Induction of Grp78/BiP by Translational Block
ACTIVATION OF THE Grp78 PROMOTER BY ATF4 THROUGH AN UPSTREAM ATF/CRE SITE INDEPENDENT OF THE ENDOPLASMIC RETICULUM STRESS ELEMENTS*

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Shengzhan Luo‡, Peter Baumeister‡, Shujie Yang§, Steve F. Abeouwer§, and Amy S. Lee¶
From the ‡Department of Biochemistry and Molecular Biology and the USC/Norris Comprehensive Cancer Center, University of Southern California Keck School of Medicine, Los Angeles, California 90089-9176 and the §Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131-0001

Mammalian cells respond to endoplasmic reticulum (ER) stress by attenuation of protein translation mediated through the PERK-eIF2α pathway and transcriptional activation of genes such as Grp78/BiP encoding ER chaperone proteins. The disruption of PERK function or the blocking of eIF2α Ser51 phosphorylation fails to attenuate translation after ER stress and also results in substantial impairment of Grp78/BiP induction by ER stress. While the activation of the Grp78 promoter by the ATF6 pathway through the endoplasmic reticulum stress elements (ERSEs) is well documented, the molecular mechanism linking PERK activation to Grp78 stress induction is unknown. We report here that ATF4, a transcription factor whose translation is up-regulated by the PERK-eIF2α pathway, can activate the Grp78 promoter independent of the ERSE. The ATF4-activating site is localized to an ATF/CRE sequence upstream of the ERSEs and is distinct from the C/EBP-ATF composite site previously identified as the ATF4 binding site in the ER stress-inducible chop promoter. In vitro translated ATF4 binding to the ATF/CRE site requires other nuclear co-factors from non-stressed cells, forming a complex that exhibits identical electrophoretic mobility as a thapsigargin-stress induced complex. Here we have identified the closely related ATF1 and CREB1 as nuclear co-factors that form in vitro complexes with endogenous ATF4. ER stress induces CREB1 phosphorylation and ATF1/CREB1 binding to the Grp78 promoter.

The use of adenoviral vector expression systems, we provide evidence that when ATF4 function is suppressed and its binding partners are not able to compensate for its function, Grp78 induction by Tg and Tu is partially inhibited. Our studies resolve a mechanism responsible for inhibition of Grp78 mRNA induction by ER stress in cells that are functionally null for PERK or devoid of eIF2α phosphorylation.

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¶ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology and the USC/Norris Comprehensive Cancer Center, University of Southern California Keck School of Medicine, 1441 Eastlake Ave., Rm. 5308, Los Angeles, CA 90089-9176. Tel.: 323-865-0507; Fax: 323-865-0094; E-mail: amylee@hsc.usc.edu.

The abbreviations used are: ER, endoplasmic reticulum; ERSE, endoplasmic reticulum stress element; UPR, unfolded protein response; Tg, thapsigargin; Tu, tunicamycin; PBS, phosphate-buffered saline.

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The abolishment of chop induction in cells devoid of PERK or with eIF2α mutated at Ser51 can be explained by the dependence of ER stress-induced chop transcription on ATF4. The transcription factor ATF4, which belongs to the ATF/CREB protein family, is absent or present in very low amounts in non-stressed cells but is efficiently translated only when Ser51 in eIF2α is phosphorylated (16, 18). By binding to the C/EBP-ATF composite site (5'-ATTGCGATCA-3') of the human chop promoter, ATF4 activates chop transcription (9, 10). A similar site (5'-GTTTCTACATCA-3') is identified on the promoter of the human asparaginase synthetase gene, which also binds ATF4 (19). However, no such site is present on the Grp78 promoter. Further, recent studies suggest that transcriptional activation of the Grp78 promoter by ER stress is strictly dependent on S2P-mediated proteolytic cleavage of the transcription factor ATF6, which specifically targets the ERSE (20, 21). All Grp promoters contain multiple copies of highly conserved ERSES with a consensus sequence 5'-CACAATN6CCACG-3' that bears no resemblance to the above ATF4 binding sites (22, 23). Other transcription factors that regulate the Grp78 promoter upon ER stress, including NF-Y, YY1, and TFIID, act through the ERSE. This raises the question of how attenuation of translation through eIF2α phosphorylation could enhance Grp78 transcription in response to ER stress. To resolve this, it is necessary to identify the effector molecule that mediates this regulation.

