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Stress Protein GRP78 Prevents Apoptosis Induced by Calcium Ionophore, Ionomycin, But Not by Glycosylation Inhibitor, Tunicamycin, in Human Prostate Cancer Cells

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Abstract GRP78 induction has recently been shown to play a critical role in maintaining cell viability against several kinds of stress, including depletion of endoplasmic reticulum Ca^{2+} and accumulation of unglycosylated proteins, under specific experimental conditions. However, the functional significance of GRP78 induction after stressful treatment has not been well defined. This article characterizes the different biological features associated with GRP78 induction by two kinds of stress agents, calcium ionophore, ionomycin (IM), and glycosylation inhibitor, tunicamycin (TM), focusing on the association with apoptosis in human prostate cancer cells. Both IM and TM treatment resulted in marked induction of GRP78 transcription in androgen-dependent prostate cancer LNCaP cells maintained in medium without androgen, but not in medium containing androgen, as measured by Northern blotting and nuclear run-off assays. After pretreatment with tumor necrosis factor-α, which has potent cytotoxic effects on LNCaP cells, both IM and TM could induce substantial increases in GRP78 transcription in LNCaP cells, even in medium containing androgen. Under both experimental conditions described, DNA fragmentation assays showed a direct correlation between the onset of apoptosis in LNCaP cells after IM treatment and the initiation of GRP78 transcript induction, while induction of GRP78 expression preceded TM-induced apoptosis. To elucidate the functional differences of GRP78 induction by IM and TM, an antisense oligodeoxynucleotide (ODN) targeted against the grp78 gene was designed to reduce GRP78 expression in a sequence-specific and dose-dependent manner. Antisense GRP78 ODN treatment substantially enhanced apoptosis of LNCaP cells induced by IM compared with mismatch control ODN treatment, whereas no marked differences were observed in apoptotic features induced by TM with antisense GRP78 and mismatch control ODN treatment. Studies of additional androgen-independent prostate cancer PC3 cells also demonstrated a correlation between GRP78 induction and resistance to apoptosis after IM treatment, but not after TM treatment. These findings suggest that there are at least two GRP78 signaling pathways, which play different roles in resistance against stress-induced apoptosis. J. Cell. Biochem. 77:396–408, 2000.

Key words: GRP78; apoptosis; calcium ionophore; glycosylation inhibitor; prostate cancer

The stress response of mammalian cells represents a protective mechanism against adverse conditions that threaten their survival. These conditions include systemic infection, tissue inflammation, and deregulated growth of malignant cells. Cells respond to these stressful conditions by inducing synthesis of evolutionarily conserved proteins, such as glucose-regulated proteins (GRPs) [Lee, 1992]. GRP78, the most well-characterized GRP, also known as the immunoglobulin heavy chain binding protein BiP, has been demonstrated to be a molecular chaperone and Ca^{2+}-binding protein. GRP78 has been found to be expressed in various cell types and localized in endoplasmic reticulum (ER) [Munro, 1986; Lee, 1992; Morimoto, 1993]. grp78 gene expression is markedly increased in response to cellular stresses, including glucose starvation, depletion of ER Ca^{2+} pool, accumulation of unglycosylated proteins, and oxygen deprivation [Lee, 1987; Li et al., 1993].

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Recently, several investigators reported that GRP78 induction is a protective response to a variety of stress conditions [Li et al., 1992; Jamora et al., 1993; Sugawara et al., 1993; Koong et al., 1994]. For example, inhibition of GRP78 induction in Chinese hamster ovary (CHO) cells has been shown to cause a decrease in cell viability after treatment with calcium ionophore [Li et al., 1992] and enhanced cell death during chronic hypoxia [Koong et al., 1994]. Furthermore, suppression of GRP78 induction in B/C10ME fibrosarcoma cells inhibited tumor progression in vivo through increases in sensitivity to cytotoxic T lymphocytes and tumor necrosis factor-α (TNF-α) [Jamora et al., 1993; Sugawara et al., 1993]. However, a recent study showed that the GRP78 stress response is differentially regulated in different types of cells and that the various response patterns of GRP78 expression are induced by different stress agents in WEHI7.2 mouse lymphoma cells [McCormick et al., 1997]. Although these findings suggest the protective role of grp78 gene induction against stress agents in several specific conditions, whether GRP78 expression after different stressful treatments has the same biological significance in resistance to apoptotic cell death remains unknown.

In epithelial cells, expression of the grp78 gene is regulated through Ca^{2+}-responsive promoter elements that respond to ER Ca^{2+} depletion [Lee, 1987]. Under physiological conditions, the ER Ca^{2+} pool is maintained by an associated Ca^{2+}-ATPase that pumps Ca^{2+} into the ER lumen from the cytoplasm [Thastrup et al., 1990]. The ER Ca^{2+} pool can be depleted by treatment of cells with a calcium ionophore, ionomycin (IM), which releases the ER Ca^{2+} pool and blocks ER protein processing. The result is an accumulation of partially folded proteins and subsequent activation of transcription of the ER chaperone gene, grp78 [Lytton et al., 1991]. By contrast, tunicamycin (TM), an inhibitor of N-linked glycosylation, also induces a substantial increase in grp78 gene expression, although the drug by itself does not appear to have any direct effects on Ca^{2+} homeostasis [McCormick et al., 1997].

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related death in men in Western industrialized countries. Accumulating evidence has strongly suggested that the incidence and progression of prostate cancer are enhanced by several kinds of stress-inducing agents [Fleshner et al., 1999]. The objectives of this study were to analyze possible differences of the functional significance of grp78 gene induction by two well-documented agents [Lee, 1987], IM and TM, using two kinds of human prostate cancer cells: androgen-dependent (AD) LNCaP and androgen-independent (AI) PC3 cells [Isaacs, 1987]. We showed a direct correlation between the onset of apoptosis in the cells after IM treatment and the initiation of GRP78 transcript induction, while induction of GRP78 expression and apoptosis by TM treatment were not well correlated. We also demonstrated that antisense GRP78 oligideoxynucleotide (ODN) inhibited induction of grp78 gene expression by IM, resulting in enhancement of apoptotic cell death, whereas antisense GRP78 ODN had no significant effect on induction of apoptosis by TM treatment. These results provide new insights into the different signal transduction pathways mediating the GRP78 stress responses as well as the different roles of grp78 gene expression in protecting against apoptotic cell death.

