In Vivo Regulation of Grp78/BiP Transcription in the Embryonic Heart

ROLE OF THE ENDOPLASMIC RETICULUM STRESS RESPONSE ELEMENT AND GATA-4

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The transcriptional activation of GRP78, which controls multiple signaling pathways of the unfolded protein response, has been used extensively as an indicator for the onset of endoplasmic reticulum stress in tissue culture systems. Here we investigate the mechanism of GRP78 induction during mouse embryonic development. Our results reveal that in transgenic mouse models, reporter gene activity driven by the Grp78 promoter is strongly activated during early embryonic heart development but subsides in later stages. This activation is strictly dependent on a 100-base pair region of the Grp78 promoter containing the endoplasmic reticulum stress response elements (ERSEs). Previous studies establish that endoplasmic reticulum stress induces in vivo binding of YY1 and the nuclear form of ATF6 to the ERSE. Since the expression of YY1 as well as ATF6 is ubiquitous in the mouse embryo, activation of the Grp78 promoter in the early embryonic heart may involve a specific mechanism. Here we report that GATA-4, a transcription factor essential for heart development, binds to the Grp78 promoter in vivo and activates the ERSE, which does not contain a consensus GATA binding site. GATA-4 cooperatively activates the Grp78 promoter with YY1, and the DNA binding domain of YY1 is necessary and sufficient for this cooperation. In addition, GATA-4 activation of the Grp78 promoter is enhanced by the nuclear form of ATF6, and this synergy is further potentiayed by YY1. These results suggest that during early heart organogenesis, Grp78 can be activated through cooperation between the cell type-specific transcription factors and ERSE-binding factors.

Glucose-regulated proteins (GRPs) are endoplasmic reticulum (ER) calcium-binding chaperone proteins (1). The ER is a cellular organelle where secretory and membrane proteins are synthesized and modified and is also a major intracellular calcium storage compartment inside the cell. Under physiological or pharmacological ER stresses, such as a block in protein glycosylation, accumulation of malfolded proteins, or depletion of ER calcium storage, the cells have developed an evolutionarily conserved defense mechanism called the unfolded protein response (UPR) (2). As a protective arm of the UPR, the GRPs are induced under these stress conditions to maintain ER homeostasis. The most abundant and best characterized GRP is GRP78, a 78-kilodalton protein also referred to as immunoglobulin-binding protein (BiP) (3). The induction of Grp78 has been used widely as an indicator of ER stress and the onset of the UPR.

The transcriptional regulation of Grp78 under ER stress is mediated largely through the evolutionarily conserved ER stress element (ERSE) present on the promoters of UPR target genes (4, 5). The classic ERSE has a tripartite structure of CCAAT(N)9CCACG (where N9 represents a 9-bp GC-rich region). The CCAAT-binding factor NF-Y binds constitutively to the CCAAT motif of the ERSE and is required for transcription of the Grp78 promoter under both normal and stress conditions (5, 6). The N9 GC-rich region of the ERSE binds the transcription factor TFII-I, which is autoregulated by ER stress and tyrosine phosphorylation and is known to facilitate protein-protein interactions (7, 8). YY1 (Yang Yang 1), a 65-kDa zinc finger transcription factor, binds to the CCACG motif and activates the Grp78 promoter under ER stress (9, 10). YY1 is ubiquitously expressed during mouse development, and homozygous deletion of YY1 is embryonically lethal (11).

Under ER stress conditions, a fraction of ATF6, a 90-kDa ER transmembrane transcription factor, relocates to the Golgi and is proteolytically cleaved. The 50-kDa N terminus of ATF6 that is generated migrates to the nucleus, binds to the CCACG element, and activates target gene transcription such as Grp78 (12, 13). Both YY1 and TFII-I are co-activators of ATF6 (7, 10). Additionally, in mammalian cells, upon ER stress, XBP-1 mRNA is alternatively spliced by the ER transmembrane kinase Ire1p, and the spliced form of XBP-1 binds to the ERSE, resulting in target gene activation (14, 15).

