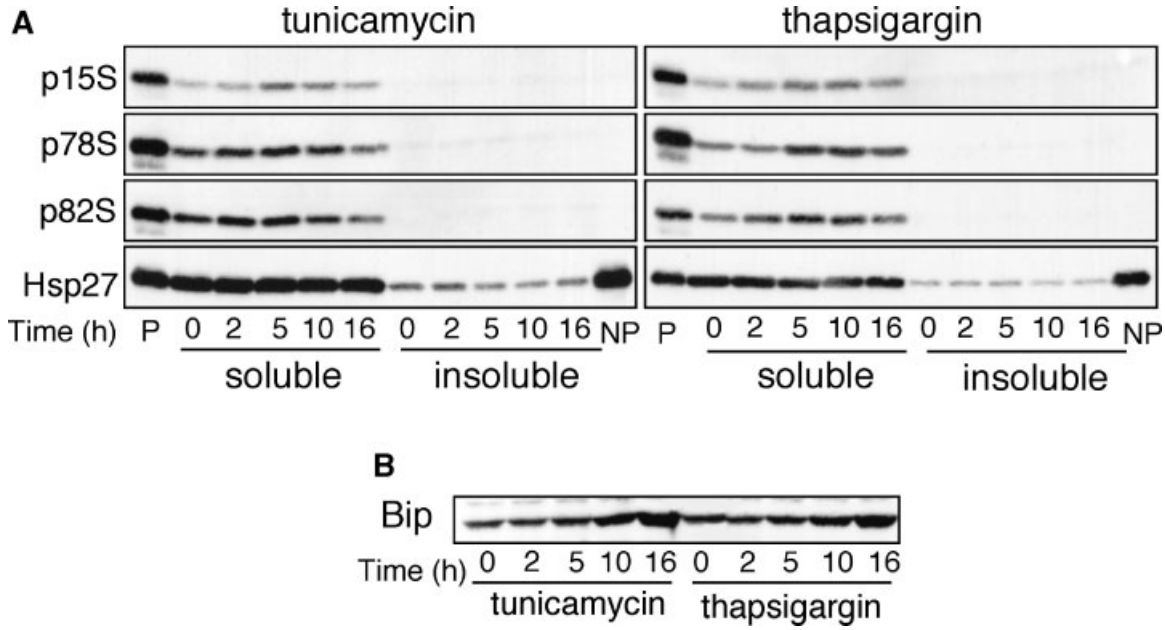

Fig. 1. Effects of treatment with tunicamycin or thapsigargin on phosphorylation of Hsp27 (**A**) and levels of Bip (**B**) in U373 MG cells. A: Tunicamycin (2 μg/ml) or 2 μM thapsigargin were added to medium of confluent cultures of U373 MG cells followed by incubation at 37°C for the indicated periods. Cells were harvested, and soluble and insoluble fractions were prepared as described in the Materials and Methods. Aliquots of soluble and insoluble extracts containing 20 μg of proteins for detection of phosphorylated Ser-15 and Ser-78, 10 μg for phosphorylated Ser-82, and 5μg for total Hsp27 were subjected to SDS–PAGE and subsequent Western blot analysis using antibodies against phosphorylated Ser-15 (p15S), Ser-78 (p78S), and Ser-82 (p82S) in Hsp27 and human Hsp27. NP, purified non-phosphorylated form of Hsp27; P, purified phosphorylated form of Hsp27. B: U373 MG cells were treated with 2 μg/ml tunicamycin or 2 μM thasigargin for the indicated periods, harvested and whole cell extracts were prepared. Twenty micrograms of aliquots were subjected to SDS–PAGE and subsequent Western blot analysis using antibodies against Bip.

