Endoplasmic reticulum (ER) stress plays a critical role in multiple diseases, and pharmacologically active drugs can induce cell death through ER stress pathways. Stress-induced genes are activated through assembly of transcription factors on ER stress response elements (ERSEs) in target gene promoters. Gel mobility shift and chromatin immunoprecipitation assays have confirmed interactions of NF-Y and YY1 with the distal motifs of the tripartite ERSE from the glucose-related protein 78 (GRP78) gene promoter. The GC-rich non-anucleotide (N9) sequence, which forms the ER stress response binding factor (ERSF) complex binds TFII-I and ATF6; however, we have now shown that in Panc-1 pancreatic cancer cells, this complex also binds Sp1, Sp3, and Sp4 proteins. Sp proteins are constitutively bound to the ERSE; however, activation of GRP78 protein (or reporter gene) by thapsigargin or tunicamycin is inhibited after cotransfection with small inhibitory RNAs for Sp1, Sp3, and Sp4. This study demonstrates that Sp transcription factors are important for stress-induced responses through their binding to ERSEs.

The endoplasmic reticulum (ER) plays a critical role in protein folding, and diseases such as Parkinson, Alzheimer, and Huntington disease and obesity are linked to misfolded proteins (1–5). Various chemical and biological stressors also induce ER stress, resulting in the unfolded protein response, which both activates and deactivates gene/protein expression to restore the cell to homeostasis (6–11). Failure to counteract induced ER stress can result in activation of apoptosis (12), and several pharmacologically active drugs act through this pathway (13–15). The unfolded protein response is accompanied by activation of several possible intracellular signaling pathways (16, 17). The unfolded protein response binding factor (ERSF) complex also interacts with the ERSE non-anucleotide sequence and contains ATF6 and TFII-I proteins (21, 22, 28). This study now demonstrates that activation of the stress response by thapsigargin (Tg) and tunicamycin (Tm) (8, 9, 30) is attenuated by Sp family proteins, and using a combination of gel mobility shift and chromatin immunoprecipitation (ChIP) and transactivation assays, we now demonstrate a role for Sp1, Sp3, and Sp4 in mediating ER stress-induced gene expression.
FIG. 1. Activation of GRP78 by ER stress requires Sp family proteins. A, Tm and Tg induction of GRP78. Panc-1 cells were treated with Tg (0.5 μM) or Tm (0.5 μg/ml) for 20 h, and GRP78 expression was determined by Western blot analysis of whole cell lysates as described under "Materials and Methods." Results are expressed as means ± S.D. for at least three separate determinations for each treatment group and significant (*p < 0.05) induction of GRP78 protein (normalized to β-tubulin protein) is indicated (*).