MATERIALS AND METHODS

Cell Culture and Treatment Conditions

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless stated otherwise. AD human prostate cancer LNCaP and AI human prostate cancer PC3 cells [Isaacs, 1987] were maintained in RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc., Burlington, ONT, Canada), respectively, supplemented with 5% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 g/ml streptomycin. When the plates were approximately 70% confluent with LNCaP cells, the culture medium was changed to serum-free medium containing vehicle or 10 nM R1881. Cells between the 38th and 47th generation were used in these experiments.

In this investigation, 1-mg/ml, 5-mg/ml, and 100-g/ml stocks of IM, TM, and TNF-α, respectively, were made in dimethylsulfoxide (DMSO) and stored at −20°C. The agents were diluted with fresh culture medium to achieve the desired final concentrations. Untreated cultures were received the same volumes of DMSO without agents.

Northern Blot Analysis

Total RNA was isolated from cultured LNCaP and PC3 cells by the acid-guanidium thiocyanate-phenol-chloroform method. A total of 20 μg of total RNA from each sample was subjected to electrophoresis on 1.2% agarose-formaldehyde gels and transferred to nylon membranes (Amersham Life Science, Arlington Heights, IL) overnight, using standard procedures [Miyake et al., 1999]. The RNA blots were hybridized at 42°C overnight with a human GRP78 or GRP94 cDNA probe labeled with [α-^{32}P]dCTP, using a random primer labeling kit (Life Technologies). The filters were then washed twice with 0.1× SSC and 0.1% sodium dodecyl sulfate (SDS) for 30 min at 65°C. After washing, filters were exposed to Kodak XAR-5 film. After stripping, the membranes were rehybridized with a human glyceraldehyde-phosphate dehydrogenase (GAPDH) cDNA probe. These probes were generated by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA of human kidney, using primers 5′-TTGATGAAATTGTTCTTGTTGGT-3′ (sense) and 5′-AACAATTTCTTC-CAGTTCCTTCTT-3′ (antisense) for GRP78, 5′-GTGGCGGACCGCGCGGCTGAGG-T-3′ (sense) and 5′-CGAAGGTCAGCAGGACGCA-3′ (antisense) for GRP94, and 5′-TGCTTTTAACTCCTGGTAAAGT-3′ (sense) and 5′-ATATTTGGCAGGTTTTTCT-AGA-3′ (antisense) for GAPDH. Densities of bands for GRP78 or GRP94 mRNA were normalized relative to that for GAPDH mRNA by laser densitometric analysis.

Western Blot Analysis

Amount of protein in each sample was measured using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Samples containing 15 μg of protein from cultured LNCaP cells were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose filter (Amersham Life Science). The filters were blocked in phosphate-buffered saline (PBS) containing 10% nonfat milk powder at 4°C overnight and then incubated for 1 h with a 1:200-diluted anti-human GRP78 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 0.03% Tween 20, and washed 3 times for 7 min each time with PBS containing 0.3% Tween 20. The filters were then incubated for 30 min with horseradish peroxidase (HRP)-conjugated anti-goat IgG (Santa Cruz Biotechnology); specific proteins were detected using an enhanced chemiluminescence (ECL) Western blot analysis system (Amersham Life Science).

DNA Fragmentation Assay

Nucleosomal DNA degradation was analyzed as described previously with a minor modification [Miyake et al., 1998]. Briefly, 1×10^5 cultured LNCaP or PC3 cells were seeded in 5-cm culture dishes and allowed to adhere overnight. After treatment with agents, cells were harvested and then lysed in a solution containing 100 mM NaCl, 10 mM Tris at pH 7.4, 25 mM EDTA, and 0.5% SDS. After centrifugation, the supernatants were incubated with 300 μg/ml proteinase K for 5 h at 65°C and extracted with phenol-chloroform. The aqueous layer was treated with 0.1 vol of 3 M sodium acetate; the DNA was precipitated with 2.5 vol of 95% ethanol. After treatment with 100 μg/ml RNase A for 1 h at 37°C, the sample was electrophoresed on a 25 agarose gel and stained with ethidium bromide.

Nuclear Runoff Assay

Nuclear runoff assays were performed as described previously with a minor modification [McCormick et al., 1997]. After culturing with agents, 5×10^7 cultured LNCaP and PC3 cells were harvested, washed twice with ice-cold PBS, and resuspended in 40 ml of cell lysis buffer containing 10 mM Tris pH 7.4, 3 mM $CaCl_2$, and 2 mM $MgCl_2$. The cells were centrifuged at 1,000 *g* for 5 min, resuspended in 1 ml of cell lysis buffer, added to 1 ml of a buffer containing 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $CaCl_2$, and 0.5% Nonidet P-40 (NP-40), and homogenized using a Dounce homogenizer. The nuclear pellet was collected by centrifugation of the homogenized cells at 500 *g* for 5 min, resuspended in 100 μl of a buffer containing 50 mM Tris pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA), and stored at −80°C. Runoff transcription was initiated by resuspending the frozen nuclei in 100 μl of a reaction mixture containing 10 mM Tris pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 1 mM dithiothreitol, 40 U/ml RNasin, 1 mM ATP, 1 mM GTP, 1 mM CTP, and 25 μl of [α-^{32}P]UTP at 30°C for 30 min. The DNA was then digested by adding 1 μl of 20,000 U/ml RNase-free DNase. Newly transcribed RNA was purified by adding 500 μl

of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate at pH 7.0, 0.5% N-lauroylsarcosine, and 0.1 M β-mercaptoethanol. The ^{32}P-labeled RNA was isolated by phenol-chloroform, followed by precipitation with 3 M sodium acetate and 1 vol of isopropyl alcohol. After centrifugation, the RNA pellet was washed with 70% ethanol and repelleted by centrifugation. The ^{32}P-labeled RNA was denatured by heating at 65°C in 6× SSPE and Denhardt's solution containing 0.5% (w/v) SDS and 50% (v/v) formamide. The ^{32}P-labeled RNA was then hybridized to slot-blotted GRP78, GRP94, and GAPDH cDNAs for 60 h at 42°C in 6× SSPE and Denhardt's solution containing 0.5% (w/v) SDS, 50% (v/v) formamide, and 50 g/ml salmon sperm DNA. After hybridization, the filters were washed twice with 0.1× SSPE and 0.1% SDS at 65°C. After washing, filters were exposed to Kodak XAR-5 film. Densities of bands for GRP78 and GRP94 were normalized relative to that for GAPDH by densitometric analysis. The densities of bands for GRP78 or GRP94 mRNA were normalized relative to that for GAPDH by laser densitometric analysis.