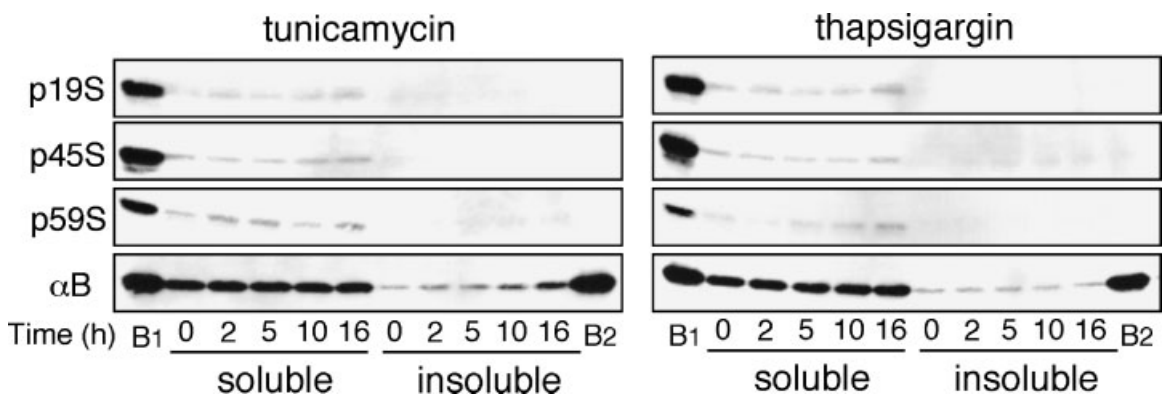

Fig. 2. Effects of treatment with tunicamycin or thapsigargin on the phosphorylation of αB-crystallin in U373 MG cells. Tunicamycin (2 μg/ml) or 2 μM thapsigargin were added to medium of confluent cultures of U373 MG cells followed by incubation at 37°C for the indicated periods. Cells were harvested, and soluble and insoluble fractions were prepared as described in the Materials and Methods. Aliquots of soluble and insoluble extracts containing 20 μg of proteins for detection of phosphorylated Ser-19, Ser-45, and Ser-59 in αB-crystallin and 5μg for total αB-crystallin were subjected to SDS–PAGE and subsequent Western blot analysis using antibodies against phosphorylated Ser-19 (p19S), Ser-45 (p45S), and Ser-59 (p59S) in αB-crystallin and carboxyl terminal αB-crystallin (αB). B_1, purified phosphorylated form of αB-crystallin; B_2, purified non-phosphorylated form of αB-crystallin.