Despite these advances achieved through investigations using tissue culture systems and mostly through the use of pharmacological ER stress inducers, little is known of what constitutes ER stress in vivo. Previously, GRP78 protein expression has been detected as early as two-cell stage mouse embryos (16), and immunohistochemical staining with purified antibody against GRP78 showed that it is highly elevated in mouse heart during early organogenesis (17). Toward understanding how Grp78 is regulated in vivo during mouse development, we have created transgenic mouse models whereby the LacZ reporter gene is driven by 3 kilobases of the rat Grp78 promoter (18). Initial analysis showed that the ERSE-LacZ model reflects the endogenous expression profile of Grp78 with highest expression in the early embryonic heart and that the promoter region containing the ERSE is critical for the LacZ activity (18). However, the mechanism for the activation of the Grp78 promoter in the developing embryo is unknown. It is possible that the activation of the Grp78 promoter during organogenesis may...
Grp78 Promoter Activation in Embryonic Heart

involve tissue-specific transcription factors acting in concert with known UPR signaling pathways. For example, in cardiomyocytes, YY1 cooperates with the transcription factor GATA-4 to activate the promoter of the gene encoding the B-type natriuretic peptide, a peptide hormone synthesized and secreted from the heart (19). GATA-4 is a zinc finger DNA-binding protein belonging to the GATA family of transcription factors and is highly expressed in the myocardium during embryonic heart development. In this report, we showed that GATA-4 can bind to the Grp78 promoter in vivo and activate the ERSE, which does not contain a consensus GATA-4 site. The activation of the Grp78 promoter by GATA-4 is enhanced through interaction with the DNA binding domain of YY1 and can be synergized substantially by the nuclear, active form of ATF6 generated by ER stress. These results suggest that Grp78 can be activated through cooperation between organ-specific transcription factors and ERSE binding factors during development.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions and Drug Treatments—293T, HeLa, and CV1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin antibiotics. Primary rat neonatal cardiomyocytes were prepared as previously described (20). For drug treatment, the cells were treated with either 300 nM thapsigargin or 1.5 μg/ml tunicamycin for 16 h prior to harvest.

Plasmids—The construction of the plasmids 3kb/LacZ, D170/LacZ, and D300/LacZ has been previously described (21). The expression vector pCGN-ATF6(373) and the −457/LUC and −169/LUC reporter gene has been previously described (21, 22). For the construction of ERSE/LUC, a 100-base pair DNA fragment, spanning −165 to −77 of the rat Grp78 promoter, where the ERSEs are located, was amplified by PCR using −457/CAT (8) as template. It was subcloned into the pGL3 promoter vector (Promega) between SacI and NheI sites. The expression vectors for FLAG-YY1 and its mutants were generous gifts from Dr. Edward Seto (University of South Florida) and have been previously described (23). The expression vector pCG-GATA-4 was kindly provided by Dr. Mona Nemer (Institut de Recherches Cliniques de Montréal) and has been described previously (24).

Transfection and Reporter Assays—293T, HeLa, and CV1 cells were grown to 40% confluence in 6-well plates, and co-transfections were performed using Polyfect (Qiagen). In general, the cells were harvested 24–36 h after transfection, except for siRNA treatment, when the cells were harvested 48 h after transfection. The luciferase activity was measured according to the manufacturer’s protocol (Promega). For transfection and co-transfection of primary neonatal cardiomyocytes, cells were seeded at 90% confluence in 12-well plates, and co-transfections were performed using Lipofectamine 2000 (Invitrogen). For each construct, fives transgenic lines were generated and analyzed.

Western Blot—Cell extracts from mouse embryonic fibroblasts of either the wild-type or the ATF6/βgeo homozygous mouse were separated on 7% SDS-PAGE and transferred to nitrocellulose membranes. The ATF6 protein was detected as described previously (30). Protein extracts from 293T cells 48 h after co-transfection with GATA-4 expression vector and siRNA against GATA-4 (Ambion) or GFP (Qiagen) were separated on 10% SDS-PAGE, and GATA-4 protein and β-actin were detected using the goat anti-GATA-4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and mouse anti-β-actin (Sigma) antibody, respectively.

Chromatin Immunoprecipitation Assays—293T cells were transfected with the pCG-GATA-4 plasmids and incubated for 48 h prior to fixation with formaldehyde. For primary rat neonatal cardiomyocytes, 1.4 × 10⁶ cells were cultured for 8 days prior to formaldehyde fixation. Chromatin immunoprecipitation was performed as previously described (21). An aliquot was removed to be used as the input fraction and was processed with the eluted precipitates from the cross-link reversal step. Equal amounts of chromatin from each sample were incubated with the manufacturer’s protocol (Promega, Madison, WI) and normalized against the protein amount used in the assays. All of the transfections were performed in duplicates or triplicates.

Generation of Transgenic Mice—The generation of the 3kbLacZ transgenic mouse has been described (25). Two independent lines were generated and examined. The D170LacZ and D300LacZ transgenic mice were generated identical to 3kbLacZ with the exception that the D170LacZ and D300LacZ plasmids were used to generate the BamHI fragments for injection. For each construct, fives transgenic lines were generated and analyzed.

