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Thapsigargin-Induced *Grp78* Expression Is Mediated by the Increase of Cytosolic Free Calcium in 9L Rat Brain Tumor Cells

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Abstract Exposure of 9L rat brain tumor cells to 300 nM thapsigargin (TG), a sarcoendoplasmic Ca^{2+}-ATPases inhibitor, leads to an immediate suppression of general protein synthesis followed by an enhanced synthesis of the 78-kDa glucose-regulated protein, GRP78. Synthesis of GRP78 increases significantly and continues to rise after 4 h of treatment, and this process coincides with the accumulation of *grp78* mRNA. TG-induced *grp78* expression can be suppressed by the cytosolic free calcium ($[Ca^{2+}]_c$) chelator dibromo-1,2-bis(aminophenoxy)ethane *N,N,N′,N′*-tetraacetic acid (BAPTA) in a concentration-dependent manner. Induction of *grp78* is completely abolished in the presence of 20 μM BAPTA under which the TG-induced increase of $[Ca^{2+}]_c$ is also completely prevented. By adding ethyleneglycol bis(β-aminoethyl)ether-*N,N,N′,N′* tetraacetic acid in the foregoing experiments, in a condition such that endoplasmic reticulum calcium ($[Ca^{2+}]_{ER}$) is depleted and calcium influx from outside is prevented, TG-induced *grp78* expression is also abolished. These data lead us to conclude that increase in $[Ca^{2+}]_c$, together with the depletion of $[Ca^{2+}]_{ER}$, are the major causes of TG-induced *grp78* expression in 9L rat brain tumor cells. By using electrophoretic mobility shift assays (EMSA), we found that the nuclear extracts prepared from TG-treated cells exhibit an increase in binding activity toward the extended *grp78* promoter as well as the individual cis-acting regulatory elements, CRE and CORE. Moreover, this increase in binding activity is also reduced by BAPTA. By competitory assays using the cis-acting regulatory elements as the competitors as well as the EMSA probes, we further show that all of the tested cis elements—CRE, CORE, and C1—are involved in the basal as well as in the TG-induced expression of *grp78* and that the protein factor(s) that binds to the C1 region plays an important role in the formation and maintenance of the transcription complex. J. Cell. Biochem. 78:404–416, 2000. © 2000 Wiley-Liss, Inc.

Key words: thapsigargin; cytosolic free calcium; endoplasmic reticulum; glucose-regulated protein; regulatory elements; gene regulation

Cytosolic free calcium ($[Ca^{2+}]_c$) plays crucial roles in a variety of cell signaling pathways that are involved in cytoskeleton dynamics, gene regulation, and cell proliferation [Resendez et al., 1986; Cole and Kohn, 1994; Clapham, 1995; Ghosh and Greenberg, 1995]. $[Ca^{2+}]_c$ homeostasis is maintained by a concerted action of various Ca^{2+} transporters (pumps) located on the endoplasmic reticulum (ER) membrane, the mitochondrial inner membrane, and the plasma membrane [Rizzuto et al., 1993; Clapham, 1995]. Therefore, the disturbance of Ca^{2+} homeostasis in one of the cellular compartments may affect the Ca^{2+} homeostasis in the other. For instance, treatment with thapsigargin (TG), a widely used inhibitor of the ubiquitous sarcoendoplasmic reticulum Ca^{2+}-ATPases, would lead to a depletion of intraluminal Ca^{2+} ($[Ca^{2+}]_{ER}$) and a concurrent increase of $[Ca^{2+}]_c$ [Thastrup et al., 1994; Treiman et al., 1998]. Moreover, because the release of Ca^{2+} from intracellular storage pools and the entry of extracellular Ca^{2+} are often coupled, treatment with TG, while blocking Ca^{2+} accumulation and allowing Ca^{2+} release from ER, induces a rapid increase of $[Ca^{2+}]_c$ followed by a sustained increase mediated by a specific Ca^{2+} influx pathway [Ikari et al., 1997; Wei et al., 1998]. Depending on cell types and treatment protocols, exposure to TG may induce the expression of various genes, including the 78-kDa

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glucose-regulated protein (GRP78) in Chinese hamster ovary cells [Li et al., 1993] and human rhabdomyosarcoma cells [Delpino et al., 1998], stress proteins in mammalian chondrocytes [Cheng and Benton, 1994], immediate early gene VL30 [Magun and Rodland, 1995] and interleukin-6 in murine macrophages [Bost and Mason, 1995], zinc finger transcription factor, EGR-1, in human melanoma cells [Muthukkumar et al., 1995], c-fos in keratinocytes [Harmon et al., 1996], as well as the growth arrest gene, gadd153, in prostate cancer cells [Lin et al., 1997]. Recently, it has been suggested that TG-induced gene expression in nonexcitable cells is dependent on calcium influx [Rodland et al., 1997].

ER residing GRP78 (also known as BiP) [Shiu et al., 1977] functions as a molecular chaperone by associating transiently with nascent proteins as they traverse the ER and aiding in their folding and transport [Lee, 1992; Little et al., 1994]. In this compartment, GRP78 may also function as a Ca^{2+} storage protein [Lievremont et al., 1997]. Additionally, GRP78 is also found to be associated with the aberrant p53 protein [Merrick et al., 1996] and cytokeratin [Liao et al., 1997] in the cytoplasm as well as with the plasma membrane with unidentified functions [Delpino et al., 1998]. GRP78 expresses constitutively, and its expression is upregulated in response to disturbance of Ca^{2+} homeostasis, i.e., treatment with A23187 and TG [Kim et al., 1987; Wooden et al., 1991; Lee, 1992; Little et al., 1994; Cao et al., 1995]. Furthermore, the protein can also be induced under various stress conditions such as glucose starvation [Shiu et al., 1977], inhibition of protein glycosylation by tunicamycin and 2-deoxyglucose [Watowich and Morimoto, 1988], blockage of vesicular trafficking by brelfeldin A [Liu et al., 1992], and inhibition of protein phosphatases by okadaic acid (OA) and calyculin A [Hou et al., 1993; Chen et al., 1999]. Furthermore, GRP78 is rapidly induced in 9L rat brain tumor (RBT) cells sequentially treated with OA and heat shock [Chen et al., 1996, 1997, 1998]. With regard to its regulation, the nucleotide sequence of the promoter of *grp78* has been extensively analyzed and the functionality of several transcriptional elements has been identified. It has been shown that conserved transcription elements/motifs in the *grp78* promoter such as a CRE-like element [Alexandre et al., 1991; Chen et al., 1997, 1998], a CORE element [Resendez et al., 1988; Li et al., 1997], and the proximal CCAAT box (C1 element) [Roy and Lee, 1995] have been shown to be essential in the basal and/or induced expression of *grp78* (Fig. 1). Recently, an ER stress response element, with a consensus of CCAATN9CCACG, which encompasses the proximal CCAAT box in *grp78* promoter, has been shown to be necessary and sufficient for the induction of GRPs [Yoshida et al., 1998]. It has been shown that TG-induced transactivation of the *grp78* promoter requires both tyrosine and serine/threonine kinases [Cao et al., 1995], and the process has been suggested to be regulated through Ca^{2+}-responsive promoter elements that respond to ER Ca^{2+} depletion [Li et al., 1993]. Nevertheless, the exact Ca^{2+} signaling events that mediated the TG-induced *grp78* are not completely elucidated, and the causal relationship between the increase in $[Ca^{2+}]_c$ and transactivation of *grp78* has not been directly addressed.