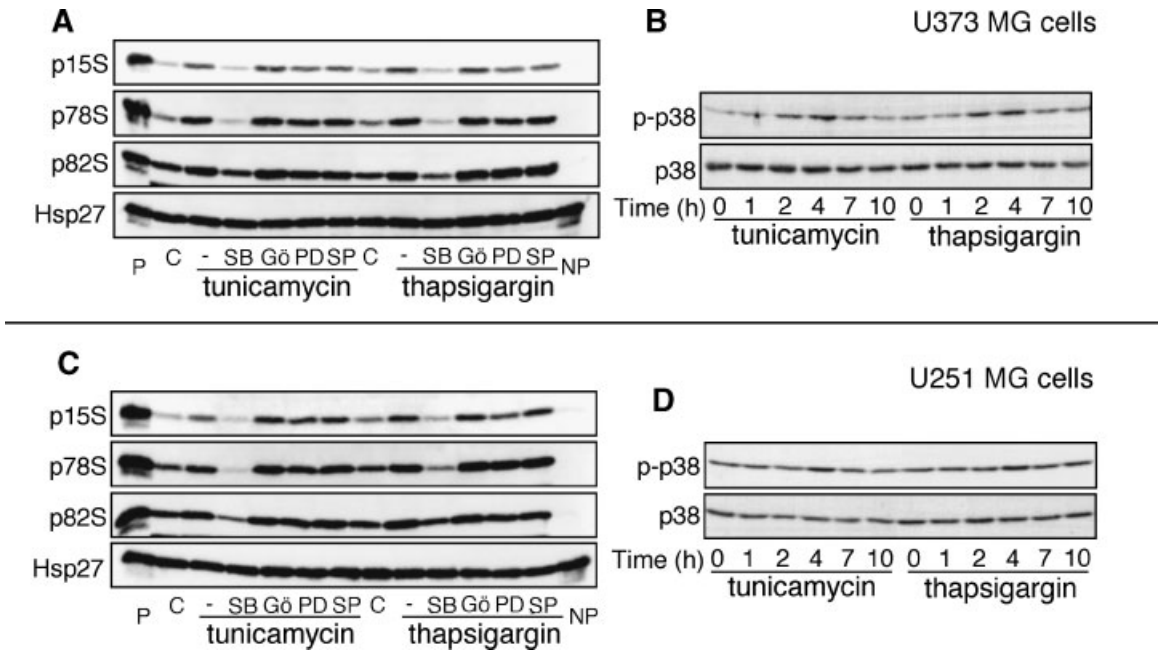

Fig. 3. Activation of the p38 MAP kinase pathway is involved in the ER stress-induced phosphorylation of Hsp27 in U373 MG and U251 MG cells. (**A**, **C**) U373 MG (A) or U251 MG (C) cells were cultured with 2 μg/ml tunicamycin or 2 μM thapsigargin in the absence (−) or presence of 10 μM SB203580 (SB), 5 μM Gö6983 (Gö), 10 μM PD98059 (PD), or 10 μM SP600125 (SP) at 37°C for 7 h. Cells were harvested and aliquots of soluble extracts containing 20 μg of proteins for detection of phosphorylated Ser-15 and Ser-78, 10 μg for phosphorylated Ser-82, and 5μg for total Hsp27 were subjected to SDS–PAGE and subsequent Western blot analysis using antibodies against phosphorylated Ser-15 (p15S), Ser-78 (p78S), and Ser-82 (p82S) in Hsp27 and human Hsp27. C, untreated control cells; NP, purified non-phosphorylated form of Hsp27; P, purified phosphorylated form of Hsp27. **B**, **D**: U373 MG (B) or U251 MG (D) cells were cultured with 2 μg/ml tunicamycin or 2 μM thapsigargin at 37°C for the indicated periods, then harvested and aliquots of whole cell extracts containing 40 μg of protein were subjected to SDS–PAGE and subsequent Western blot analysis using antibodies against phophorylated p38 MAP kinase (p-p38) and total p38 MAP kinase (p38).

Effects of Prolonged Treatment With Tunicamycin and Thapsigargin on the Phosphorylation State and Cellular Localization of Hsp27

Long exposure to ER stress inducers is known to cause cell death in some types of cells [Zinszner et al., 1998; Nakagawa et al., 2000]. In U373 MG cells, phosphorylation of Hsp27 was observed at 7 h after treatment with tunicamycin and thapsigargin, and the amount of phosphorylated Hsp27 had declined to the control level at 24 and 48 h (Fig. 4A). Translocation of Hsp27 from the soluble fraction to insoluble fraction was not observed at any time point of treatment (Fig. 4A). In U251 MG cells, the level of phosphorylated Hsp27 also increased after 7 h and declined to control levels at 24 h after treatment with both agents (Fig. 4B). However, prolonged treatment with tunicamycin, but not thapsigargin, for up to 48 h again induced phosphorylation of Hsp27 (Fig. 4B). Small amounts of Hsp27 were found in the insoluble fraction at 48 h after treatment with tunicamycin or thapsigargin (Fig. 4B).

The second induction of the phosphorylation of Hsp27 at 48 h after treatment with tunicamycin was inhibited by addition of SB203580 (Fig. 5A), and to a much lesser degree by PD98059 and SP600125 (Fig. 5A). Gö6983 did not visibly suppress the phosphorylation of Hsp27 (Fig. 5A). Recovery of increase of the phosphorylated form of p38 MAP kinase raised after 7 h of treatment and declining to control levels after 24 h was also observed at 48 h after treatment with tunicamycin (Fig. 5B).