Generation of ATF6/βgeo Mouse and β-Galactosidase Staining—The ATF6/βgeo insertion mouse was generously provided by Dr. William Skarnes (Wellcome Trust Sanger Institute), and its generation has been described (26). Embryos from wild-type C57 mouse mated with ATF6/βgeo mouse were isolated, and whole-mount β-galactosidase staining was performed.

Whole-mount β-Galactosidase Staining, Embryo Clearing, and Plastic Sectioning—Mouse embryos were isolated and fixed in 4% paraformaldehyde for 15 min and washed with PBS for 15 min three times. The embryos were then subjected to β-galactosidase staining as previously described (27). DNA from embryo yolk sacs was prepared for genotyping of mouse embryos as described (28). For clearing, β-galactosidase-stained embryos were dehydrated by methanol and immersed in 2:1 benzyl benzoate/benzyl alcohol (28). For plastic sectioning, whole-mount β-galactosidase stained embryos were embedded in JB-4 medium according to the manufacturer’s protocol (Polysciences, Inc.). The embryos were then subjected to plastic sectioning at 4 μm in a JB-4 microtome section machine (SORVALL porter-blum). Pictures of the sections were taken and digitized under dark field with Nomarski optics.

RNA Blot and RT-PCR—Total RNAs from whole embryos at different stages or different tissues of either embryos or adult mice were extracted, and Northern blots were performed as described (22). For RT-PCR assays, first strand cDNA was synthesized with Superscript III kit (Invitrogen). The primers used for the detection of the mouse YY1 mRNA were 5′-CAGAA GCAGG TGCG ATCAA GACCC (forward) and 5′-TTCCC GCAGC CCTTCG AATGT GCAGTA (reverse), yielding a 460-bp product. The primers used for the detection of mouse ATF6 mRNA were 5′-TGCTA GGACT GGAGG CCAGG CTCAA (forward) and 5′-CATGT CTATG AACCC AGCCT CGAAGT (reverse), yielding a 350-bp product. The PCR primers against both spliced and unspliced forms of XBP-1 were as described by Iwawaki et al. (29).

Western Blot—Cell extracts from mouse embryonic fibroblasts of either the wild-type or the ATF6/βgeo homozygous mouse were separated on 7% SDS-PAGE and transferred to nitrocellulose membranes. The ATF6 protein was detected as described previously (30). Protein extracts from 293T cells 48 h after co-transfection with GATA-4 expression vector and siRNA against GATA-4 (Ambion) or GFP (Qiagen) were separated on 10% SDS-PAGE, and GATA-4 protein and β-actin were detected using the goat anti-GATA-4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and mouse anti-β-actin (Sigma) antibody, respectively.

Chromatin Immunoprecipitation Assays—293T cells were transfected with the pCG-GATA-4 plasmids and incubated for 48 h prior to fixation with formaldehyde. For primary rat neonatal cardiomyocytes, 1.4 × 10⁶ cells were cultured for 8 days prior to formaldehyde fixation. Chromatin immunoprecipitation was performed as previously described (21). An aliquot was removed to be used as the input fraction and was processed with the eluted precipitates from the cross-link reversal step. Equal amounts of chromatin from each sample were incubated at 4°C overnight with 10 μg of goat or rabbit anti-GATA-4 antibody or, for the detection of NF-Y/CBF, 3 μg each of anti-CBF-A, -B, and -C antibodies (Santa Cruz Biotechnology). Cross-linking was reversed overnight at 65°C and was treated with proteinase K for 1 h at 45°C, and the DNA was then purified by phenol/chloroform extraction and ethanol precipitation and subjected to 40 cycles of PCR, and the
products were run on a 2% agarose gel with ethidium bromide. The P1/P2 primers used for the detection of the human Grp78 promoter were 5'-H11032-GTGAA CGTTA GAAAC GAATA GCAGCCA (forward) and 5'-H11032-GTCGA CCTCA CCGTC GCCTA (reverse), yielding a 213-bp product. The P3/P4 primers used for the detection of human Grp78 exon 8 were 5'-H11032-CCTCT GAAGA TAAGG AGACC ATGGAA (forward) and 5'-H11032-TGCTG TATCC TCTTC ACCAG TTGG (reverse), yielding a 187-bp product. The rP1/rP2 primers used for the detection of the equivalent rat Grp78 promoter region were 5'-H11032-GCGTA CCAGTGACGT GAGTT (forward) and 5'-H11032-CAGTG TAGTC ACAGC CAGTA TCG (reverse), yielding a 225-bp product.