Antisense ODN and Treatment of Cells With ODN

The phosphorothioate oligodeoxynucleotides (ODNs) used in this study were obtained from Takara (Kusatsu, Japan). The sequence of the antisense GRP78 ODN corresponding to the human grp78 translation initiation site was 5′-CACCAGGGAGAGCTTCAT-3′. The two-base GRP78 mismatch ODN (5′-CACCAGGGAGAGGTTGAT-3′) was used as a control.

Lipofectin, a cationic lipid (Life Technologies, Gaithersburg, MD) was used to increase the ODN uptake of cells. LNCaP and PC3 cells were treated with various concentrations of ODN after preincubation for 20 min with 4 μg/ml lipofectin in serum-free OPTI-MEM (Life Technologies). At 4 h after the beginning of the incubation, the medium containing ODN and lipofectin was replaced with the standard culture medium described above.

RESULTS

Changes in GRP78 Expression in LNCaP Cells After Treatment With IM or TM

We initially characterized changes in grp78 gene expression after treatment with IM or TM in AD human prostate cancer LNCaP cells maintained in medium both with and without androgen. As shown by Northern blot analysis in Figure 1A, the GRP78 mRNA levels in LNCaP cells maintained without androgen increased after treatment with 100 nM IM in a time-dependent manner; upregulation of 1 μM tunicamycin-induced GRP78 mRNA peaked by 24 h posttreatment and then began decreasing by 48 h posttreatment. However, in the presence of androgen, no changes in GRP78 mRNA levels were observed after 100 nM IM treatment, and 1 μM TM did not induce the GRP78 mRNA in LNCaP cells as rapidly or markedly as it did in cells without androgen. We then analyzed the effects of different doses of IM or TM on changes in grp78 gene expression in LNCaP cells 48 h posttreatment. In the absence of androgen, both IM and TM induced increases in the GRP mRNA in LNCaP cells in a dose-dependent manner at concentrations of up to 100 nM and 1 μM, respectively, whereas the induction of the GRP mRNA in LNCaP cells maintained with androgen was not as marked as that in cells maintained without androgen (Fig. 1B).

To determine whether the changes in GRP78 mRNA levels induced by IM or TM were accompanied by corresponding increases in protein levels, Western blot analysis was used to evaluate changes in the GRP78 protein levels in LNCaP cells after treatment with 100 nM IM or 1 μM TM. Staining confirmed that in an SDS-polyacrylamide gel with Coomassie Brilliant Blue (Bio-Rad Laboratories), equal amount of protein sample was applied in each lane (data not shown). As shown in Figure 2, consistent with the changes in transcriptional levels, 100 nM IM and 1 μM TM induced strong upregulation of GRP78 protein in LNCaP cells maintained without androgen, but not as rapidly and substantially in cells maintained with androgen.

GRP94, which shares significant sequence homology with GRP78, has been shown to have similar biological function to GRP78 [McCormick et al., 1997]. To analyze whether GRP94 is regulated in the same manner as GRP78 in LNCaP cells by IM and TM, we evaluated the changes in GRP94 mRNA levels after treatment with 100 nM IM or 1 μM TM. The elevation of GRP94 mRNA levels occurred in a pattern similar to that of GRP78 mRNA (Fig. 3); that is, both 100 nM IM- and 1 μM TM-induced GRP78 mRNA in LNCaP cells cultured with-

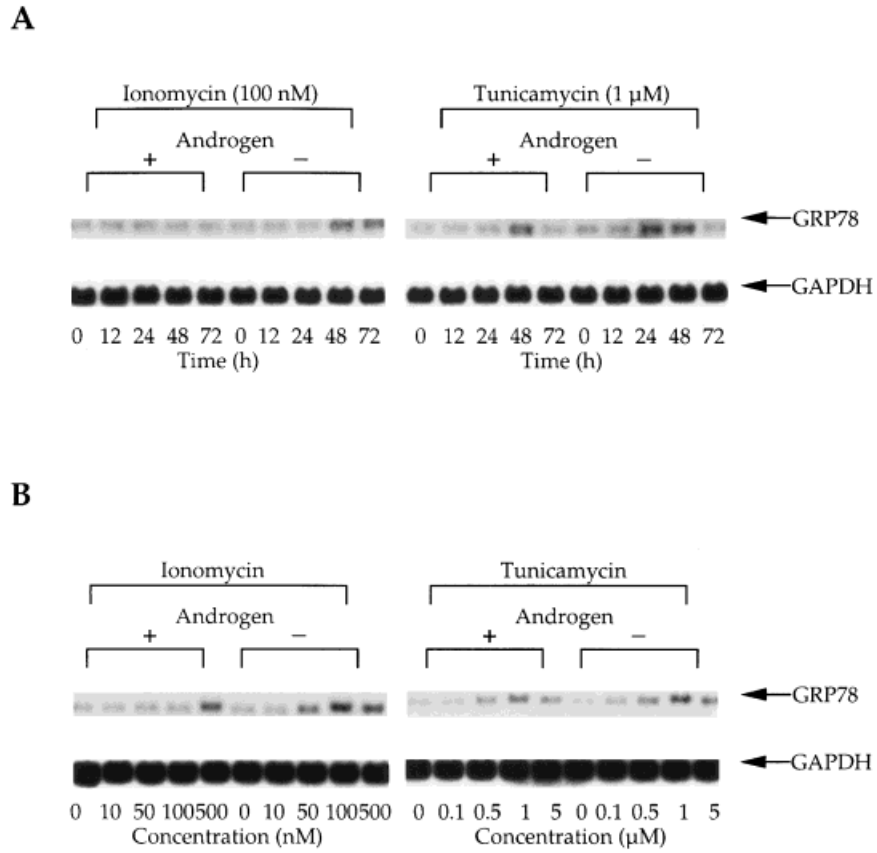

Fig. 1. GRP78 mRNA induction in LNCaP cells after treatment with ionomycin (IM) or tunicamycin (TM). **A:** Exponentially growing cells maintained in medium both with and without androgen were treated with 100 nM IM or 1 μM TM. RNA was extracted at the indicated times after treatment and analyzed by Northern blotting, using radiolabeled GRP78 cDNA probe (top) and GAPDH cDNA probe (bottom). **B:** Exponentially growing cells maintained in medium both with and without androgen were treated with the indicated concentrations of IM or TM. RNA was extracted 48 h after treatment and analyzed by Northern blotting, using radiolabeled GRP78 cDNA probe (top) and GAPDH cDNA probe (bottom).