We next examined the cellular localization of Hsp27 in U251 MG and U373 MG cells by immunocytochemistry. Hsp27 was observed mainly in the cytosol in control U251 MG cells, and the staining pattern was not changed at

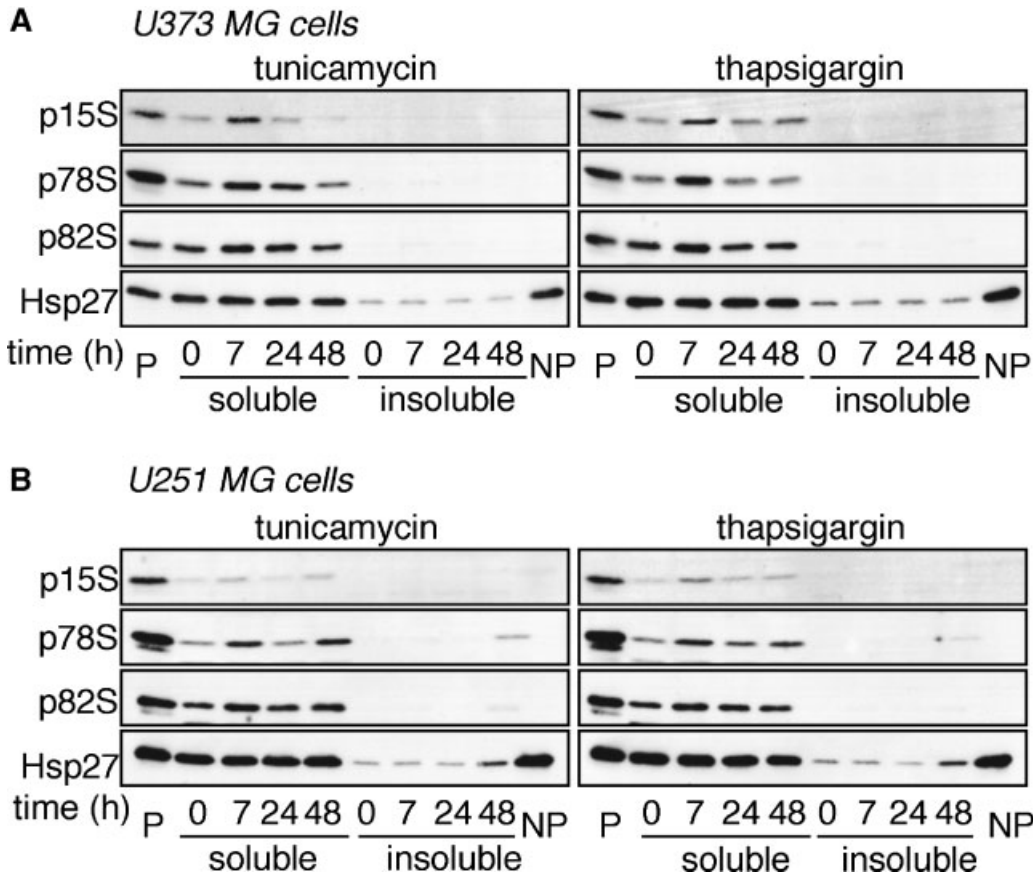

Fig. 4. Effects of prolonged exposure on the phosphorylation state of Hsp27 in U373 MG cells (**A**) and U251 MG cells (**B**). Tunicamycin (2 μg/ml) or 2 μM thapsigargin was added to medium of confluent cultures of U373 MG cells (A) or U251 MG cells (B) followed by incubation at 37°C for the indicated periods. Cells were harvested and soluble, and insoluble fractions were prepared as described in the Materials and Methods. Aliquots of soluble and insoluble extracts containing 20 μg of proteins for detection of phosphorylated Ser-15 and Ser-78, 10 μg for phosphorylated Ser-82, and 5 μg for total Hsp27 were subjected to SDS–PAGE and subsequent Western blot analysis using antibodies against phosphorylated Ser-15 (p15S), Ser-78 (p78S), and Ser-82 (p82S) in Hsp27 and human Hsp27. NP, purified non-phosphorylated form of Hsp27; P, purified phosphorylated form of Hsp27.