For the studies of calcium mobilization in cultured cells, influx of extracellular calcium can be prevented by using calcium-free medium, i.e., specialized medium containing ethyleneglycol bis(β-aminoethyl)ether-*N,N,N'* tetraacetic acid (EGTA), a highly effective Ca^{2+} chelator [Smith et al., 1984; Harrison and Bers, 1987]. On the other hand, $[Ca^{2+}]_c$ can be effectively chelated by dibromo-1,2-bis(aminophenoxy)ethane *N,N,N'*-tetraacetic acid (BAPTA), which enters cells as an ester derivative, BAPTA-acetoxymethyl ester (BAPTA-AM) [Collatz et al., 1997]. In this report, we focus our investigation on the role of $[Ca^{2+}]_c$ on TG-induced transactivation of *grp78.* By exploiting the use of BAPTA-AM and calcium-free medium, which either reduce $[Ca^{2+}]_c$ or prevent calcium influx, we herein show that TG perturbs intracellular Ca^{2+} homeostasis and that the increase of $[Ca^{2+}]_c$ is essential for the induction of *grp78.* These results for the first time provide direct evidence to show that an increase in $[Ca^{2+}]_c$ is essential in the TG-induced *grp78* transactivation. Furthermore, our data suggest that the protein factor(s) that binds to the C1 element in the *grp78* promoter plays an important role in this regulatory process.

MATERIALS AND METHODS

Materials

TG was purchased from Calbiochem (San Diego, CA). Indo-1-acetoxymethyl ester and

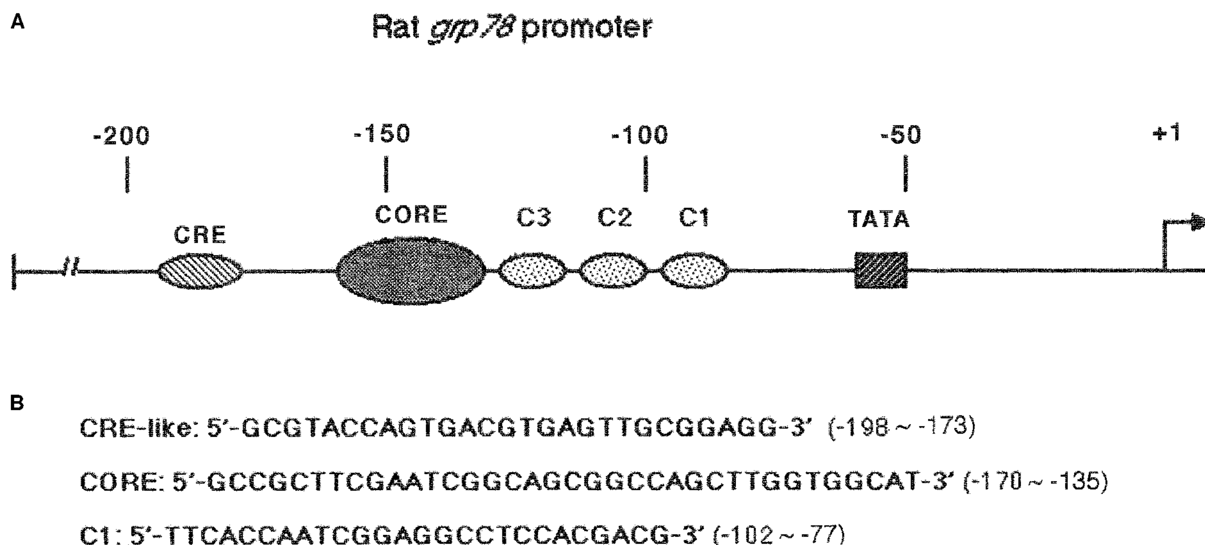

Fig. 1. Schematic drawing of the rat *grp78* promoter and oligonucleotide sequences synthesized. The nucleotide sequence of the *grp78* promoter was obtained from the Genebank and the sequence from +1 to −250 is presented. **A:** The symbols represent the approximate locations of the putative regulatory element of interest, including a series of CCAAT-containing elements (C1, C2, C3) as well as a *grp* CORE element flanked by GC-rich motifs, and a CRE element. **B:** Oligonucleotide sequences of the above elements that were synthesized and used as EMSA probes.

BAPTA-AM were purchased from Molecular Probes, Inc. (Junction City, OR). Cultureware was obtained from Corning (Corning, NY), and culture medium components were purchased from Gibco Laboratories (Grand Island, NY). [^{35}S]methionine (800 Ci/mmol) and [γ-^{32}P]ATP (5,000 Ci/mmol) were purchased from Amersham (Buckinghamshire, England). Other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Cell Culture and Drug Treatment

The 9L RBT cells, originated from a rat gliosarcoma [Weizsaecker et al., 1981], were a gift from Dr. M. L. Rosenblem, University of California at San Francisco. The 9L cells were maintained in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Before each experiment, stock cells were plated at a density of 6×10^4 cells/cm^2. Exponentially growing cells at 80–90% confluency were used.

All drugs were diluted to appropriate concentrations with culture medium before adding to the cells. The drug treatments were maintained at 37°C according to the protocols as specified in the following sections and figure legends.