B, Effects of RNA interference of Sp1, Sp3 and Sp4 protein on induction of GRP78 by Tg and Tm. These experiments were carried out essentially as described for A except that Panc-1 cells were transfected with iScr (nonspecific control), iSp1, iSp3, or iSp4. Tg and Tm significantly induced GRP78 expression in all treatment groups; however, significant (*p < 0.05) decreases after transfection with iSp1, iSp3, or iSp4 are indicated (*). The specificity of iSp3 is indicated in the Western blot for Sp3 protein in the various treatment groups. C, specificities of iSp3 and iSp4. This experiment was identical to that described in Fig. 1B and shows Western blot analysis of Sp4 (left) and Sp1 (right) proteins in cells transfected with iSp1 or iSp4. D, Sp protein knockdown decreases Tg-induced activation of pGRP78. Panc-1 cells were treated with Tg (0.5 μM); cotransfected with iScr, iGL2 (luciferase), iSp1, iSp3, or iSp4; and luciferase activity determined as described under "Materials and Methods." Results are expressed as means ± S.D. for three separate determinations for each treatment group, and significant (*p < 0.05) induction by Tg (*) and inhibition by siRNAs (**) are indicated.
FIG. 2. Protein interactions with ERSE and related oligonucleotides. A, 32P-labeled ERSE binding to nuclear extracts from Panc-1 cells treated with Tg. Nuclear extracts from Me2SO or Tg-treated cells were incubated with 32P-labeled ERSE alone or in combination with 100-fold excess unlabeled ERSE (100X or 100 times excess) oligonucleotide, YY1 (αYY1), or TFII-I (αTFII-I) antibodies and separated in a gel mobility shift assay as described under “Materials and Methods.” The specifically bound ERSE, NF-Y, and YY1 complexes are indicated by arrows. B, induction of TFII-I by Tg and gel mobility shift assay. Whole cell lysates from untreated (αIgG), or Tg-treated Panc-1 cells were analyzed by Western blot analysis for TFII-I expression as described under “Materials and Methods.” Nuclear extracts from Tg-treated Panc-1 cells were analyzed by gel mobility shift assays as described in Fig. 2A; however, increasing amounts of TFII-I antibody were used. C, supershift of ERSE-protein complex by Sp protein antibodies. Panc-1 cells were treated with Me2SO and Tg. Nuclear extracts were incubated with 32P-labeled ERSE and Sp1 (αSp1), Sp3 (αSp3), or Sp4 (αSp4) antibodies and separated in a gel mobility shift assay as described under “Materials and Methods.” ERSE, NF-Y, YY1, and Sp protein complexes...
HEPES, 0.5 mM sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 µl/ml of protease inhibitor mixture (Sigma). The lysates from the cells were incubated on ice for 1 h with intermittent vortexing followed by centrifugation at 40,000 × g for 10 min at 4 °C. Equal amounts of protein from each treatment group were diluted with loading buffer, boiled, and loaded onto 10% SDS-polyacrylamide gel. Samples were electrophoresed, and proteins were detected by incubation with polyclonal primary antibodies Sp1 (PFP2), Sp3 (D-20), Sp4 (V-20), TFII-I, GRP78 (H-129), and β-tubulin (H-235) followed by blotting with appropriate horseradish peroxidase-conjugated secondary antibody as previously described (31, 32). After autoradiography, band intensities were determined by a scanning laser densitometer (Sharp Electronics Corp., Mahwah, NJ) using Zero-D Scannalytics software (Scannalytics Corp., Billerica, MA).

**EMSA**—Wild-type ERSE (wtERSE), mutant ERSE (mERSE), and GC-rich oligonucleotides were synthesized and annealed, and 5-pmol aliquots were denatured by boiling and loaded in 8% polyacrylamide gel electrophoresis as previously described (31, 32). Specific DNA-protein and antibody-supershifted complexes were resolved by 5% polyacrylamide gel electrophoresis as previously described (31, 32). Specific DNA-protein and antibody-supershifted complexes were observed as retarded bands in the gel. wtERSE and mERSE sequence used in gel shift analysis are given below. The NF-Y/CBF and YY1 motifs are underlined, and the mutation is in bold: human GRP78, GGG CCA ATG AAC GGC CTC CAA CGA (wt) and GGG CCA ATG AAC AAG GCC CTC CAA CGA (mut); human p53, TCG AAC AGG AGG AGC AGA GAG CGA (wt) and TCG AAC AGG AGG AGC AGA GAG GC (mut). The order of effectiveness of these siRNAs in reducing Sp1, Sp3, and Sp4 was determined in Panc-1 cells transfected with iSp1, iSp3, or iSp4 (Fig. 1B). The order of effectiveness of these siRNAs in reducing GRP78 protein levels was iSp4 > iSp1 > iSp3. The results in Fig. 1, B and C, also demonstrate that neither Tg or Tm affected Sp protein levels and that the RNA interference assay for Sp protein knockdown is highly selective for Sp1, Sp3, and Sp4 as reported previously (31–33). These data clearly demonstrate that ER stress-induced GRP78 protein expression is dependent on Sp1, Sp3, and Sp4.