7 and 24 h of treatment with tunicamicin and thapsigargin (Fig. 6A). In contrast, intense juxtanuclear staining of Hsp27 was observed in 10%–20% of U251 MG cells exposed to tunicamycin for 48 h (Fig. 6A, arrows). This dramatic change in localization was not evident with thapsigargin (Fig. 6A). The aggregates resembled the previously described cytoplasmic inclusions, aggresomes. Staining with anti-β-tubulin revealed that intracellular staining of Hsp27 after long exposure to tunicamycin was localized to microtuble organizing centers (Fig. 6B). Vimentin also accumulated in perinuclear regions with Hsp27 (Fig. 6B). Staining of ubiquitin was not positive within the structures, although the proteasome inhibitor MG-132 induced aggresomes containing ubiquitinated proteins (Fig. 6B,C). In U373 MG cells, Hsp27 was also observed mainly in the cytosol and change in the localization was not observed at any time point (data not shown).

Effect of Tunicamycin or Thapsigargin on Viability of U251 MG Cells

As described above, prolonged exposure with tunicamycin, but not thapsigargin, for 48 h caused second induction of the phosphorylation and accumulation of Hsp27 in aggresomes. To ascertain whether tunicamycin is more toxic reagent than thapsigargin, cell death was investigated with the annexin-V-FITC assay, which divides cells into four classes, living

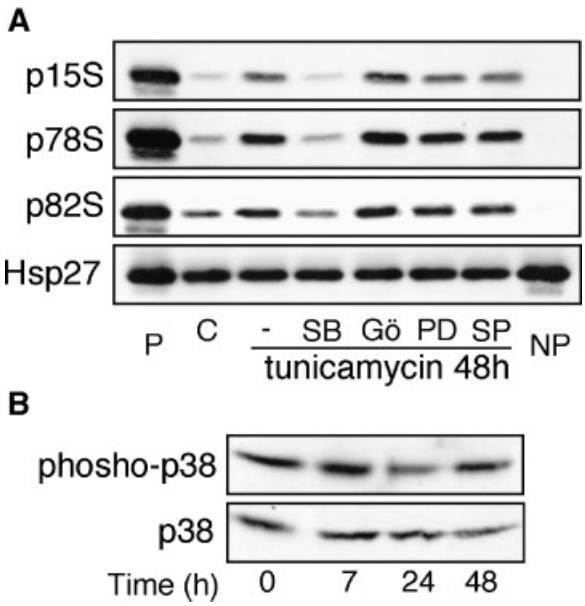

Fig. 5. Phosphorylation of Hsp27 after prolonged exposure to tunicamycin is mediated by activation of p38 MAP kinase. **A**: U251 MG cells were cultured with 2 μg/ml tunicamycin in the presence or absence (−) of 10 μM SB203580 (SB), 5 μM Gö6983 (Gö), 10 μM PD98059 (PD), or 10 μM SP600125 (SP) at 37°C for 48 h. Cells were harvested and aliquots of soluble extracts containing 20 μg of proteins for detection of phosphorylated Ser-15 and Ser-78, 10 μg for phosphorylated Ser-82, and 5 μg for total Hsp27 were subjected to SDS–PAGE and subsequent Western blot analysis using antibodies against phosphorylated Ser-15 (p15S), Ser-78 (p78S), and Ser-82 (p82S) in Hsp27 and human Hsp27. C, untreated control cells; NP, purified non-phosphorylated form of Hsp27; P, purified phosphorylated form of Hsp27. **B**: Cells were cultured with 2 μg/ml tunicamycin at 37°C for the indicated periods, then harvested and aliquots of whole cell extracts containing 40 μg of protein were subjected to SDS–PAGE and subsequent Western blot analysis using antibodies against phophorylated p38 MAP kinase (phospho-p38) and total p38 MAP kinase (p38).

(annexin/PI; −/−), apoptotic (+/−), necrotic (−/+), and secondary apoptotic (+/+) cells. In the untreated control case, living cells accounted for $93.2 \pm 0.956\%$ of the total cells (Fig. 7). Treatment with tunicamycin or thapsigargin tended to decrease the living cells in the same extent (89.1 ± 2.05 and 88.4 ± 0.866, respectively, Fig. 7). With the positive control, treatment with 1 μM staurosporine for 5 h resulted in the severe decrease of the living cells (Fig. 7).