We report here that ATF4 is a new activator of the Grp78 promoter. ATF4, also referred to as CREB2, is a member of the ATF/CREB family that can bind as homodimers or heterodimers to the CAMP responsive element (CRE) within the ATF/CREB family and with members of the AP-1 transcription factor family (18). We have discovered a conserved ATF4 binding site (5'-TACGCTTCTACATCA-3') upstream of the ERSEs in the mammalian Grp78 promoters distinct from the C/EBP-ATF composite site previously described for the chop and asparaginase synthetase promoters. This ATF/CRE-like site was first identified as a binding site for a CREB-related protein, however, its role in ER stress induction of the Grp78 promoter was undetermined (28). As ATF4 protein level is up-regulated upon ER stress, it binds to the ATF/CRE-like site of the Grp78 promoter in the presence of other transcription co-factors. Here we identify the closely related ATF1 and CREB1 as the nuclear co-factors that form in vivo complex with ATF4 and ER stress induces phosphorylation of CREB1 as well as ATF1/CREB1 binding to the Grp78 promoter. Through mutational analysis of this ATF/CRE-like site with exogenously expressed ATF4, we provide evidence that ATF4 is a new activator of the Grp78 promoter independent of the ERSEs. Further, through the use of adenoviral vector expression systems, we provide evidence that when ATF4 function is suppressed and its binding partners are not able to compensate for its function, Grp78 induction by Tg and Tu is partially inhibited. These results confirm that ATF4 can contribute to the ER stress induction of Grp78.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Conditions and Drug Treatment**—NIH3T3 and 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The HeLa S3 cells were grown as described (28). For stress treatment, the cells were treated with 300 nM of thapsigargin (Tg) or 1.5 μg of LaCZ reporter (3kb/LacZ, D170/LacZ or D300/LacZ) were co-transfected with either 0.5 μg of empty vector or 0.5 μg of pMyc-ATF4 using 10 μl of superfect or polyfect, in accordance with the manufacturer's protocol (Qiagen, Valencia, CA). For each well of a 6-well plate of NIH3T3 cells, 0.5 μg of Ad/Luc, 0.5 μg of LaCZ reporter (3kb/LacZ, D170/LacZ or D300/LacZ) was confirmed by DNA sequencing and subcloned in-frame into the XbaI and BamHI site of pCMV5/myc to obtain the Myc-tagged ATF4 expression plasmid, pMyc-ATF4.

**Transfection and Reporter Assays**—NIH3T3 or 293 cells were grown to 80% confluence in 6-well plates and co-transfected with pMyc-ATF4 or the empty vector pCMV5/myc with different reporter genes using superfect or polyfect, in accordance with the manufacturer's protocol (Qiagen, Valencia, CA). Each well of a 6-well plate of NIH3T3 cells, 0.5 μg of Ad/Luc, 0.5 μg of LaCZ reporter (3kb/LacZ, D170/LacZ or D300/LacZ) was co-transfected with either 0.5 μg of empty vector or 0.5 μg of pMyc-ATF4 using 10 μl of superfect or polyfect. For 293 cells, 0.7 μg of each plasmid was used instead. Twenty-four hours after transfection, the cells were harvested and assayed for luciferase activity according to the manufacturer's instructions (Promega, Madison, WI) or β-galactosidase activity as described (31). The results were determined in duplicates or triplicates and repeated 3–4 times. The activity levels were analyzed by Student's t test (*p < 0.05; **p < 0.01).

**In Vitro Protein Translation**—The full-length ATF4 cDNA was subcloned into XbaI and BamHI of the pBlueScript KS+ vector. In vitro translation was carried out using a TNT T7-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions, either in the absence or presence of [35S]methionine. The translated proteins were separated on 12% SDS-PAGE. The non-radio labeled proteins were subjected to Western blot and the radiolabeled proteins were subjected to autoradiography.