Metabolic Labeling and Gel Electrophoresis

Synthesis of GRP78 was monitored by [^{35}S]methionine labeling. For labeling of de novo synthesized proteins, cells were incubated with [^{35}S]methionine (20 μCi/ml) for 1 h before harvesting. After labeling, the cells were washed with phosphate-buffered saline (PBS) and then lysed in sample buffer [Laemmli, 1970]. Equal amounts of cell lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [Laemmli, 1970] with 10% (wt/vol) acrylamide for resolving gels and 4.75% acrylamide for stacking gels.

RNA Isolation and Northern Hybridization

Total RNA was isolated from 9L RBT cells by using a commercial RNA extraction kit (RNAzol, Gibco Laboratories). Five micrograms of RNA was loaded onto 1% formaldehyde agarose gel, electrophoresed, and blotted on a nylon membrane by a rapid optimized downward alkaline protocol [Koetsier et al., 1993]. Hybridization and detection were performed by using the DIG high prime DNA labeling and detection system (Boehringer Mannheim, Mannheim, Germany). Probes for *grp78* mRNA hybridization were polymerase chain reaction (PCR) product from isolated 9L genomic DNA,

where the primers of the PCR reaction were 5′-TGGCTGTGACTACACTGACTTGG-3′ (sense) and 5′-CAGGAATAGGTGGTCC-3′ (antisense) according to the exon 1 of the rat *grp78* gene. In each sample, cellular 18S and 28S rRNAs were used as the internal control for respective transcripts of *grp78*.

Nuclear Extract Preparation

Nuclear extracts were prepared form 9L RBT cells by a rapid fractionation protocol [Hennighausen and Lubon, 1987]. Approximately 2×10^7 cells were washed, trypsinized, and collected by centrifugation. The cell pellet was suspended in 3 ml of nuclear extraction buffer-1 [NE-1: 250 mM sucrose, 15 mM Tris-HCl, pH 7.9, 140 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermidine, 1 mM dithiothreitol (DTT), 0.4 mM PMSF, 25 mM KCl, 2 mM $MgCl_2$, and 0.5% NP-40] and was incubated on ice for 5 min. After incubation, the cells were disrupted by 10 strokes with a Dounce grinder. The nuclei and cell debris were collected by centrifugation at 3,000 *g* for 10 min, and the resulting pellet was lysed in 200 μl of NE-2 buffer (NE-1 buffer containing 350 mM KCl) at 4°C for 5 min, followed by a 25-stroke homogenization. The homogenate was transferred to 1.5-ml microcentrifuge tubes and was centrifuged at 14,000 *g* for 90 min. The supernatant was dialyzed for 3 h at 4°C against dialysis buffer (20 mM HEPES, 100 mM KCl, 0.1 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 20% glycerol) and kept frozen at −70°C until used.

Electrophoretic Mobility Shift Assay

The synthetic oligonucleotide probes used in the EMSA studies (Fig. 1) were purchased from MDBio (Taiwan). Additionally, the EMSA probe representing the extended *grp78* promoter was a PCR product that was generated by the following primers: 5′-TGGACGGTT-ACCGGCGGAAA-3′ (sense) and 5′-TAGTCA-CAGCCAGTATCGAGCGCG-3′ (antisense). The EMSA probes were end-labeled with $[\gamma\text{-}^{32}P]$ATP by T4 polynucleotide kinase (New England BioLabs, Beverly, MA). The reaction mixture were applied to a CHROMA SPIN Column (Clonetech, CA) to remove free $[\gamma\text{-}^{32}P]$ATP and stop the labeling reaction. Each gel shift reaction was carried out in 20-μl reaction volume in binding buffer [15 mM HEPES, pH 7.9, 100 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 10% glycerol, and 2 mg poly(dI-dC)]. Nuclear extract was added to the binding buffer, and the samples were incubated on ice for 15 min. After incubation, the DNA probe was added and incubated at room temperature for 20 min. The reaction mixtures were then loaded onto a 5% or 6% (30:0.8) polyacrylamide gel that had been prerun for 1 h in 0.5 × TBE at 200 V. Gels were run for 3 h at 200 V at 4°C. After electrophoresis, the gels were dried and exposed to x-ray film. Competition experiments were performed by preincubating the extracts with unlabeled oligonucleotides for 10 min, and labeled oligonucleotides were then added for 10 or 20 min before electrophoresis.

Measurement of Cytosolic Free Calcium

$[Ca^{2+}]_c$ was determined by fluorescence ratio imaging of indo-1. The cells used for calcium tracing were cultured on coverslips in six-well plates. Before the experiments, the cells were incubated in the medium containing 1 μM indo-1-AM in darkness at 37°C for 30 min. After dye loading, excess of the probe was removed from the cells by washing twice in PBS. The cells were then allowed to recover in fresh medium at 37°C for 30 min.

Coverslips with dye-loaded cells were mounted on a 35-mm culture dish with a hole placed on the stage of an inverted Zeiss microscope. The cells were then illuminated by epifluorescence through a 40× oil immersion objective (NA = 1.3) at excitation wavelength 340 nm. The fluorescent intensities at emission wavelength of 405 nm (Ca^{2+}-bound form) and 490 nm (free form) were measured simultaneously by two photomultipliers and were integrated in 100-ms intervals. Signals were digitally converted and processed on a computer equipped with a PhoCal Pro system (Life Science Resources, Cambridge UK). The concentration of cytosolic free calcium was estimated from the ratio R of the two emitted fluorescence intensities according to the following equation: $[Ca^{2+}]_c = K_d * (R - R_{min})/(R_{max} - R) * (S_{f2}/S_{b2})$ [Grynkiewicz et al., 1985], where R_{min} is the limiting value of the ratio R when all the indicator is in the Ca^{2+}-free form and the R_{max} is the limiting value of R when the indicator is saturated with calcium. Experimentally, the factor S_{f2}/S_{b2} is simply the ratio of the fluorescence intensity measured when all the indicators is free (S_{f2}) to intensity measured and when all the indicator is Ca^{2+} bound (S_{b2}): both

measurements are taken at 490 nm. A k_d value of 250 nM is predetermined in vitro [Grynkiewicz et al., 1985].