DISCUSSION

In this study, we found representative ER stress inducers, tunicanycin and thapsigargin, to induce phosphorylation of the small heat shock protein Hsp27 after several hours in U373 MG, U251 MG, and HeLa cells (Fig. 1A and data not shown). The phosphorylation was almost completely blocked by SB203580, a selective inhibitor of p38 MAP kinase (Fig. 3A,C), in line with earlier findings for downstream kinases of p38 MAP kinase such as MAPKAP kinase-2/3 [Stokoe et al., 1992; McLaughlin et al., 1996] and PRAK [New et al., 1998]. Activation of p38 MAP kinase was also observed after treatment with tunicamycin and thapsigargin (Fig. 3B,D), further indicating that it mediates their effects. Under some conditions, the delta isoform of PKC can catalyze the phosphorylation of Hsp27 [Maizels et al., 1998] and we have reported that the PKC inhibitor Gö6983 blocks that induced by phorbol 12-myristate 13-acetate (PMA) [Kato et al., 2001] but in the present case, it was without major influence. Slight decrease in phosphorylation of Hsp27 was noted with inhibitors of p44/42 MAP kinase and JNK, but these kinases have not been reported to directly target this heat shock protein.

Several reports have appeared describing prolonged exposure to ER stressors can lead to cell death [Zinszner et al., 1998; Nakagawa et al., 2000]. We have found that prolonged exposure to tunicamycin, although not thapsigargin, caused a second rise in phosphorylation of Hsp27 in U251 MG cells (Fig. 4). The two agents are known to have different mechanisms of action, tunicamycin inhibiting glycosylation of protein and thapsigargin acting on ER Ca^{2+}-ATPase [Mahoney and Duksin, 1979; Thastrup et al., 1990]. The former may be more stressful. Tunicamycin-induced second induction of the phosphorylation of Hsp27 was not observed in U373 MG cells (Fig. 4A), indicating a difference in susceptibility that remains to be clarified.

The long exposure to tunicamycin induced perinuclear aggregates containing Hsp27 in U251 MG cells (Fig. 6A). Johnston et al. reported expression of misfolded proteins and proteasome inhibition to induce the formation of intracellular inclusions called "aggresomes" [Johnston et al., 1998] are generally defined as pericentriolar membrane-free, cytoplasmic inclusions containing misfolded, ubiquitinated proteins ensheathed in a cage of intermediate filaments. The present structures were caged by vimentin and formed at microtubule organizing centers (MTOC), but did not contained ubiquitinated protein (Fig. 6B). However, García-Mata et al. described aggresomes formed by