The direct effects of Sp proteins on GRP78 was further investigated in Panc-1 cells transfected with the plasmid pGRP78, which contains a −374-bp insert derived from the proximal region of the human GRP78 gene promoter. This promoter sequence contains three ERSEs, and treatment with Tg significantly induced transactivation (>3-fold) (Fig. 1D). This paralleled the induction of GRP78 protein (Fig. 1A). In cells treated with Tg and cotransfected with pGRP78 and iSp1, iSp3, or iSp4, both basal and induced luciferase activities were significantly decreased (Fig. 1D). These results further confirm that Sp1, Sp3, and Sp4 play an important role in regulation of a prototypical stress induced gene/protein GRP78. We further examined the role of Sp proteins in stress responses by gel mobility shift assays using the 32P-labeled ERSE, which was identical to ERSE-2 from the GRP78 promoter. The pattern of three retarded bands associated with the YY1 and NF-Y or ERSE-DNA complexes in extracts from control (Me2SO) or Tg (Fig. 2A) was similar to that reported previously (28). All three bands were decreased after coinubcation with 100-fold excess of unlabeled ERSE (lane 5) and YY-1 antibodies immunodepleted the YY-1-ERSE complex (lane 7). Previous studies report that TFII-I was induced by ER stress and bound the ERSE-ERSE complex, and this contributed to the enhanced intensity of the retarded band (28). Incubation of stress-induced Panc-1 cell lysate with 32P-labeled ERSE and TFII-I antibodies decreased intensity of the ERSE-ERSE band (immunodepletion), although a supershifted band was not detected. Tg also induces TFII-I protein levels in Panc-1 cells as reported previously in other cell lines (28), and gel mobility shift assays show that the intensity of the Tg-induced ERSE-DNA complex (lane 6) is significantly decreased after coinubcation with increasing amounts of TFII-I antibody (lanes 3–5) (Fig. 2B). Even under these conditions, a supershifted complex was not observed.

Sp proteins interact directly or indirectly with other transcription factors to modulate gene/protein expression (34). Direct interaction of Sp1 protein with the ERSE was investigated in a gel mobility shift assays using 32P-labeled ERSE extracts from Tg-treated Panc-1 cells and Sp1, Sp3, and Sp4 antibodies (Fig. 2C, lanes 4–6). Distinct supershifted bands were observed using Sp1, Sp3, and Sp4 antibodies (lanes 4–6), and the most pronounced decrease in the intensity of the ERSE-ERSE complex was observed with the Sp1 antibody suggesting that Sp1
protein is the major Sp protein bound to the ERSF-ERSE complex. These results indicate that Sp proteins may directly bind the ERSE, and this was further confirmed in gel mobility shift assays using a series of radiolabeled oligonucleotides and recombinant Sp1 protein. Previous studies indicate that the consensus GC-rich oligonucleotide binds Sp1, Sp3, and Sp4 with high affinity (31–34), and the results illustrated in Fig. 2D compare the binding of recombinant human Sp1 protein with 32P-labeled GC (lanes 1–4), ERSE (lanes 7, 8, and 10–12), and mutant ERSE (lane 9). Using the same amount of Sp1 protein, it was clear that the Sp1-GC retarded band was significantly more intense than the Sp1-ERSE band reflecting different Sp protein-DNA binding affinities. However, the results show that Sp1 directly binds the GC and ERSE oligonucleotides, and both retarded bands were supershifted by Sp1 antibody (lanes 4 and 10) but not by non-specific IgG (lane 11). The mutant ERSE, which did not bind Sp1 (lane 9), contained three specific mutations (underlined) in the nonanucleotide sequence (GAA CTT ACT) in which a TTA sequence replaced the wild-type GCC. Parker et al. (28) have previously demonstrated the high conservation of the GC-rich sequences in ERSEs from GRP78 and other stress-related genes, and mutation of the GC sequence greatly decreased stress-induced responses in transactivation assays (16, 35). The transactivation studies correlated with the loss of Sp protein-ERSE binding observed in this study (Fig. 2D) and complement the functional studies showing that Sp proteins are critical for activation of stress-induced GRP78 (Fig. 1).