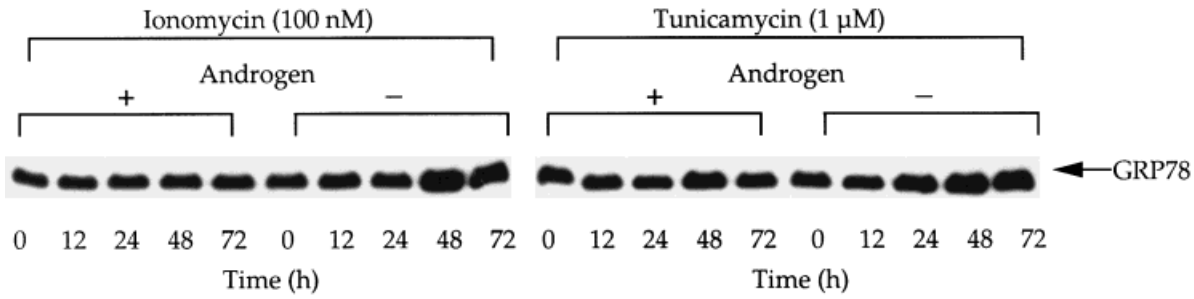

Fig. 2. Changes in GRP78 protein levels in LNCaP cells after treatment with ionomycin (IM) or tunicamycin (TM). Protein was isolated from exponentially growing cells at the indicated times after treatment with 100 nM IM or 1 μM TM; 15 μg of each sample was separated by SDS-PAGE. Western blots were probed with a polyclonal antibody to GRP78; the specific binding of the antibody was detected using an enhanced chemiluminescent (ECL) detection system.

out androgen peaked by 48 h posttreatment and began decreasing by 72 h posttreatment. By contrast, no significant differences in GRP94 mRNA levels were observed after 1 μM IM treatment of LNCaP cells cultured with androgen; 1 μM TM also induced only a modest upregulation of GRP94 mRNA in cells cultured with androgen.

These findings suggest that androgen decreases the inducibility of GRP78 and GRP94

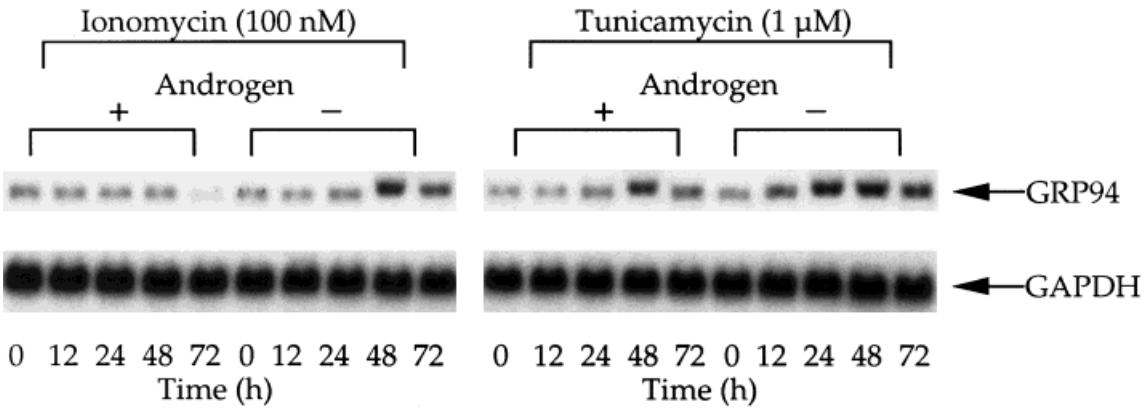

Fig. 3. GRP94 mRNA induction in LNCaP cells after treatment with ionomycin (IM) or tunicamycin (TM). Exponentially growing cells maintained in medium both with and without androgen were treated with 100 nM IM or 1 μM TM. RNA was extracted at the indicated times after the treatment and analyzed by Northern blotting, using radiolabeled GRP94 cDNA probe (top) and GAPDH cDNA probe (bottom).

expression in AD LNCaP cells by IM and TM. To confirm these results, we measured the effects of treatment with IM or TM on the transcription rates of grp78 and grp94 genes by nuclear runoff assays using nuclei isolated from LNCaP cells. As shown in Figure 4, without androgen, 100 nM IM and 1 μM TM induced a significant increase in GRP78 and GRP94 transcripts, which was detectable 12 h and 6 h after treatment, respectively. However, in the presence of androgen, no increase in newly expressed GRP78 or GRP94 transcripts after 100 nM IM treatment was observed, and a modest elevation of these transcripts was detected 6 h after 1-μM TM treatment. This finding shows that the presence of androgen in the culture medium affects the GRP78 and GRP94 transcription in LNCaP cells.

Apoptotic Features in LNCaP Cells After Treatment With IM or TM

It is well documented that despite the different patterns in various cell types, and under various experimental conditions, a close relationship between the GRP78 induction and apoptotic features is generally observed [Li et al., 1992; Jamora et al., 1993; Sugawara et al., 1993; Koong et al., 1994]. Therefore, DNA fragmentation assays were performed under the same conditions as the Northern blot analysis described in Figure 1A. Interestingly, a direct correlation was found between the onset of apoptosis in LNCaP cells after 100 nM IM treatment and initiation of GRP78 mRNA induction; that is, after 100 nM IM treatment, neither upregulation of GRP78 mRNA nor apoptotic DNA ladder was detectable in LNCaP cells cultured with androgen over a 72-h period, whereas in LNCaP cells cultured without androgen, GRP78 mRNA upregulation and apoptosis were detected 48 h after 100 nM IM treatment (Fig. 5). However, GRP78 induction in LNCaP cells after 1 μM TM treatment preceded the onset of apoptosis in both media with and without androgen, suggesting that GRP78 may be an upstream factor of TM-induced apoptosis.

Effects of TNF-α Treatment on IM- or TM-Induced GRP78 Expression and Apoptosis in LNCaP Cells

To determine whether changes in GRP78 expression in AD LNCaP cells after IM or TM treatment are directly associated with apoptosis, or partially reflect repression of the gene expression by androgen, we assessed the effects of 100 nM IM or 1 μM TM on GRP78 mRNA levels and apoptotic features in LNCaP cells cultured in medium containing androgen and pretreated with 1 ng/ml TNF-α, which has been shown to have a potential cytotoxic effect on LNCaP cells [Sensibar et al., 1995]. As shown by Northern blot analysis in Figure 6A, after pretreatment with 1 ng/ml TNF-α, both 100 nM IM and 1 μM TM induced GRP78 mRNA upregulation in LNCaP cells earlier and to higher levels than without TNF-α. This finding suggests that induction of grp78 gene expression is not directly repressed by androgen but is regulated by apoptotic stimuli.