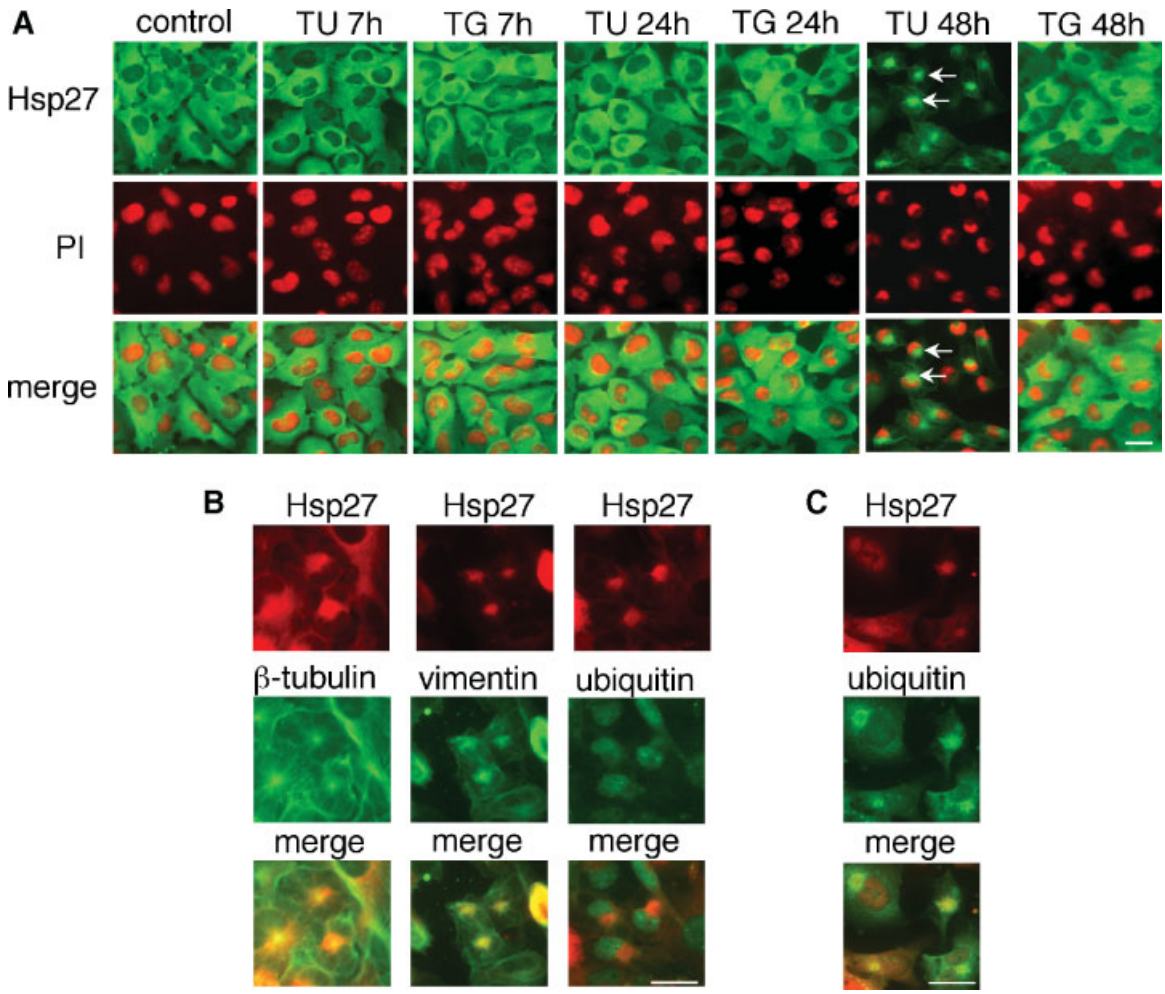

Fig. 6. Immunocytochemical analysis of Hsp27 in U251 MG cells exposed to tunicamycin or thapsigargin. **A**: U251 MG cells were incubated with 2 μg/ml tunicamycin (TU) or 2 μM thapsigargin (TG) at 37°C for the indicated periods, fixed and then subsequently stained with antibodies against Hsp27. The nuclei were stained with propidium iodide (PI). **B**: Cells treated with tunicamycin for 48 h were stained with antibodies against Hsp27 and β-tubulin, Hsp27 and vimentin, or Hsp27 and ubiquitin. (**C**) Cells treated with 5 μM MG-132 for 16 h were stained with antibodies against Hsp27 and ubiquitin. All images were obtained using fluorescence microscopy. All bars show 20 μm.

accumulation of misfolded GFP-250 protein to not contain ubiquitinated protein [García-Mata et al., 1999] and we consider the term appropriate for the structures. The significance of the formation of aggresomes and recruitment of Hsp27 after prolonged exposure with tunicamycin is not clear. The formation of aggresome is now considered as a protective response against the misfolded toxic proteins in the cell [Kopito, 2000]. The second phosphorylation of Hsp27 and formation of aggresomes appear to be closely related events, and Hsp27 may play an important causative role.

In conclusion, we here found that ER stress induces early (in several hours) reversible phosphorylation of Hsp27 in various cells. Furthermore, a representative ER stress inducer, tunicamycin, caused late phosphorylation and recruitment of Hsp27 to aggresomes in U251 MG cells. Although it is reported to be involved in the ubiquitin-dependent degradation of proteins [den Engelsman et al., 2003; Parcellier et al., 2003], a quality control system in cells, our findings are the first to our knowledge implicating Hsp27 in ER stress-induced cellular responses.