RESULTS

Treatment with TG Induces the Expression of *Grp78* in 9L RBT

Induced synthesis of GRP78 and accumulation of *grp78* mRNA by TG were respectively monitored by metabolic labeling with [^{35}S]methionine and by Northern blot analysis. Cells were incubated with 300 nM TG, and de novo synthesized GRP78 as well as cumulative *grp78* mRNA level were determined at different periods. In the presence of TG, the general translational activity in the treated cells was drastically suppressed and virtually ceased after 2 h of treatment (Fig. 2A, lane 2). Protein synthesis recovered gradually as the treatment time was extended to 10 h. During this period, the synthesis of GRP78 was much more preferred and thus resulted in an apparent induction of this protein (Fig. 2A, lanes 2–6). The level of *grp78* mRNA was simultaneously determined, and it was found that enhanced synthesis of *grp78* mRNA can be detected on the TG treatment and that the cumulative level increased significantly in a time-dependent manner (Fig. 2B). Quantitative analysis showed that the induction kinetics of GRP78 and its mRNA are similar, although the absolute values were different (Fig. 3).

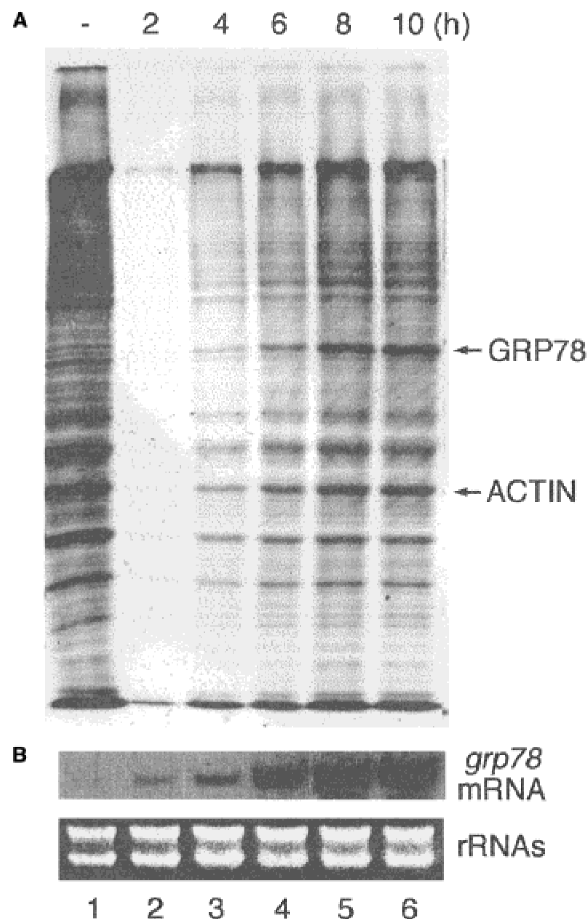

Fig. 2. Enhanced synthesis of GRP78 and its mRNA in thapsigargin (TG)-treated 9L rat brain tumor cells. Cells were treated with 300 nM TG for various durations as indicated. **A:** The cells were metabolically labeled with [^{35}S]methionine for 1 h before harvesting. The treated and labeled cells were lysed in sample buffer and the cell lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. De novo synthesized proteins were visualized by autoradiography. **B:** Total RNA was extracted from the treated cells and analyzed for the expression of *grp78* mRNA by Northern blotting. Actin and rRNAs were used as internal standards.

Effect of BAPTA on *Grp78* Induction in TG-Treated 9L RBT Cells

Calcium chelator BAPTA has been widely used for studying the role of intracellular calcium. In an attempt to determine whether the TG-induced *grp78* and the rise of $[Ca^{2+}]_c$ are casually related, we examined the effect of BAPTA on the enhanced transcription of *grp78* in TG-treated cells. Figure 4A shows that treatment of BAPTA-AM alone at up to 20 μM would not affect the basal transcriptional activity of the *grp78* gene. However, TG-induced synthesis of *grp78* mRNA was significantly suppressed if the cells were pretreated with and further incubated with BAPTA-AM. This suppression effect was concentration-dependent, induction of *grp78* mRNA by 300 nM TG was completely abolished by 20 μM BAPTA-AM (Fig. 4B,C). To confirm the effectiveness of BAPTA at the concentration applied, increased of $[Ca^{2+}]_c$ in TG-treated cells was monitored microspectrophotometrically with or without addition of BAPTA-AM. Treatment with TG alone produced a gradual rise in $[Ca^{2+}]_c$ from a resting level of 80 nM to 400 nM, followed by a slow return to the basal level (Fig. 5). In the presence of 10 μM BAPTA-AM, however, the increase in $[Ca^{2+}]_c$ in TG-treated cells was reduced to 250 nM and was almost completely abolished when 20 μM BAPTA-AM was applied (Fig. 5). These results suggested that BAPTA-AM inhibits *grp78* induction in

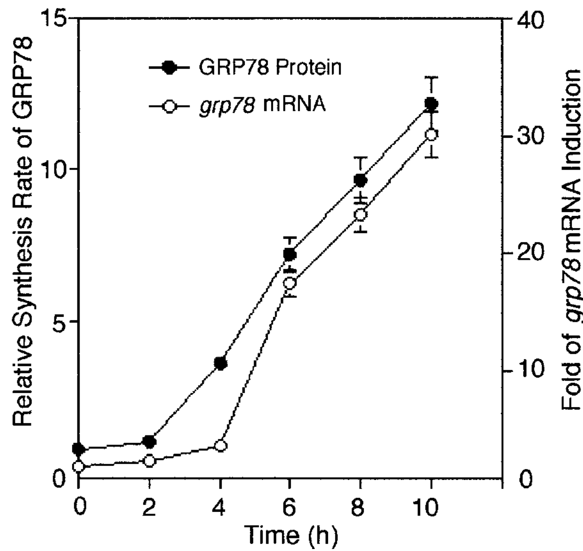

Fig. 3. Induction kinetics of GRP78 and its mRNA in thapsigargin (TG)-treated 9L rat brain tumor cells. Autoradiographs and Northern blots as shown in Figure 2 were analyzed by densitometric scanning. For each experiment, the pixel values representing GRP78 synthesis and mRNA accumulation were respectively normalized against that of actin and rRNAs in the same lane. The data are presented as fold of induction relative to that of untreated cells, and they are means ± SD from three independent experiments.

response to TG treatment by abolishing the increase of intracellular calcium.