RESULTS

High Grp78 Promoter Activity in the Embryonic Heart Requires the ERSE—To investigate Grp78 expression in vivo and define the regulatory region of the Grp78 promoter mediating the expression, we generated transgenic (TG) mice using three LacZ reporter constructs, 3kbLacZ, D300LacZ, and D170LacZ (Fig. 1A). 3kbLacZ contains 3 kilobases of the rat Grp78 promoter driving the expression of the LacZ reporter gene. D300LacZ contains a 230-bp internal deletion spanning from -300 to -70, which eliminates the known ER stress-inducible elements of the Grp78 promoter, including both the ERSE and the cAMP-response element (CRE). For D170LacZ, the internal deletion was narrowed down to a 100-bp region spanning -170 to -70, which eliminates primarily the three tandem copies of the ERSE. Previously, in NIH3T3 cells, it was confirmed that 3kbLacZ is inducible by ER stress, whereas the induction was substantially suppressed in D170LacZ and completely abolished in D300LacZ (21).

For each construct, 2–5 independent TG mouse lines were generated and analyzed. Embryos and organs of adult mice from transgenic mice and their nontransgenic (non-TG) siblings were stained for β-galactosidase activity. For the 3kbLacZ TG mouse, β-galactosidase activity was strongly detected in the heart and to a lesser extent in other tissues, such as the somites and neural tube during early embryonic development (E9.5–E11.5) (Fig. 1, B–D) (data not shown). As a negative control, no β-galactosidase activity was detected in non-TG E11.5 siblings (Fig. 1E). For the D300LacZ mice, no β-galactosidase activity was detected at E11.5 or other stages during embryonic development (Fig. 1F) (data not shown). The LacZ transgene directed by D300 is functional, since strong β-galactosidase activity was detected in the hypothalamus of the adult mice and not in the non-TG sibling control (Fig. 1G). For the D300LacZ mice, no β-galactosidase activity was detected at E11.5 or other stages during embryonic development (Fig. 1F) (data not shown). The LacZ transgene directed by D300 is functional, since strong β-galactosidase activity was detected in the hypothalamus of the adult mice and not in the non-TG sibling control (Fig. 1G). The Grp78 promoter region required for heart expression is further narrowed down to the 100-bp region containing the ERSEs, since the reporter activity of D170LacZ is completely quiescent from E9.5 to E11.5 (Fig. 1F) (data not shown). D170LacZ in the TG mice is functional, since LacZ activity can be detected in preimplantation embryos (data not shown). Identical results were observed in multiple, independently derived transgenic mouse lines for all three constructs. These results provide the first evidence that the in vivo induction of Grp78 transcrip-
tion during early mouse organogenesis, in particular the embryonic heart, is mediated largely through the ERSEs.

Grp78 Transcription Is Activated in Early Embryonic Cardiomyocytes but Subsides Later in Development—To obtain a clearer view of the β-galactosidase staining pattern of 3kbLacZ, E11.5 embryos were dehydrated in methanol and cleared in 2:1 benzyl benzoate/benzyl alcohol after whole mount β-galactosidase staining. As shown in Fig. 2A, β-galactosidase activity was most intense at the heart, with activity also detected in the somites. To obtain higher resolution of the β-galactosidase expression pattern, plastic sections of the embryos after β-galactosidase staining were prepared, and images were taken under dark field conditions where the β-galactosidase staining turned to a pink color (Fig. 2B). It is evident that the heart showed high β-galactosidase activity. The LacZ staining is predominant in the trabeculae, and at higher magnification, a majority of the staining was observed in cardiomyocytes (Supplementary Fig. S1). In addition, β-galactosidase staining was visible in other organs, such as the mandibular component. Within the developing embryonic heart, β-galactosidase staining was clearly detected in the myocytes of both the atrium and the ventricle of the embryonic heart (Fig. 2C).

To determine directly whether transcription of the endogenous Grp78 gene is induced in the embryonic mouse heart, as predicted from the reporter gene approach, a Northern blot was performed with RNA extracted from whole embryo, heart, brain, or somites of E11.5 embryos of non-TG siblings. In agreement with the β-galactosidase staining data, the level of Grp78 transcript was highest in the embryonic heart (Fig. 2D, lanes 1–4). Thus, endogenous Grp78 transcription correlates with LacZ reporter gene activity driven by the Grp78 promoter.