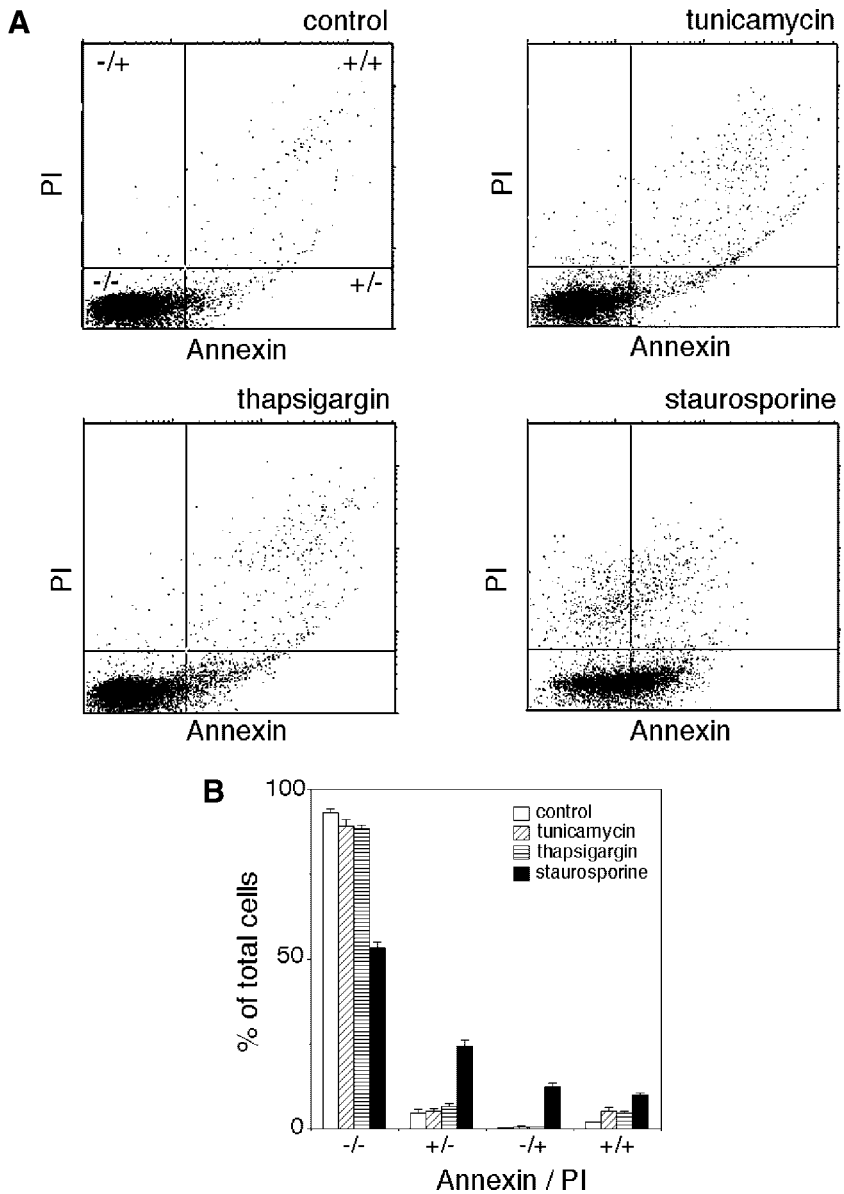

Fig. 7. Effect of tunicamycin or thapsigargin on viability of U251 MG cell. **A**: U251 MG cells were incubated with 2 μg/ml tunicamycin or 2 μM thapsigargin at 37°C for 48 h. Cells were prepared for annexin-V-FITC assay and analyzed by flow cytometry as described in the Materials and Methods. Representative histograms are shown. Results for cells treated with 1 μM staurosporine for 5 h as a positive control are also presented. **B**: The results of annexin-V-FITC assay were summarized and graphed. The data are percentages of the total cells counted. Each bar shows the mean ± SD of the results for three to four dishes.

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