Role of Ca^{2+} Homeostasis in Basal and TG-Induced *Grp78* Expression in 9L RBT Cells

Treatment with TG would lead to a concurrent depletion of $[Ca^{2+}]_{ER}$ and increase in $[Ca^{2+}]_c$. In an attempt to directly differentiate the roles of $[Ca^{2+}]_c$ and $[Ca^{2+}]_{ER}$ in *grp78* induction, we further examined the induction process in cells under a calcium-free environment. EGTA was thus used. As shown in Figure 6, treatment of 9L RBT cells with 20 μM BAPTA in the presence of 5 mM EGTA, when both $[Ca^{2+}]_c$ and $[Ca^{2+}]_{ER}$ are depleted, resulted in the suppression of basal expression of *grp78* (Fig. 6, lane 3). Moreover, under this condition, induction of *grp78* in TG-treated cells was also completely abolished (Fig. 6, lane 4). The data suggested that proper balance among $[Ca^{2+}]_o$, $[Ca^{2+}]_c$, and $[Ca^{2+}]_{ER}$ is important for both basal and induced expression of *grp78*. The results also supported the notion that depletion of $[Ca^{2+}]_{ER}$ per se could not be the only cause of

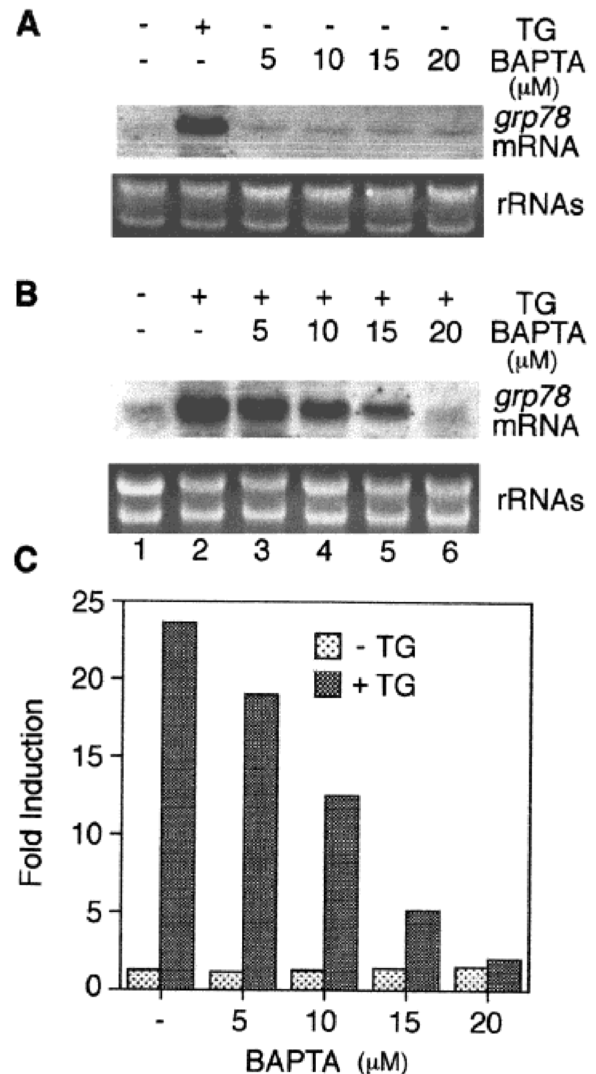

Fig. 4. Dose-dependent effect of dibromo-1,2-bis(aminophenoxy) ethane *N,N,N′,N′*-tetraacetic acid (BAPTA) on *grp78* mRNA accumulation in thapsigargin (TG)-treated 9L rat brain tumor cells. **A:** Cells were treated with BAPTA-acetoxymethyl ester (AM) for 1 h at various concentrations as indicated. **B:** Cells were preincubated with BAPTA-AM for 1 h at various concentrations and then treated with 300 nM TG for 8 h in the presence of BAPTA-AM. After treatment, total RNA was isolated and proceeded for Northern blotting analysis. **C:** Northern blots as shown in (A) and (B) were quantified by densitometric scanning, and the level of *grp78* mRNA was assessed as described. Data present the means of two independent experiments.

grp78 induction in TG-treated cells. It should be noted that the effect of EGTA on TG-induced *grp78* expression could not be assessed because the combined treatment of TG and EGTA would lead to rapid cell death (data not shown).

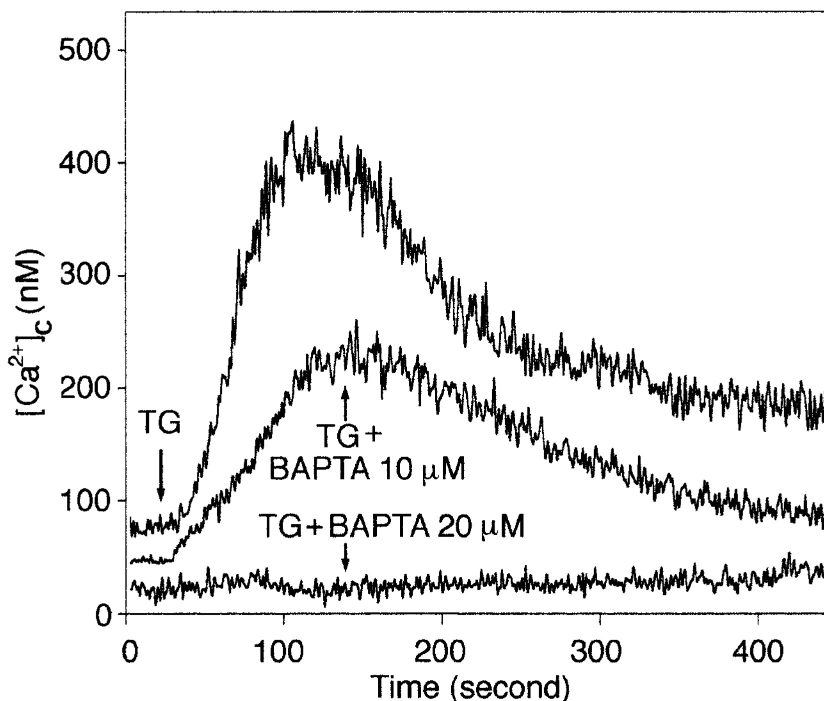

Fig. 5. Effect of dibromo-1,2-bis(aminophenoxy)ethane *N,N,N′,N′* tetraacetic acid (BAPTA) on thapsigargin (TG)-induced changes in intracellular calcium levels. Cells grown on coverslips were loaded with Indo-1-AM, preincubated with 10 or 20 μM BAPTA-acetoxymethyl ester for 1 h, and then stimulated with 300 nM TG. Changes in $[Ca^{2+}]_c$ were determined by fluorescence microphotospectrometry. Results are representative of those obtained in five separate experiments.