To determine whether Grp78 transcription is sustained later in development and in postnatal mice, an additional Northern blot was performed with RNA extracted from whole embryos at E9.5–E14.5 or from hearts of E18 embryos or 3-month-old mice. We observed that, compared with embryonic tissues, Grp78 transcript level was dramatically reduced in the heart tissue of E18 embryos and in 3-month-old mice (Fig. 2E, lanes 1–5). This is in agreement with our observation that β-galactosidase staining in 3kbLacZ mice was below the detection limit in the E18 embryonic heart as well as the heart of adult mice (25) (data not shown).

LacZ Reporter Activity Driven by the ATF6α Promoter Is Widely Expressed in Developing Embryos—Since high level expression of the Grp78 promoter during early organogenesis requires the ERSE, it is possible that the Grp78 induction is mediated by transcription factors known to bind and activate the ERSE. These include ATF6, XBP-1, and YY1, and in tissue culture systems, these transcription factors are able to activate the Grp78 promoter in a manner dependent on the ERSE. There are two forms of ATF6, α and β, encoded by two different genes, both of which are processed by ER stress to yield an active, N-terminal protein (31). The ATF6/βgeo mice created through gene trap generate an ATF6α fusion protein driven by the endogenous ATF6 promoter (26). This fusion protein contains 601 amino acids of ATF6 linked to β-galactosidase and neomycin transferase (Fig. 3A). Western blot performed with anti-ATF6 antibody on protein extracts from embryonic fibroblasts of wild-type and ATF6/βgeo homozygous mice confirmed the expression of a 220-kDa ATF6 fusion protein in the ATF6/βgeo homozygous mouse (Fig. 3B). As expected, the 220-kDa protein was also detected by antibody against β-galactosidase (data not shown). Thus, the ATF6/βgeo mouse provides a useful reporter system to examine the expression pattern of ATF6α during embryonic development. To compare with the Grp78 expression pattern, E9.75 and E11.5 embryos from the ATF6/βgeo mouse were stained for β-galactosidase and neomycin transferase (Fig. 3A). Western blot performed with anti-ATF6 antibody on protein extracts from embryonic fibroblasts of wild-type and ATF6/βgeo homozygous mice confirmed the expression of a 220-kDa ATF6 fusion protein in the ATF6/βgeo strain, in contrast to the 90-kDa protein in the non-TG sibling (Fig. 3B).

Previously, in situ hybridization experiments showed that YY1 is ubiquitously expressed during early organogenesis (E7.5–E12.5) (11).
Through RT-PCR, we confirmed that the YY1 transcript level was readily detectable in the heart, brain, and somites of E11.5 embryos (Fig. 3D). Our results also showed that despite the high level of XBP-1 mRNA expression in these embryonic tissues, no XBP-1 mRNA splicing was detected (Fig. 3E, lanes 3–5). As a positive control, XBP-1 mRNA was spliced in NIH3T3 cells treated with the ER stress inducer thapsigargin (Fig. 3E, lanes 1 and 2). Collectively, these results show that ATF6/H9251 and YY1 are ubiquitously expressed in mouse embryos during early organogenesis. Whereas they can contribute to Grp78 induction through activation of the ERSE, it is possible that specific, high level expression of
Grp78 in the developing heart is further facilitated by heart-specific transcription factors in these stages, either directly or through functional interactions with YY1 or ATF6.1.

GATA-4 Binds to the Grp78 Promoter in Vivo and Activates the Promoter through the ERSE—One such candidate is GATA-4, a transcription factor highly active in the embryonic heart and a known interactive partner of YY1 in promoter activation. Within the 100-bp region deleted in D170LacZ, which results in elimination of reporter gene activity in the developing heart, we did not detect any consensus GATA sequence; thus, there is no obvious high affinity site for GATA-4 within the ERSE region. To examine the possibility that GATA-4 can bind the Grp78 through indirect interactions as has been reported for other promoters, chromatin immunoprecipitation (ChIP) assays were performed with human embryonic kidney 293T cells transiently transfected with an expression plasmid for GATA-4. Two sets of PCR primers were used. The P1/P2 set of primers detects factors binding to a region of the Grp78 promoter containing the ERSE, whereas the P3/P4 set of primers detects factors binding to exon 8 of the Grp78 promoter (Fig. 4A).