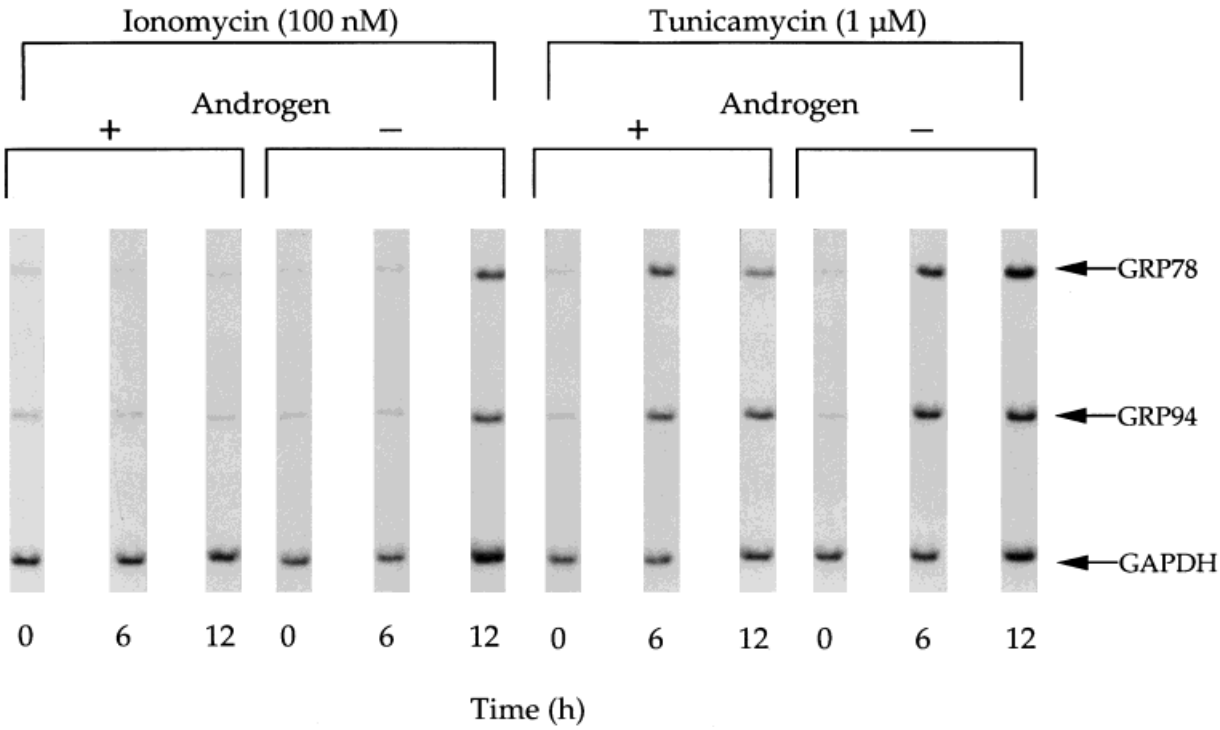

Fig. 4. Effects of ionomycin (IM) or tunicamycin (TM) treatment on GRP78 and GRP94 transcription. Nuclei were isolated from LNCaP cells after treatment with 100 nM IM or 1 μM TM for the indicated times. Equal amounts of radiolabeled nuclear runoff RNA were hybridized to slot blots containing 5 μg of GRP78 and GRP94 cDNAs. Hybridization of runoff RNA to slot blots containing GAPDH cDNA was used as a control. The times when samples were taken after treatment with IM or TM are indicated.

Furthermore, as shown in Figure 6B, DNA fragmentation assays demonstrated that after pretreatment with 1 ng/ml TNF-α, a direct correlation was found between onset of apoptosis in LNCaP cells treated with 100 nM IM and initiation of GRP78 mRNA induction, while induction of GRP78 expression and apoptosis by 1 μM TM treatment were not well correlated.

Antisense GRP78 ODN Enhanced Apoptosis Induced by IM, But Not by TM

To analyze the functional significance of GRP78 upregulation in LNCaP cells after treatment with IM or TM, antisense GRP78 ODN corresponding to the human grp78 translation initiation site and mismatch control ODN containing two base changes were used in this study. The effects of treatment with antisense GRP78 ODN on GRP78 expression were evaluated by Northern blot analysis. As shown in Figure 7A, daily treatment of LNCaP cells with antisense GRP78 ODN (100, 500, and 1,000 nM) for 2 days reduced the GRP78 mRNA levels in a dose-dependent manner. By contrast, GRP78 mRNA levels were not affected by the two-base mismatch control ODN at the employed concentrations. We then evaluated the effects of antisense GRP78 ODN on GRP78 mRNA levels after IM or TM treatment. Although GRP78 induction was still present several times after 1 μM antisense GRP78 ODN treatment when GRP78 mRNA was shown to be highly upregulated by IM or TM (Fig. 1A), 1 μM antisense GRP78 ODN treatment substantially inhibited GRP78 mRNA expression after 100-nM IM or 1-μM TM treatment in LNCaP cells (Fig. 7B).

We also evaluated the effects of IM or TM treatment on apoptotic features of LNCaP cells pretreated with antisense GRP78 ODN. As shown in Figure 7C, the reduction of GRP78 expression by 1 μM antisense GRP78 ODN was associated with enhancement of 100 nM IM-induced apoptosis in LNCaP cells maintained in medium with or without androgen, whereas no significant differences in 100 nM IM-induced apoptosis were observed between LNCaP cells pretreated with 1 μM mismatch control