Interaction of Nuclear Extracts From TG-Treated 9L RBT Cells With the Extended *Grp78* Promoter

To investigate the interaction between the nuclear factors and the *grp78* promoter, the PCR product of the extended *grp78* promoter was firstly used as an EMSA probe. On being mixed with the nuclear extracts prepared from control and TG-treated 9L cells, a major DNA–protein complex was found in each of the samples (Fig. 7A). After TG treatment, the formation of this complex increased significantly (Fig. 7A, lane 2). The enhanced binding activity was abolished in the presence of BAPTA (Fig. 7A, lane 3). The protein–DNA complex was specific to the *grp78* promoter because its stability was not affected by excess molar of heat shock element, an unrelated transcription element (Fig. 7B). To further examine the effects of individual cis-regulatory elements on the complex formation, the nuclear extracts from TG-treated cells were respectively mixed with excess molar of specified unlabeled elements—CRE, CORE, and C1—before they were allowed to react with the EMSA probe, the radiolabeled extended *grp78* promoter. In the presence of excess molar of CRE and CORE elements, formation of the complex was slightly reduced. However, excess molar of C1 was able to completely abolish the increased binding activity at low dosages (Fig. 7C). These results suggested that the regulatory mechanism of *grp78* induction in TG-treated cell is mediated through the enhanced binding of transcription factors to its cis-regulatory elements and that the C1 binding protein(s) plays an important role in the formation and maintenance of the transcription complex.

Interaction of Individual cis-Regulatory Elements With Nuclear Extracts From TG-Treated 9L RBT Cells

From the foregoing data, we found that the interaction between nuclear factors and *grp78* promoter was increased after TG treatment, and that the increased binding was abolished by BAPTA. We further examined the interaction of nuclear factors with each of the cis-

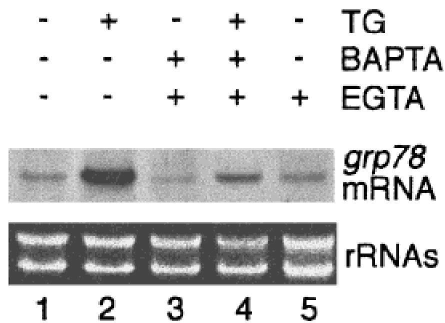

Fig. 6. Effects of EGTA on thapsigargin (TG)-induced accumulation of *grp78* mRNA in 9L rat brain tumor cells loaded with dibromo-1,2-bis(aminophenoxy)ethane *N,N,N′,N′* tetraacetic acid acetoxymethyl ester (BAPTA-AM). Cells were loaded with 20 μM BAPTA-AM in calcium-free culture medium containing 5 mM EGTA for 1 h and then treated with 300 nM TG for 8 h in the presence of both Ca^{2+} chelators. After treatment, total RNA was isolated and the level of *grp78* mRNA was assessed as described.

regulatory element by using CRE, CORE, and C1 elements as EMSA probes. As shown in Fig. 8, nuclear factors prepared from untreated cells were able to form complexes with all three cis-regulatory elements (Fig. 8, lanes 2, 6, and 10). These observations support the notion that there are basal binding of the transcription factors to the elements and thus may confer the basal expression of *grp78.* Interestingly, a significant difference in complex formation was observed between the control and the TG-treated samples when CRE and CORE, but not C1 elements, were used (Fig. 8, lanes 3, 7, and 11). These enhanced bindings between nuclear factors and CRE as well as CORE elements were reduced to the control level in the presence of BAPTA (Fig. 8, lanes 4 and 8). With regard to the C1 element, the largest (uppermost) protein–DNA complex was even weaker in TG-BAPTA-treated cells compared to that of untreated cells (Fig. 8, lane 12). The specificity of these bindings was confirmed by a chasing experiment (Fig. 9). The data showed that, in the cases of CRE and CORE, all complexes detectable in the previous experiment were specific bindings because their binding intensities were diminished by the adding of the unlabeled probes (Fig. 9, lanes 1–6). On the other hand, only the uppermost protein–DNA complex in the C1 experiments could be chased by the unlabeled C1 elements (Fig. 9, lanes 7–9). Taken together with our previous findings, these data further supported the notion that all three regulatory elements, i.e., CRE, CORE, and C1, participate in the basal and TG-induced expression of *grp78* in 9L RBT cells.

DISCUSSION

This is the first report to address the role of intracellular calcium signaling involved in TG-induced *grp78* expression. We have shown that treatment of 9L RBT cells with 300 nM TG would lead to a time-dependent induction of *grp78* and that this process was abolished by BAPTA, an effective $[Ca^{2+}]_c$ chelator, indicating that TG-induced *grp78* expression in 9L RBT cells is dependent on the increase in cytoplasmic free calcium. Calcium signaling in *grp78* induction has been studied in a variety of experimental systems. By using TG as well as A23187, a calcium ionophore, it was suggested that TG-induced *grp78* expression is caused by the depletion of $[Ca^{2+}]_{ER}$ and that the induction is not dependent on the rise of $[Ca^{2+}]_c$ in Chinese hamster lung cells [Drummond et al., 1987]. More recently, in an in vitro experiment, it was shown that depletion of nuclear calcium would also result in the transactivation of the *grp78* promoter [Roy and Lee, 1995; Roy et al., 1996]. Calcium is an ubiquitous intracellular messenger and regulator of cellular activity. Disruption of intracellular calcium homeostasis would lead to different cellular effects, which are dependent on sources and routes. For instance, recent studies of calcium signal-transduction mechanisms have revealed that, depending on the route of entry into a neuron, calcium differentially affects processes that are central to the development and plasticity of the nervous system [Ghosh and Greenberg, 1995]. It has been shown that TG elevates intracellular calcium levels by a combination of calcium release from intracellular stores and calcium influx [Takemura et al., 1989] and they have differential contribution to TG-induced gene expression [Rodland et al., 1997]. Other classic GRP78 inducers, such as the protein glycosylation inhibitors 2-deoxyglucose and tunicamycin, may also exert their induction effects through calcium signaling because treatment of cells with these compounds would also lead to both increased calcium release and calcium influx across the plasma membrane [Tekkok and Krnjevic, 1996; Buckley and Whorton, 1997]. Therefore, understanding the relative contribution of calcium release versus influx in TG-induced *grp78* expression is very important. In