Using the anti-GATA-4 antibody as the immunoprecipitating antibody and P1/P2 as the PCR primers, we detected GATA-4 binding to the Grp78 promoter region containing the ERSE in cells transfected with the GATA-4 expression vector (Fig. 4B). ChIP analysis performed with nontransfected cells did not show a signal above the IgG negative control (data not shown). As a positive control, a ChIP assay was also performed using anti-NF-Y antibody, confirming constitutive binding of NF-Y to the Grp78 promoter as previously reported. In the negative control, only low background binding was detected when IgG was used for the immunoprecipitation. With equal amounts of input DNA, no signal was detected in any of the ChIP assays using the P3/P4 primers flanking exon 8 of human Grp78, which is in agreement with the expected binding of the transcription factors to the Grp78 promoter but not its exon. Further, serial dilution of the PCRs confirmed that the PCRs were performed in the linear range (Fig. 4C).

Similarly, in primary rat neonatal cardiomyocytes, ChIP assays showed that endogenous GATA-4 and NF-Y bind to the ERSE region of the rat Grp78 promoter (Fig. 4D). The specificity of endogenous GATA-4 binding to the Grp78 promoter was demonstrated by using two independent preparations of antibodies against GATA-4, which showed signals considerably above the IgG negative controls. Thus, GATA-4 binds to the Grp78 promoter in vivo.

To test whether GATA-4 is able to activate the Grp78 promoter, −457/LUC and −169/LUC were constructed as described and co-transfected with an expression vector for GATA-4 into CV1 cells. We observed that GATA-4 can activate the Grp78 promoter in a dosage-dependent manner for both constructs (Fig. 5A). To test directly the effect of GATA-4 on the ERSE, we constructed ERSE/LUC, in which the 100-bp region of the rat Grp78 promoter containing three tandem copies of the ERSEs is subcloned into the pGL-3 promoter vector (Fig. 5B).
In SV40/LUC, luciferase activity is driven by SV40 early promoter and enhancer elements. Our results confirmed that the ERSE is a target of GATA-4 activation, since the ERSE/LUC showed 5-fold stimulation by GATA-4 compared with 1.6-fold for SV40/LUC (Fig. 5B). To test whether endogenous GATA-4 is required for the Grp78 promoter activation, siRNA against the rat GATA-4 was synthesized, and its ability to suppress rat GATA-4 protein expression was validated in 293T cells by co-transfecting the cells with the rat GATA-4 expression vector and siGATA-4 or siGFP, which served as a negative control. The GATA-4 protein level was significantly decreased when comparing to the cells co-transfected with siGFP (Fig. 4E). The reporter plasmid −169/LUC and siRNA against either GATA-4 or GFP were then co-transfected into rat neonatal cardiomyocytes, and luciferase activities were measured. We observed that the Grp78 promoter activity was suppressed by siGATA-4 in a dosage-dependent manner (Fig. 4F).

The DNA Binding Domain of YY1 Is Necessary and Sufficient for Its Synergy with GATA-4 to Activate Grp78 Promoter—One mechanism for GATA-4 induction of the ERSE is through cooperation with YY1. To test this, CV1 cells were co-transfected with −169/LUC and an expression plasmid for GATA-4, in the presence or absence of YY1. Two forms of YY1 were tested, the full-length form and the YY1Δ mutant, YY1Δ. β-galactosidase activity in CV1 cells with YY1 was co-transfected into CV1 cells with GATA-4 and −169/LUC (Fig. 7A). Our results showed that the DNA binding domain spanning 261–414 is both sufficient and necessary for its stimulatory effect on GATA-4. This is based on the observation that subdomains 1–170, 1–261, and 261–333, all lacking the full DNA binding domain, were ineffective, whereas subdomain 261–414 alone was as active as the full-length protein 1–414 (Fig. 7B).

The Nuclear Form of ATF6α Synergistically Activates the Grp78 Promoter with GATA-4, and This Synergy Is Further Potentiated by YY1—The active, nuclear form of ATF6α is a potent activator of the Grp78 promoter. We sought to determine whether GATA-4 synergizes with ATF6 to activate Grp78 transcription. The nuclear form of ATF6α was expressed in the form of ATF6(373), which contains the proteolytically cleaved portion of ATF6 that relocates to the nucleus of ER stressed cells. CV1 cells were co-transfected with −169/LUC and various combinations of GATA-4 and ATF6(373) expression vectors, and luciferase activities were determined. As expected, the Grp78 promoter was activated by ATF6(373) to about 10-fold in a dosage-dependent manner (Fig. 8A). When increasing amounts of ATF6(373) were added to a constant amount of GATA-4, the -fold induction was elevated to about 40-fold, which was significantly higher than the additive effect of ATF6(373) and GATA-4, since GATA-4 alone only induced the Grp78 promoter about 6-fold. These results established that GATA-4 and ATF6 can synergistically activate the Grp78 promoter.