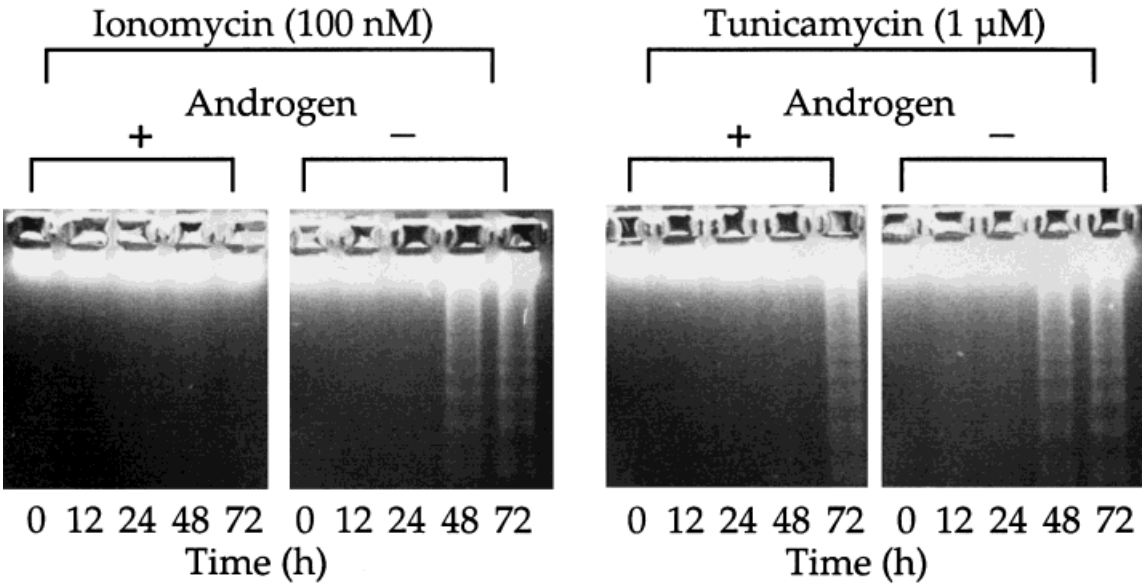

Fig. 5. DNA fragmentation in LNCaP cells induced by ionomycin (IM) or tunicamycin (TM). Cellular DNA was extracted from LNCaP cells at the indicated times after treatment with 100 nM IM or 1 μM TM, and analyzed by electrophoresis on a 2% agarose gel to detect DNA fragmentation.

ODN and untreated LNCaP cells (data not shown). By contrast, pretreatment with 1 μM antisense GRP78 ODN had no significant effect on 1 μM TM-induced apoptosis in LNCaP cells (Fig. 7C).

Effects of Changes in GRP78 Expression on Apoptosis Induced by IM or TM in PC3 Cells

To determine whether the differential effects of reduced GRP78 expression induced by IM and TM on apoptosis are observed in other prostate cancer cells, we analyzed the effects of IM or TM treatment on human AI prostate cancer PC3 cells pretreated with antisense GRP78 ODN. As shown in Figure 8A,B, both 100 nM IM and 1 μM TM induced increases in GRP78 mRNA expression levels in PC3 cells in a time-dependent manner, and 1 μM antisense GRP78 ODN significantly inhibited the upregulation of GRP78 mRNA expression by 100 nM IM or 1 μM TM. Furthermore, as observed in experiments using LNCaP cells, 1 μM antisense GRP78 ODN treatment enhanced 100 nM IM-induced apoptosis in PC3 cells, but not 1 μM TM-induced apoptosis (Fig. 8C).

DISCUSSION

Recent studies have demonstrated the critical role of GRP78 induction in maintenance of cell viability following exposure to stress-inducing agents [Li et al., 1992; Jamora et al., 1993; Sugawara et al., 1993; Koong et al., 1994]. However, the functional significance of GRP78 upregulation remains controversial and undefined, as several investigators have reported conflicting findings. For example, thapsigargin (TG), an inhibitor of Ca^{2+} uptake into the ER, failed to upregulate GRP78 expression in some types of cell lines [McCormick et al., 1997]. Enhanced GRP78 expression in V79 CHO cells by 6-aminonicotinamide or 2-deoxyglucose treatment was correlated with increased sensitivity to DNA cross-linking agents, including melpharan, 1,3-bis(2-chloroethyl)-1-nitrosourea, and cisplatin [Chatterjee et al., 1997]. Collectively, these findings suggest that the functional roles of GRP78 expression may differ depending on the cell type and the agent inducing GRP78 expression. Accordingly, in the present study, we analyzed the functional significance of GRP78 induction by two kinds of agents, IM and TM, in AD and AI prostate cancer cells, focusing on the association with apoptotic features.

Initially, we characterized the effects of IM or TM treatment on changes in GRP78 expression in AD human prostate cancer LNCaP cells maintained in medium with and without androgen. Northern and Western blot analyses showed that GRP78 expression was remarkably upregulated after treatment

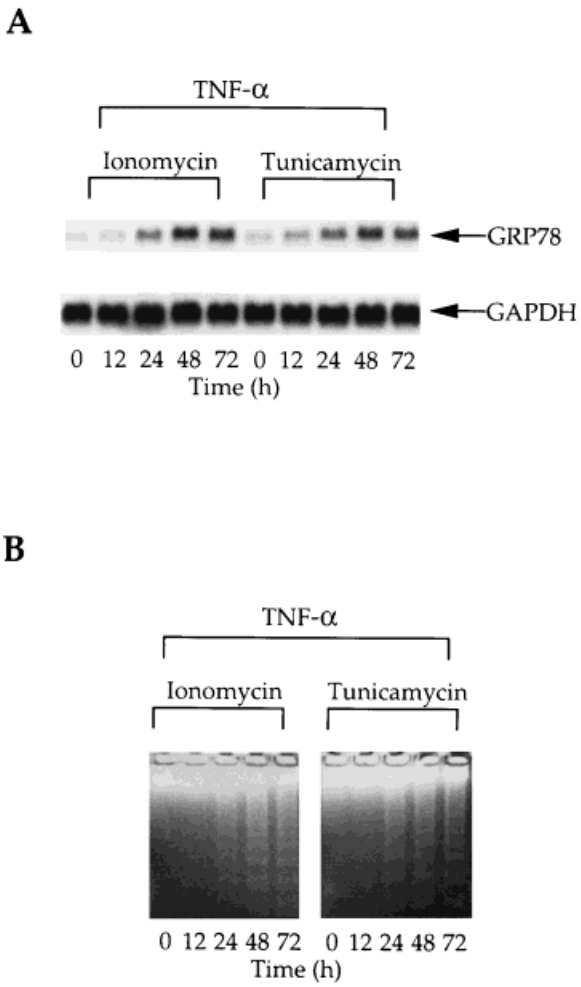

Fig. 6. Effects of pretreatment with tumor necrosis factor-α (TNF-α) on GRP78 induction and apoptosis in LNCaP cells treated with ionomycin (IM) or tunicamycin (TM). **A:** Exponentially growing cells, maintained in medium containing androgen, were pretreated with 1 ng/ml TNF-α and then treated with 100 nM IM or 1 μM TM. RNA was extracted at the indicated times after treatment and analyzed by Northern blotting, using radiolabeled GRP78 cDNA probe (top) or GAPDH cDNA probe (bottom). **B:** Cellular DNA was extracted from LNCaP cells at the indicated times after the same treatment as described in **A** and analyzed by electrophoresis on 2% agarose gels to detect DNA fragmentation.

with both IM and TM in LNCaP cells cultured without androgen; however, the respective changes in LNCaP cells cultured in medium without androgen were quite modest. We also found similar patterns of changes in the GRP78 transcription rate in LNCaP cells after IM or TM treatment by using nuclear runoff assays. These results suggest an inhibitory effect of androgen on GRP78 induction in LNCaP cells, whereas pretreatment with TNF-α, a potent inducer of apoptosis in LNCaP cells [Sensibar et al., 1995], rapidly and significantly enhanced GRP78 expression by IM or TM in LNCaP cells cultured with androgen. Collectively, these findings demonstrate that GRP78 expression in AD LNCaP cells is directly associated with apoptotic stimuli, rather than partially regulated by androgen repression.