**Western Blot**—Cell extracts or in vitro translated ATF4 were separated on 12% SDS-PAGE, transferred to nitrocellulose membranes. The ATF4 protein was detected using a rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 1:1000. For detection of the closely related ATF1/CREB1, an anti-ATF1 mouse monoclonal antibody (Santa Cruz Biotechnology Inc.) that recognizes both proteins was used at 1:500 dilution. For detection of the phosphorylated forms, an anti-phosphoATF1/CREB1 goat polyclonal antibody (Santa Cruz Biotechnology Inc.) was used at 1:200 dilution. The same filter was Western-blotted with a monoclonal antibody against β-actin (Sigma) at a dilution of 1:3000 to monitor for protein loading.

**Chromatin Immunoprecipitation Assays**—The gel shift and supershift assays were performed as described (28). The oligonucleotides used for probe were 5'-gtgagGCTGGGGGGCCGCTAACAAGTGGGACGAAAAG-3' and 5'-gtggattCTCTCTGCGACCATGACGTAACCTGTTGACG-3'. The CRE-like site is underlined. For assays with in vitro translated ATF4, 5 μl of in vitro translated ATF4 was added into the reaction mixture. The CRE-mutant competitor fragment spanning −300 to −170 was generated by PCR using the CRE-mutant/LUC plasmid as template. All antibodies for supershift assays were from Santa Cruz Biotechnology Inc.

**Chromatin Immunoprecipitation Assays**—NIH3T3 cells were grown in 15-cm plates under normal cell culture conditions to 80% confluence and treated with 300 nm Tg for 2 or 4 h or 10 μm dithiothreitol for 4 h and then the fixed by addition of formaldehyde to the growth media to a final concentration of 1%. To harvest NIH3T3 cells, plates were rinsed with cold PBS, covered with 10 ml of 5% fetal bovine serum in PBS, and then scraped. Chromatin was prepared using a kit from Upstate Biotech Inc. (Lake Placid, NY) according to the recommendations of the manufacturer, with twenty-four 5-s sonication pulses at 10-s intervals, followed by incubation of an apoptotic factor. An aliquot from each sample representing 5% of the total volume was used to be the input fraction and was processed with the CRE-mutant/LUC plasmid as template. All antibodies for supershift assays were from Santa Cruz Biotechnology Inc.

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gel and visualized with EtBr staining. The primers used for the endogenous Grp78 promoter were: 5′-CATGGTGCGGCTTAAAGATGACAG (forward) and 5′-AGTATGGACGGCGCCTGCGC (reverse), yielding a 223-bp product.

Co-immunoprecipitation—NIH3T3 cells were grown in 15-cm plates to about 80–90% confluency. The cells were either non-treated or treated with Tg or Tu for 6 h and then harvested in Triton X-100 lysis buffer and the immunoprecipitation with an anti-ATF1 mouse monoclonal antibody (Santa Cruz Biotechnology Inc.) was carried out as described (29). The immunoprecipitated complexes were then resolved by 12% SDS-polyacrylamide gel electrophoresis and subjected to Western blot with an anti-ATF4 rabbit polyclonal antibody. The same membrane was re-blotted with anti-ATF1 antibody to control for immunoprecipitation of ATF1 in each of the samples.