Since the active, nuclear form of ATF6α complexes with YY1, and the two transcription factors act as co-activators in the induction of the Grp78 promoter (10), we tested whether YY1 is able to potentiate the synergy between GATA-4 and ATF6 in activating the Grp78 promoter. CV1 cells were co-transfected with increasing amounts of YY1 expression vector and fixed amounts of GATA-4 and ATF6(373). Under these transfection conditions, YY1 alone produced a modest induction of the Grp78 promoter of around 3-fold (Fig. 8A). Strikingly, when the same amounts of YY1 were expressed in the presence of both GATA-4 and ATF6(373), the -fold induction was increased to about 80-fold, which was significantly higher than the 40-fold induction by GATA-4 and ATF6(373). Similarly, in primary rat neonatal cardiomyocytes, ATF6(373) strongly stimulated GATA-4 activation of the Grp78 promoter, which was potentiated by YY1 (Fig. 8B).

To test further the role of GATA-4 in ER stress induction of the Grp78 promoter, CV-1 and HeLa cells were co-transfected with the −169/LUC and the GATA-4 expression vector. The transfected cells were treated with thapsigargin, which depleted ER intracellular calcium store, or tunicamycin, which blocked protein N-linked glycosylation (Fig. 9, A and B). In both cell types, GATA-4 enhanced the basal and ER stress-induced Grp78 promoter activity in a dosage-dependent manner.

DISCUSSION

The expression and regulation of GRP78, which controls multiple signaling pathways of the unfolded protein response through a bind and release mechanism, has been broadly investigated in tissue culture systems (32, 33). These have led to the discovery of the ERSE in the promoter of UPR target genes and the identification of several unique signaling pathways and transcription factors that mediate ER stress-induced transcription of Grp78 and other UPR target genes (34). Despite these advances derived from tissue culture model systems, very little is known about how these pathways operate in vivo to respond to physiological ER stress. One approach to resolve this issue is to use transgenic mouse models to query under what conditions the ERSE will be activated and determine the mechanism for its activation.

Toward this goal, we created TG mice models bearing LacZ reporter genes driven by 3 kb of the rat Grp78 promoter and examined the
β-galactosidase activity in various tissues. The choice of 3 kb for the promoter length is important, since the longer promoter fragment size helps buffer the transgene from the position effect of the integration site. Our attempts to create transgenic mouse strains bearing LacZ reporter gene driven by only 169 bp of the same promoter containing primarily the ERSE resulted in uneven expression patterns among different TG strains.4 Consistently, we observed that in the 3kbLacZ TG lines, Grp78 promoter activity as measured by β-galactosidase activity is most prominent in the developing heart from E9.5 to E11.5 and subsides considerably during later stages and in the adult mouse. Our results are consistent with the previous observation that GRP78 protein, as detected by immunohistochemical staining, is highly expressed in the embryonic heart during organogenesis but the level diminishes rapidly in postnatal mice (17). Further, the lack of β-galactosidase expression in the embryonic heart in transgenic mouse strains bearing LacZ driven by the Grp78 promoter with a 100-bp deletion in the region containing the ERSEs directly showed that Grp78 induction during early organogenesis is strictly dependent on the ERSE. Another interesting point of note is that while 3kbLac is largely quiescent in major adult mouse organs, suggesting a low rate of Grp78 transcription, the Grp78 promoter is reactivated in cancer tissues and inflammatory cells such as macrophages in the vicinity of the cancer in the TG mice (25). Our observations support the emerging theory that cancer bears similarities with developmental stages, an energy shift occurs such that the adult heart utilizes glucose and lactate as the major energy source, whereas at later developmental stages, an energy shift occurs such that the adult heart uses fatty acids as the energy source (39). It is possible that a high rate of proliferation and differentiation coupled with fast consumption of glucose may cause low glucose stress, which will in turn activate glucose-regulated protein genes such as Grp78 and Grp94 as a protective measure. In support of this hypothesis, Grp78 and Grp94 expression were increased when the embryonic heart was exposed to hypoglycemia in vivo (17, 40). Another stress in the microenvironment of the developing heart is hypoxia (41). Under anoxia conditions in vitro and in the microenvironment of solid tumors where hypoxia and hyperglycemia can develop, Grp78 transcription and transgene activity driven by the Grp78 promoter are highly activated (25, 42, 43).