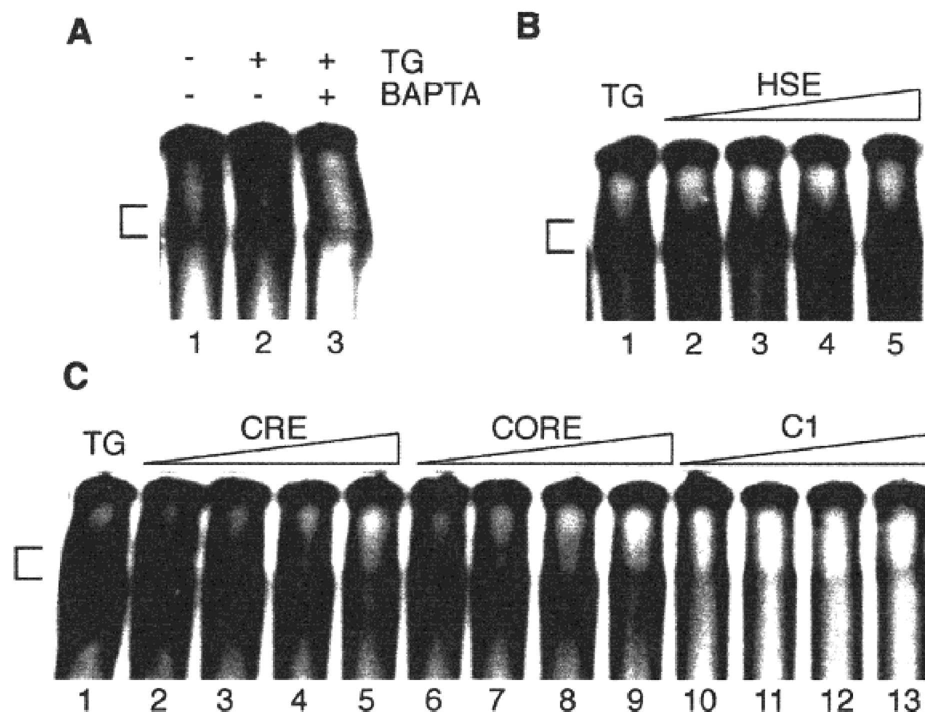

Fig. 7. Interaction of nuclear factors extracted from thapsigargin (TG)-treated cells with the extended promoter region of rat *grp78.* **A:** Polymerase chain reaction product of the extended promoter region of *grp78* were end labeled and mixed with the nuclear extracts prepared from untreated cells, cells treated with 300 nM TG for 2 h, or cells preloaded with 20 μM dibromo-1,2-bis(aminophenoxy)ethane *N,N,N′,N′* tetraacetic acid acetoxymethyl ester (BAPTA-AM) for 1 h and then treated with 300 nM TG in the presence of BAPTA-AM for 2 h. The DNA–protein complexes were analyzed by EMSA. **B:** Nuclear extracts prepared from TG-treated cells were incubated with 10-, 20-, 50-, and 100-fold molar excess of the HSE derived from human *hsp70* before being mixed with the radiolabeled extended *grp78* promoter EMSA probe. **C:** Nuclear extracts prepared from TG-treated cells were incubated with 10-, 20-, 50-, and 100-fold molar excess of the cis-regulatory elements CRE, CORE, and C1 derived from the rat *grp78* promoter before being mixed with the radiolabeled extended *grp78* promoter EMSA probe. Brackets indicated the position of the specific DNA–protein complex. Similar results were observed in three independent experiments.

the present studies, we have shown that the TG-induced *grp78* expression could not happen in the presence of BAPTA and EGTA, i.e., the cells were in a calcium-free medium when influx of extracellular calcium is also prevented. Unfortunately, the effect of EGTA alone on TG-induced *grp78* expression could not be assessed because this combined treatment would cause severe cell death. Nevertheless, these results lead us to conclude that depletion of $[Ca^{2+}]_{ER}$ alone is not sufficient for the induction of *grp78* in the TG-treated cells, so the rise of $[Ca^{2+}]_c$ must also play a crucial role in this process.

As mentioned previously, basal and induced expression of *grp78* has been attributed to a concerted action of a number of conserved regulatory elements including CRE, CORE, and C1. In Chinese hamster cells, it has been suggested that TG-induced *grp78* transactivation is regulated through redundant elements containing CCAAT boxlike motifs flanked by a GC-rich region on the *grp78* promoter, i.e., the CORE and C1 regions, but not basal-level regulatory element CRE [Li et al., 1993, 1997; Roy and Lee, 1995]. By contrast, it has been shown that the CRE element is essentially involved in rapid induction of *grp78,* in 9L RBT cells subjected to a combined treatment of OA and heat shock [Chen et al., 1996, 1997]. Herein, we showed that all of the aforementioned regulatory regions participate in the basal expression of *grp78* in 9L RBT cells because basal binding activities toward the extended *grp78* promoter as well as the individual elements are detected in control samples. Moreover, a significant increase in binding activities toward the extended *grp78* promoter, CRE, and CORE elements was observed in TG-treated samples.