ATF4 Adenoviral Vector Construction—Wild-type and DN mutant ATF4 cDNAs were expressed using the AdEasy adenoviral vector system (32) provided by Bert Vogelstein (Howard Hughes Institute, Johns Hopkins University). Expression plasmids pEFmATF4/4m and pEFmATF4 M were kindly provided by Jawed Alam (Department of Biochemistry and Molecular Biology, Louisiana State University Health Science Center). These plasmids contain wild-type murine ATF4 cDNA and a DN mutant (ATF4ARK) encoding murine ATF4 protein with a seven amino acid substitutions within the DNA-binding domain (292RYRQKKR to 292GYLEAAA) (33). To clone into the AdEasy system, the wt and ARK coding sequences were PCR amplified from these vectors using a 5′-NotI-containing (underlined) forward primer (5′-AACAACACGGCCGCGTCGTGAAACACCATGGACCG3′) and a 5′-HindIII-containing (underlined) reverse primer (5′-GTTGTTGGTAAACCTTGAAGCTTCGGGCCATCGAC3′). The PCR reaction was carried out with Taq polymerase (Applied Biosystems, Foster City, CA) under the following conditions: 95 °C 1 min, 55 °C 2 min, 72 °C 2 min, 35 cycles followed by a 7 min 72 °C incubation. The PCR products were cloned into the pAdTrack-CMV adenoviral shuttle vector encoding kanamycin resistance and containing a second expression cassette encoding green fluorescent protein (GFP). The shuttle vectors were then linearized with the restriction enzyme PmeI and electroporated into DY329 electro-competent cells along with the adenoviral baculovirus plasmid pAE-ATF4 and pAE-ATF4ARK, formed by homologous recombination, were subsequently selected for kanamycin resistance and identified by plasmid size in conjunction with endonuclease analysis. The coding sequences and insertion points of pAE-ATF4 and pAE-ATF4ARK inserts were confirmed by DNA sequencing. Recombinant adenoviral vectors were generated by transfecting the recombinant plasmid into the mammalian 293 packaging cell line. Transfected cultures were maintained until the percentage of cells exhibiting green fluorescence approached 100%. Virus was obtained by freeze-thaw lysis of the cells in PBS, followed by centrifugation at 6000 × g for 10 min. Lysate was used to infect large-scale cultures of 293 cells, and similar results were observed (data not shown).

Collectively, these results suggest that ATF4 can act as a transcription activator for the Grp78 promoter, but the transcription activation is not mediated through the ERSEs.

ATF4 Activates Grp78 through a Cis-element Located between −300 and −170 of the Promoter—To locate the ATF4 target site on the Grp78 promoter and to confirm that ATF4 activation of the Grp78 promoter does not require the ERSEs, we constructed D170/LacZ, D300/LacZ and D170m/LacZ (Fig. 2A). D170/LacZ, with a deletion spanning −170 to −70, is identical to 3kb/LacZ with the only difference being the internal deletion eliminating all three ERSEs. D300/LacZ extended the 5′-border of the internal deletion to −300, resulting in the elimination not only of the three ERSEs but also of the CRE-like site, three CCAAT elements and two SpI sites. D170m/LacZ is identical to D170/LacZ with the additional mutation of the CRE site through site-directed mutagenesis.

First, we determined whether the Grp78 promoter absolutely requires the ERSEs for ER stress induction. For these experiments, either 3kb/LacZ, D170/LacZ, D170m/LacZ, or SV40/LacZ was transfected separately into NIH3T3 cells. The SV40/LacZ reporter was used for normalization of cell viability upon stress treatment. The transfected cells were treated with Tg, which depletes the ER calcium store and is a potent ER stress inducer of the Grp78 promoter (34). In the context of the 3-kb Grp78 promoter, elimination of all three ERSEs resulted in a substantial drop in fold induction (from 4.7–1.8-fold) by Tg, nonetheless there is residual stress inducibility which was largely eliminated when the CRE element upstream of the ERSEs was destroyed (Fig. 2B). While these results reaffirm the importance of the ERSEs toward stress induction of Grp78, they also reveal a potential role of the CRE element in allowing the Grp78 promoter to respond to ER stress.

We next examined the effect of exogenously transfected ATF4 on the wild-type and the two internal deletion mutations of the Grp78 promoter by performing co-transfections in NIH3T3 cells. We noted that the internal deletions of the promoter affected the basal level promoter activity, with D170 and D300 reduced to 60 and 20% respectively of that observed for 3kb/LacZ (Fig. 2C). However, while the exogenously expressed ATF4 could still activate D170/LacZ, it had no effect on the D300/LacZ (Fig. 2C). Further calculation showed that the fold induction of 3kb/LacZ and D170/LacZ by ATF4 were comparable, such that both promoters were activated by about 3-fold (Fig. 2D). Thus, these results suggest that the ATF4 responsive element might be localized in the region between −300 and −170 of the rat Grp78 promoter.