We then analyzed the relationship between the apoptotic features and the induction of GRP78 expression in LNCaP cells. Interestingly, DNA fragmentation assays clearly demonstrated a close correlation between the onset of apoptotic cell death and GRP78 upregulation induced by IM, but not by TM. These findings suggest that there may be two different pathways that mediate the GRP78 stress response: one is closely regulated by apoptotic stimuli through ER Ca^{2+} depletion and the other, a response to inhibition of protein glycosylation, is not directly associated with apoptosis. A recent report indicated that the GRP78 signaling pathway activated in response to TM treatment appears to be operative in mouse lymphoma cells, but not that which is activated in response to TG, suggesting the presence of at least two signal transduction pathways mediating GRP78 induction [McCormick et al., 1997]. Since it has been shown that Ca^{2+} mobilization and inhibition of glycosylation induce GRP78 transcription through common promoter elements [Wooden et al., 1991], the differences in GRP78 induction are likely to reside downstream from the promoter level.

Antisense ODN, a chemically modified stretch of single-stranded DNA that is complementary to mRNA regions of a target gene, and that therefore effectively inhibits gene expression by forming RNA/DNA duplexes [Crooke, 1992], offers one strategy to specifically target grp78 gene expression. Phosphorothioate ODNs are water-soluble, stable agents manufactured to resist nuclease digestion [Saijo et al., 1994]. In this study, to examine whether the upregulation of GRP78 induced by IM has a different significance from that induced by TM in protecting cells against apoptosis, phosphorothioate antisense GRP78 ODN, corresponding to the human grp78 translation initiation site, was designed and shown to reduce GRP78 mRNA levels in a sequence-specific and dose-

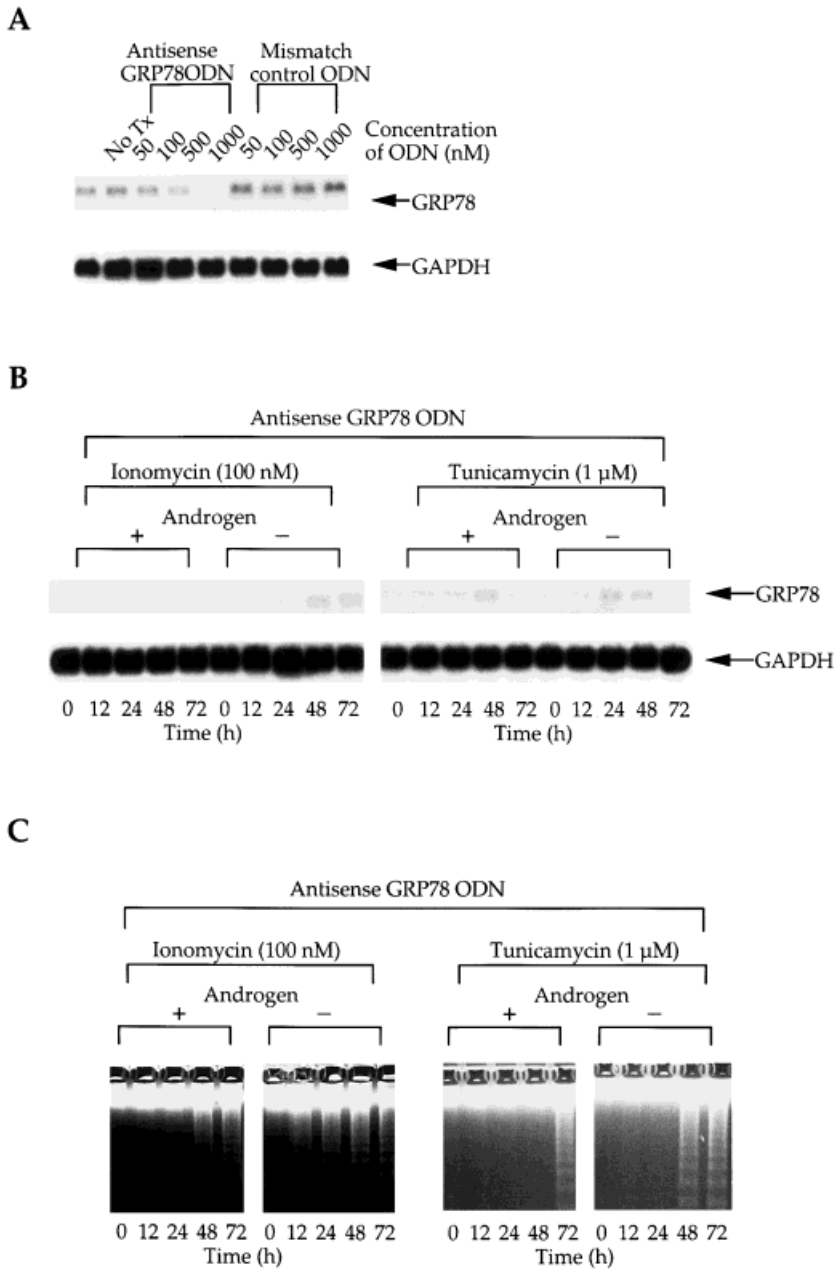

Fig. 7. Effects of antisense oligodeoxynucleotide (ODN) targeted against GRP78 on GRP78 mRNA expression and apoptosis in LNCaP cells treated with ionomycin (IM) or tunicamycin (TM). **A:** Exponentially growing cells were treated daily with various concentrations of an antisense GRP78 ODN or a two-base GRP78 mismatch ODN as a control for 2 days. RNA was extracted 72 h after initiation of treatment and analyzed by Northern blotting using radiolabeled GRP78 cDNA probe (top) and GAPDH cDNA probe (bottom). **B:** Exponentially growing cells were pretreated with 100 nM IM or 1 μM TM; 24 h after treatment, the cells were then treated with 1 μM antisense GRP78 ODN under the same conditions described in **A.** RNA was extracted at the indicated times after the treatment and analyzed by Northern blotting, using radiolabeled GRP78 cDNA probe (top) or GAPDH cDNA probe (bottom). **C:** Cellular DNA was extracted from LNCaP cells at the indicated times after the same treatment as described in **B** and analyzed by electrophoresis on 2% agarose gels to detect DNA fragmentation.