Another question that arises is how Grp78 expression is regulated in vivo during mouse development. Whereas the precise physiological stresses that activate the Grp78 promoter may be very difficult to identify, our results obtained with the deletion mutants of the Grp78 pro-

\[ \text{Grp78 Promoter Activation in Embryonic Heart} \]

* C. Mao, W.-C. Tai, Y. Bai, C. Poizat, and A. S. Lee, unpublished data.
Grp78 Promoter Activation in Embryonic Heart

moter in the TG models clearly showed that the signaling pathways that mediate the activation act through the 100-bp region containing the ERSEs. This discovery suggests that transcription factors that interact with the ERSE of the Grp78 promoter may play a major role in the activation of the Grp78 promoter in the developing embryos. Taking advantage of the ATF6/βgeo mouse (26), we found that ATF6α was ubiquitously expressed in many tissues in early embryonic development, including the heart in E9.5-E11.5 embryos where Grp78 induction appears to be most prominent. Similarly, YY1 is also ubiquitously expressed in early embryonic development (11). Another signaling pathway regulating the UPR is the IRE-1p-XBP-1 pathway. Despite a high level of XBP-1 transcript in the developing heart, XBP-1 splicing was below the detection limit in mouse embryonic hearts at the time of Grp78 induction. This result is consistent with the lack of reporter gene activity for the ERAI model in mouse embryos (29). Considering that ATF6 and XBP-1 each only regulate a subset of UPR target genes under ER stress (44) and that the ERSE may integrate other novel signaling pathways during mouse development, these known UPR regulators may not be sufficient alone but act in synergy with other organ-specific transcription factors to facilitate Grp78 induction in the developing heart.

For example, embryonic heart induction of another ER chaperone gene calreticulin is activated by NKx2.5, a transcription factor essential for cardiac development (45, 46).

Here we investigated GATA-4, a transcription factor that plays an important role in gene expression during early heart organogenesis. Homozygous knock out of the GATA-4 gene in mice resulted in embry-
Grp78 Promoter Activation in Embryonic Heart

...onic lethality due to defects in heart formation (47, 48). Using chromatin immunoprecipitation assays in the 293T cells transfected with the GATA-4 expression vector as well as in primary rat neonatal cardiomyocytes, we were able to detect GATA-4 binding to the human Grp78 promoter region containing the ERSE elements. Further, using siRNA to knockdown endogenous GATA-4 in rat neonatal cardiomyocytes, we directly showed that GATA-4 contributes to the Grp78 promoter activity. However, within the 100-bp sequence of the Grp78 promoter that is required for induction during heart organogenesis, we did not detect any consensus (A)GATA(T) sequence that could explain high affinity binding of GATA-4. One possibility is that GATA-4 cooperates with other factors that bind to the Grp78 promoter. Interestingly, it has been reported that YY1 cooperatively activates the cardiac B-type natriuretic peptide promoter with GATA-4, and the DNA binding domain of YY1 is sufficient for the synergy (19). Here we showed that GATA-4 can activate the Grp78 promoter through the ERSE. When YY1 was co-transfected with GATA-4, the -fold induction was more than the additive effects from GATA-4 or YY1 alone. Further mapping showed that the DNA binding domain of YY1 is necessary and sufficient for the cooperative effect with GATA-4. It has been postulated that the physical and functional interaction between GATA-4 and YY1 might depend on co-activators such as CREB-binding protein/p300 (19). In co-immunoprecipitation assays, we only detected weak interaction between GATA-4 and YY1, consistent with the notion that the GATA-4-YY1 complex may consist of multiple components and that the complex is unstable under the assay conditions (data not shown). Recently, it has been reported in human Panc-1 pancreatic cancer cells, which contain abundant Sp family proteins, that Sp1, -3, and -4 can bind the ERSE through the GC-rich spacer motif and contribute to Grp78 promoter activity (49). The GC-rich motif is highly conserved among the human, murine, and rat Grp78 promoters; thus, it is likely that Sp protein families also bind to the rodent Grp78 promoters. Since GATA-4 has been shown to modulate tissue-specific transcription by direct interaction with Sp1 and since the two proteins can be co-immunoprecipitated as a complex (50), Sp1 may contribute to the recruitment of GATA-4 to the ERSE in some cell types.

Finally, in co-transfection assays, we discovered that the nuclear form of ATF6α is a potent co-activator of GATA-4 in inducing the Grp78 promoter. When GATA-4, YY1, and ATF6 were expressed in combination, even stronger enhancement of induction of the Grp78 promoter was achieved. GATA-4 is also able to enhance ER stress induction of the Grp78 promoter. ER stress is known to activate the p38 MAP kinase (22). Interestingly, both GATA-4 and ATF6 can be phosphorylated by p38 MAP kinase (22, 51, 52), which enhances their transacti...
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