ATF4 Activates the Grp78 Promoter through a ATF/CRE-like Cis-element Upstream of the ERSE—Computer prediction using the TFSEARCH program (35) showed that between −300 and −170 of the rat Grp78 promoter, there is an ATF/CRE-like site. This sequence, TGAAGCTGA, spanning −190 to −183, is identical to the consensus CRE sequence TGAACGTCA with the

RESULTS

Exogenous ATF4 Activates the Grp78 Promoter but Not through the ERSEs—To investigate the functional role of ATF4 in the induction of the Grp78 promoter under ER stress conditions, we first co-transfected into NIH3T3 cells a plasmid expressing the Myc-tagged human ATF4 (pMyc-ATF4) with two Grp78 promoter reporters (Fig. 1A). The 3kb/LacZ reporter contains a 3-kb rat Grp78 promoter driving the expression of the LacZ reporter gene. In addition to the TATA element and three tandem copies of the ERSE, the 3-kb Grp78 promoter fragment also contains a CRE-like site, additional CCAAT motifs and several putative Sp1 sites. In −169/LUC, the regulatory elements upstream of the ERSEs and the TATA element were eliminated and this shorter Grp78 promoter fragment drives the expression of the luciferase reporter gene. We observed that the exogenously expressed ATF4 activated the 3kb/LacZ by about 2.5-fold (Fig. 1B). In contrast, overexpression of ATF4 had no effect on the activity of −169/LUC (Fig. 1C). To further confirm that the differences between the two reporter gene responses to ATF4 were not due to differences arising from separate transfection experiments, we co-transfected ATF4 together with both 3kb/LacZ and −169/LUC into the same cells and measured the LacZ and luciferase activities respectively. Consistent with our previous results, exogenous ATF4 only activated 3kb/LacZ but not −169/LUC (Fig. 1D). The same co-transfection experiments were also performed in NIH3T3 cells, and similar results were observed (data not shown).

To further confirm that ATF4 can act as a transcription activator for the Grp78 promoter, but the transcription activation is not mediated through the ERSEs.

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exception of one base. Since ATF4 belongs to the ATF/CREB family, and within the −300 to −170 region we did not find any other ATF/CRE-related site, one possibility is that ATF4 activates the Grp78 promoter through this CRE site. To test this, we constructed two luciferase reporter genes, −457/LUC and CRE-mut/LUC (Fig. 3A). In −457/LUC, the expression of the luciferase gene is driven by a Grp78 promoter fragment spanning from −457 to −29, which includes the TATA element, three ERSEs, the ATF/CRE site and upstream sequences up to −457. In the CRE-mut/LUC, the ATF/CRE-containing sequence was specifically mutated while the three ERSEs remained intact (Fig. 3B). The CRE-mut/LUC showed a lower induction level by Tg and to a lesser extent a lower basal level (Fig. 3C). By co-transfection with the ATF4 expression plasmid, we observed that −457/LUC is activated by ATF4 in a dosagedependent manner, whereas the activation by ATF4 was greatly impaired by mutation at the ATF/CRE site (Fig. 3D).

Thus, these results show that ATF4 activates the Grp78 promoter through the ATF/CRE element.

**ATF4 Binds to the ATF/CRE Site Following Up-regulation by ER Stress**—To determine whether ATF4 regulates the Grp78 promoter directly or through induction of other factors, we examined whether ATF4 can bind to the ATF/CRE site on the Grp78 promoter. First, we prepared ATF4 by in vitro translation using rabbit reticulocyte lysate. A major protein band of the expected 47 kDa size was observed by [35S]methionine labeling (Fig. 4A, lane 1) and its identity as ATF4 was confirmed by Western blot using antibody directed against ATF4 in the presence of a mock control (Fig. 4A, lanes 2 and 3). However, in gel shift assays, in vitro translated ATF4 by itself did not bind the reassigned synthetic oligonucleotides spanning −210 to −171 containing the ATF/CRE site, as no new complex was observed besides the nonspecific band also present with probe alone in the presence of reaction buffer containing bovine serum albumin (Fig. 4D, lanes 1 and 2).