dependent manner. After inhibition of GRP78 expression by treatment of LNCaP cells with antisense GRP78 ODN, IM-induced apoptosis in LNCaP cells was significantly enhanced compared with that after mismatch control ODN treatment; however, no marked differences were observed between the effects of antisense GRP78 and

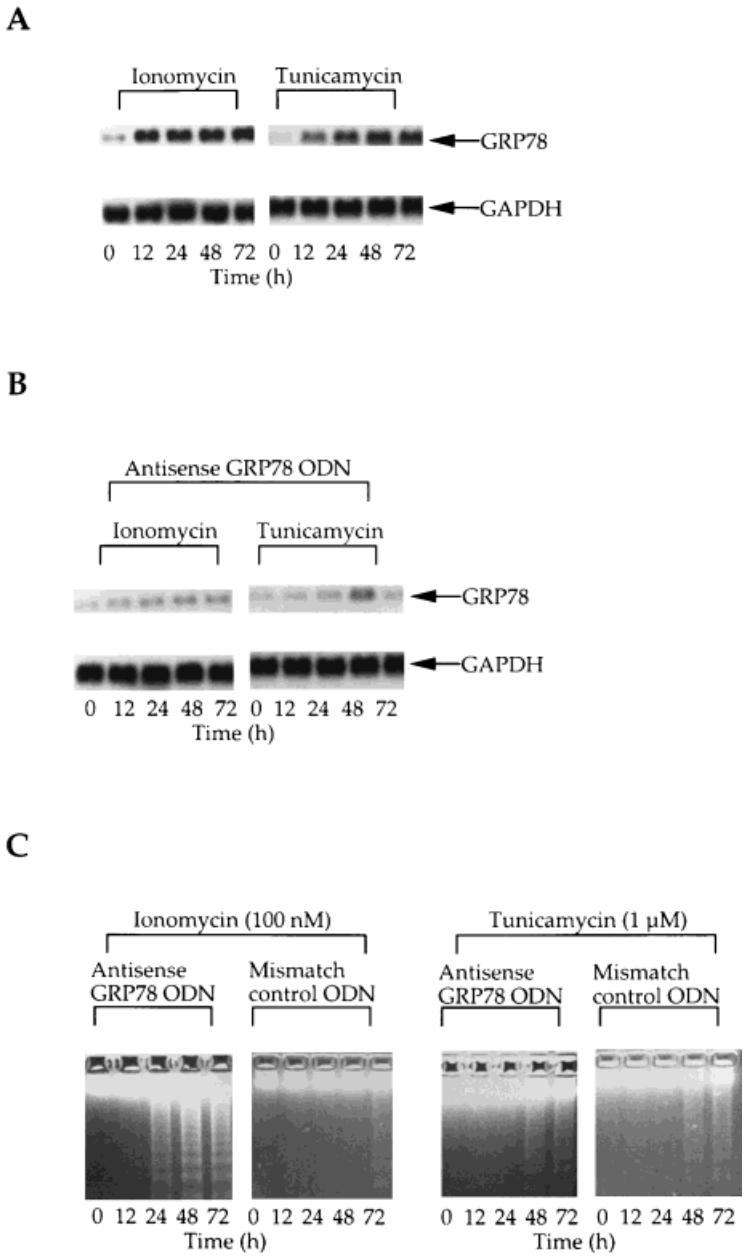

Fig. 8. Effects of antisense oligodeoxynucleotide (ODN) targeted against GRP78 on GRP78 mRNA expression and apoptosis in PC3 cells treated with ionomycin (IM) or tunicamycin (TM). **A:** Exponentially growing cells were treated with 100 nM IM or 1 μM TM. RNA was extracted at the indicated times after the treatment and analyzed by Northern blotting, using radiolabeled GRP78 cDNA probe (top) or GAPDH cDNA probe (bottom). **B:** Exponentially growing cells were pretreated with 100 nM IM or 1 μM TM; 24 h after treatment, the cells were treated with 1 μM antisense GRP ODN under the same conditions described in Fig. 7A. RNA was extracted at the indicated times after treatment and analyzed by Northern blotting, using radiolabeled GRP78 cDNA probe (top) or GAPDH cDNA probe (bottom). **C:** Cellular DNA was extracted from PC3 cells at the indicated times after the same treatment described in Fig. 7B and analyzed by electrophoresis on 2% agarose gels to detect DNA fragmentation.

mismatch control ODN treatments on the apoptotic features induced by TM. In agreement with our findings, a previous study revealed that suppression of GRP78 upregulation by a GRP78 antisense plasmid resulted in increased cell death following treatment with calcium ionophore A23187 [Li et al., 1992]. Similarly, when induction of GRP78 and

GRP94 was inhibited by ribozyme cleavage of newly transcribed GRP94 mRNA, increased sensitivity to treatment with A23187 and TG was demonstrated [Little and Lee, 1995].

Furthermore, abrogation of both the GRP78 and GRP94 stress responses has been shown to fail to enhance the cytotoxicity of TM [Little and Lee, 1995]. Collectively, these findings suggest that GRP78 upregulation may play specific roles in protection against ER Ca^{2+} depletion stress, although GRP78 expression can be induced by several kinds of stimuli, such as accumulation of unglycosylated proteins.

Better understanding of the role of GRP78 in regulating apoptotic cell death may lead to possible implications for cancer therapy. Recent studies have reported the critical relationship between prostate cancer progression and several kinds of stress-inducing agents [Fleshner et al., 1999]. In addition, some reports demonstrate that an increase in GRP78 expression confers resistance to doxorubicin on cells [Shen et al., 1987] and etoposide [Hughes et al., 1989]. Considering these findings, combined treatment of cancer cells with classical chemotherapeutic agents and antisense GRP78 ODN may achieve additive or synergistic effects through enhancement of apoptotic cell death.

In conclusion, the results reported here include the following three significant findings. First, GRP78 stress response may be regulated by the ability of each stress to induce apoptosis in target cells. Second, apoptotic features and GRP78 induction are well correlated in response to ER Ca^{2+} depletion, but not in response to inhibition of protein glycosylation, suggesting the presence of at least two different pathways mediating the GRP78 stress response. Third, upregulation of GRP78 confers protection against ER Ca^{2+} depletion on cells.

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