Previous studies indicate that induction of ER stress is accompanied by cleavage of p90 ATF-6 to p60 ATF-6, a nuclear transcription factor that interacts with NP-Y proteins (21, 22). TFII-I is also induced by ER stress and interacts with ATF-6 to form part of the ERSE-protein complex (28). We examined Tm-induced protein interactions with the ERSEs of the GRP78 gene promoter by ChIP (Fig. 3A). As a positive control for this experiment, we show that TFII-I interacts with a specific region of the human GAPDH promoter but not the CNAP1 gene exon (Fig. 3B) (33). Treatment of Panc-1 cells with Tm for 0 (Me2SO), 15, 30, and 60 min, followed by ChIP assay, showed that Sp1, Sp3, Sp4, NF-Y, and YY1 were constitutively bound to this region of the GRP78 promoter. There were only minimal temporal increases in Sp1 and YY1 binding. In contrast, TFII-I and ATF-6 were not constitutively bound to the ERSE region of the GRP78 promoter but were rapidly recruited to the promoter after treatment with Tm, and both proteins remained bound for up to 60 min. The induction of ATF6 and TFII-I by ER stress (Tm) and their recruitments to the GRP78 promoter in Panc-1 cells are consistent with the important role of these proteins in mediating a stress-induced response (21, 22, 28). We also investigated the ERSE binding of nuclear extracts from untreated (lane 1) and Tg-treated (lanes 2–5) Panc-1 cells cotransfected with nonspecific scramble RNA (iScr, lanes 1 and 2) and iSp1, iSp3, or iSp4 (lanes 3–5, respectively) (Fig. 3C). The results showed that Sp protein knockdown decreased but did not eliminate intensity of the ERSF-DNA complex, suggesting that stress-induced proteins are retained as part of the complex and do not require Sp proteins for binding.

**DISCUSSION**

ER stress has been linked to various diseases, and several drugs act through activating ER stress pathways, which can lead to growth inhibition and apoptosis (1–5, 13–15). Activation of ER stress pathways is accompanied by modulation of several transcriptional and translational pathways, which include induction of several stress-response genes including GRP78 and GADD153 (CHOP). The ERSE is a major cis-element that is involved in activation of stress-responsive genes, and interac-
tion of trans-acting nuclear transcription factors with the ERSE is responsible for target gene activation. Interaction of NF-Y/CFB and YY1 with the two end-flanking motifs of the ERSE has been well characterized (16, 17), and both ATF-6 and TFII-I have also been identified as components of the ERSE-protein complex (19–22, 28). The inner N9 sequence in most ERSEs is GC-rich and are required for maximal stress-dependent transactivation (28, 35). Sp family proteins Sp1, Sp3, and Sp4 are highly expressed in Panc-1 and other cancer cell lines (31), and we hypothesized that one or more Sp proteins, which bind GC-rich elements, may be required for ER stress-induced responses. RNA interference with siRNAs for Sp1, Sp3, and Sp4 clearly demonstrate that these proteins are involved in Tg-/Tm-induced GRP78 protein and reporter gene expression in Panc-1 cells transfected with pGRP78 (Fig. 1). Moreover, the results of both ChIP and electrophoretic mobility shift assays demonstrate that Sp proteins interact with the ERSE, and this is observed in both stress and unstressed cells (Figs. 2 and 3).

These results demonstrate for the first time that Sp1, Sp3, and Sp4 proteins play a key role in mediating stress-dependent activation of GRP78 in Panc-1 cells. Sp family proteins form a critical part of the ERSF, binding directly to the ERSE and cooperatively activating ER stress-dependent responses. RNA interference studies suggest some redundancy for Sp1, Sp3, and Sp4, and we hypothesized that one or more Sp proteins interact with the ERSE, and this is observed in both stress and unstressed cells (Figs. 2 and 3).

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