We next determined the ATF4 protein level in NIH3T3 cells following Tg treatment. In agreement with an earlier report (10), we detected a very low basal level of ATF4 in non-stressed cells, and after 4−8 h of Tg treatment, the ATF4 protein level was greatly elevated, as compared with the relatively even β-actin levels (Fig. 4B). The ATF4 level in the non-stressed HeLa nuclear extract was also very low and increased substantially upon Tg treatment (Fig. 4C). Gel shift assays using the control and Tg-treated HeLa nuclear extracts showed that while two major complexes (I and III) were present in both control and Tg-treated extracts (Fig. 4D, lanes 3 and 5), there was a third complex (II) present only in Tg-treated extract. Complex III has been previously shown by supershift assays to contain the DNA-binding protein Ku that has an affinity for DNA termini (28). While in vitro translated ATF4 by itself was unable to bind the probe (Fig. 4D, lane 2), when it was added to the control nuclear extract, a new complex was detected that exhibited identical electrophoretic mobility as the Tg stressinducible complex II in the Tg-treated nuclear extract lane (Fig. 4D, lane 4). Formation of the new complex II was further enhanced over the other complexes when the amount of the control nuclear extract was decreased to 0.2-fold and the amount of in vitro translated ATF4 was increased 2-fold (Fig. 4D, lane 6). Thus, in vitro translated ATF4 can bind to the ATF/CRE site of the Grp78 promoter in the presence of nuclear
indicated by representation of 3kb/LacZ, the two internal deletion mutants D170/LacZ and D300/LacZ, and the double mutant D170m. The deleted regions are

induction of ATF4 for each of the three reporter genes shown in

CNIH3T3 cells. Twenty-four h after transfection, the cells were harvested and assayed for

were either non-treated, or treated with 300 nM of Tg for 16 h. The cells were harvested and assayed for β-gal activity. The relative β-gal activities are plotted with

p

0.01, * indicates

p

< 0.05. C, the constructs 3kb/LacZ, D170/LacZ and D300/LacZ were co-transfected with either pCMVmyc or pMyc-ATF4 into NIH3T3 cells. Twenty-four h after transfection, the cells were harvested and assayed for β-gal activity. The relative β-gal activities are plotted with the activity of the 3 kb/LacZ transfected with pCMVmyc set as 1. The standard deviations are shown. The stars ** indicate

p

< 0.01. D, the fold induction of ATF4 for each of the three reporter genes shown in C is plotted with standard deviations.

extract from non-stressed cells. The new complex has identical electrophoretic mobility as the Tg-stress inducible complex observed with nuclear extract prepared from Tg-stressed cells. To further confirm that complex II binds specifically to the ATF/CRE site, gel shift assays using Tg-treated HeLa nuclear extract were performed in the presence of different competitors. An CRE-mutant competitor was generated by PCR using an upstream primer starting at −300 and a downstream primer at −29 of rat Grp78 promoter using −457/mut/LUC as a template. We also noted that a longer electrophoresis time separated complex II into a doublet band, referred to below as IIA and IIB. Using the unlabeled probe (−210/−171 of rat Grp78 promoter) as a competitor, as was expected, all three complexes (I, II, and III) were competed away in a dosage dependent manner (Fig. 4E, lanes 2 to 4). Complex III consisting of the abundant Ku protein was most resistant to the competition. In contrast, the CRE-mutant competitor spanning −300 to −29 was able to compete away complexes I and III but not the complex II doublet (Fig. 4E, lanes 5 and 6). These results indicate that complex II specifically binds to the CRE-site and no other site between −300 and −29 can bind ATF4. In addition, it shows that complexes I and III bind sequences outside the ATF/CRE site and therefore can be titrated away by the CRE-mutant competitor. Collectively, our results show that ATF4 is either absent or present in minimal amounts in non-stressed NIH3T3 cells; following ER stress, its level increases greatly and can bind to the Grp78 promoter at the ATF/CRE site in the presence of other nuclear factors to act as a transcription activator.