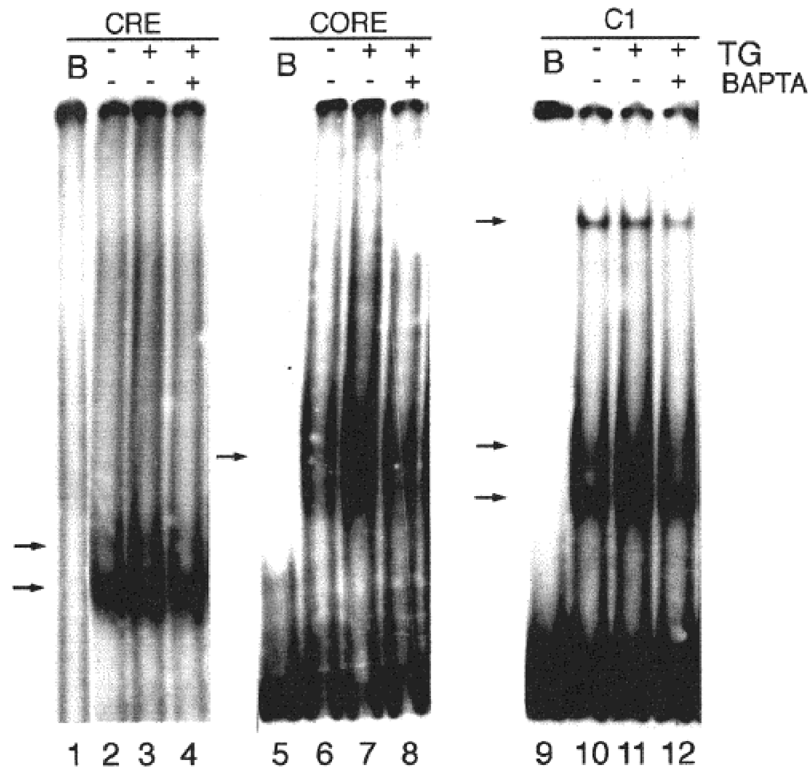

Fig. 8. Interaction of nuclear factors extracted from thapsigargin (TG)-treated cells with the oligonucleotides corresponding to the cis-regulatory elements CRE, CORE, and C1 from rat *grp78* promoter. Synthetic oligonucleotides corresponding to the cis-regulatory elements of rat *grp78* promoter were annealed, end labeled, and mixed with the nuclear extracts. The DNA–protein complexes were analyzed by EMSA. **Lane 1:** Radiolabeled DNA only (no nuclear extract). **Lane 2:** Probes reacted with nuclear extracts prepared from untreated cells. **Lane 3:** Probes reacted with nuclear extracts prepared from cells treated with 300 nM TG for 2 h. **Lane 4:** Probes reacted with nuclear extracts prepared from cells that were preincubated with 20 mM dibromo-1,2-bis(aminophenoxy)ethane *N,N,N′,N′* tetraacetic acid acetoxymethyl ester (BAPTA-AM) and then treated with TG for 2 h in the presence of BAPTA-AM. Arrows indicate the positions of specific DNA–protein complexes. Similar results were observed in three independent experiments.

These results suggested that there is a significant difference of binding to CRE and CORE elements response to TG treatment and that binding of nuclear factors to these two elements may mediate the activation of the *grp78* gene. Although treatment with TG did not enhance the binding of nuclear factor(s) toward the C1 element, the increased binding activity toward the extended *grp78* promoter was largely eliminated by unlabeled C1 elements at low dosage but was only partially reduced by unlabeled CRE and CORE elements at high dosage. These data indicate that, for some reason, the binding of factors on the C1 element is needed for the formation of stable complexes on CRE and CORE elements. This notion is supported by the finding that mutation of the C1 element would result in the loss of *grp78* induction response to a variety of inducers [Yoshida et al., 1998]. It is also conceivable that C1 might mediate *grp78* induction indirectly, via protein activation, but not increase binding with components of the binding complex, as in the case of nuclear factor-T/CCAAT binding factor (NF-Y/CBF) [Zhou and Lee, 1998]. We further showed that, in the presence of BAPTA, where both rise in $[Ca^{2+}]_c$ and induction of *grp78* were inhibited in TG-treated cells, the increased binding toward to all of the EMSA probe was also diminished. These data indicated that BAPTA-abolished TG-induced *grp78* expression is mediated by the inhibition of the binding activities of nuclear factors toward the individual cis-regulatory element and thus results in the lost of enhanced binding activity toward the extended *grp78* promoter. As previ-

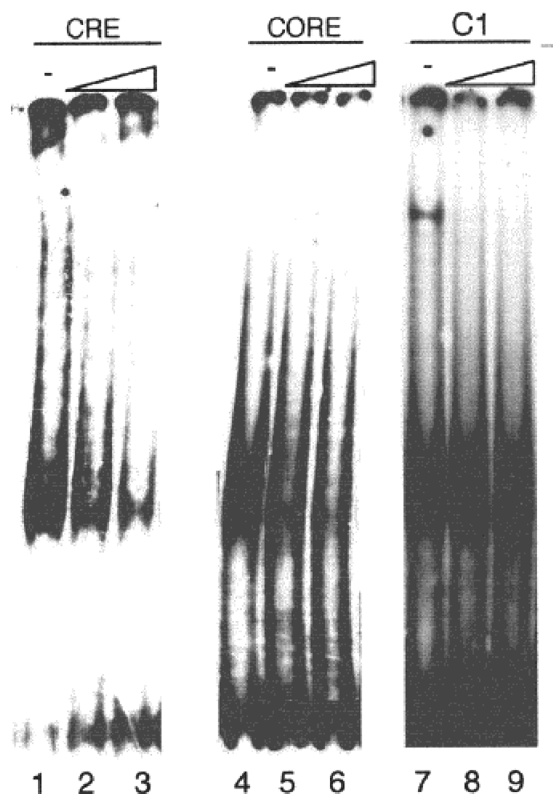

Fig. 9. Competitive assays of interaction of nuclear factors from 9L cells with cis-regulatory elements CRE, CORE, and C1 derived from the rat *grp78* promoter. Nuclear extracts were prepared from cells treated with TG for 2 h. Synthetic oligonucleotides were annealed, end labeled, and mixed with the nuclear extracts (**lane 1**). The specific binding of nuclear factors to the probes was assessed by adding 5- (**lane 2**) to 10-fold (**lane 3**) excessive amounts of nonradioactive probes 10 min before addition of radiolabeled probes. The DNA–protein complexes were analyzed by EMSA as previously described. Arrows indicated the positions of specific DNA–protein complexes. Similar results were observed in three independent experiments.

ously discussed, the reduced binding to the C1 element may further diminish the stability of the complexes formed on the extended *grp78* promoter.

The signaling pathway from the disturbance of intracellular calcium homeostasis to the transactivation of the *grp78* gene warrants further investigation. Nevertheless, we have provided the first direct evidence that increased $[Ca^{2+}]_c$ plays a major role in TG-induced *grp78* expression. Furthermore, we shown that all of the previously identified regulatory elements including CRE, CORE, and C1 are involved in the basal as well as in the TG-induced expression of *grp78* in 9L RBT cells.

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