Identification of ATF1 and CREB1 as the Nuclear Co-factors of ATF4—ATF4 has been demonstrated to form heterodimers with a variety of bZiP proteins including Fos, Jun, and C/EBP (18, 36). To determine the components of the Tg-stress inducible complex II that binds specifically to the CRE site, antibodies against ATF4, Fos, and Jun were added to the Tg-nuclear extract prior to addition of the probe in gel shift assays. We also tested for the presence of ATF1 and CREB1, which are closely related members of the ATF/CREB family, using an antibody that can recognize both proteins. Confirming that ATF4 is a component of complex II, addition of increasing amounts of anti-ATF4 antibody correlated with greater suppression of complex II formation and inverse enhancement of complex III formation (Fig. 5A, lanes 3 and 4). Strikingly, addition of anti-ATF1/CREB1 antibody specifically supershifted complex II with no effect on complex I or III (Fig. 5A, lanes 5 and 6). In contrast, addition of anti-c-Fos or anti-c-Jun antibody showed no effect on any of the complexes and addition of bovine serum albumin further enhanced the formation of complex II (Fig. 5A, lanes 2, 7, and 8). Further, whereas the anti-ATF1/CREB1 antibody was highly effective in eliminating complex II, addition of either anti-C/EBP(C19) antibody which is specific for C/EBPβ, or anti-C/EBPΔ198 antibody, which can react with C/EBPβ, C/EBPα, C/EBPδ, and C/EBPε were without effect (Fig. 5B).

Complex II consists of a doublet with a faster migrating IIA and a slower migrating IIB band (Fig. 5, A and B). Addition of the anti-ATF4 antibody suppressed formation of both bands and addition of the anti-ATF1/CREB1 antibody supershifted both bands. Western blots with whole cell extracts confirmed that the anti-ATF1/CREB1 antibody can specifically recognize the faster migrating ATF1 (35 kDa) and the slower migrating CREB1 (43 kDa) (Fig. 5C). Thus, these results are consistent with band IIA representing an ATF4/ATF1 complex and band IIB representing an ATF4/CREB1 complex, and in agreement

Induction of Grp78/BiP by Translational Block

FIG. 2. ATF4 activates the Grp78 promoter through a cis-element located between −300 and −170 of the promoter. A, schematic representation of 3kb/LacZ, the two internal deletion mutants D170/LacZ and D300/LacZ, and the double mutant D170m. The deleted regions are indicated by dashed lines. B, NIH3T3 cells were transfected with either 3kb/LacZ, D170/LacZ, D170m/LacZ, or SV40/LacZ. The transfected cells were either non-treated, or treated with 300 nM of Tg for 16 h. The cells were harvested and assayed for β-gal activity. The fold induction of Tg is plotted with standard deviations. The stars ** indicate

p

< 0.01, * indicates

p

< 0.05.
with our earlier observation that recombinant CREB1 can bind the ATF/CRE site (28). Unlike ATF4, the level of ATF1/CREB1 remained relatively constant after Tg treatment (Fig. 5C). Further, using an antibody that recognizes the phosphorylated forms of both ATF1 and CREB1, we observed strong CREB1 phosphorylation following 4 h of Tg treatment while ATF1 phosphorylation was below detection limit (Fig. 5D, lanes 1–4).

As a positive control, both CREB1 and ATF1 were phosphorylated in UV-treated cells (Fig. 5D, lane 5).

To test whether ER-stress induced binding detected in vitro can be observed in vivo, chromatin immunoprecipitation (ChIP) assays were performed with NIH3T3 cells subjected to Tg or DTT treatment. The latter creates protein with disrupted disulfide bonds, leading to malfolded protein formation in the ER. Using the anti-ATF1/CREB1 antibody as the immunoprecipitating antibody and equal amounts of input DNA, we detected minimal ATF1/CREB1 binding to the Grp78 promoter in non-stressed cells; upon 2–4 h of Tg treatment, there was gradual increase in ATF1/CREB1 binding (Fig. 6A, lanes 1–7).

High level binding of ATF1/CREB1 to the Grp78 promoter was also detected after 4 h of dithiothreitol of treatment (Fig. 6A, lane 8). In contrast, core histone H3 binding to the same promoter region was similar before and after Tg treatment (Fig. 6A, lanes 9–11). Serial dilution of the PCR reactions confirmed that the band intensities shown in Fig. 6A were within the linear range of the PCR reactions (Fig. 6B).

**Fig. 3.** **ATF4 activates the Grp78 promoter through the ATF/CRE element.** A, schematic representation of the reporter construct −457/LUC and CRE-mut/LUC. The two constructs are identical with the exception of the mutated CRE site for the latter. B, the DNA sequence of the consensus ATF/CRE site, the CRE site in the rat Grp78 promoter as contained in −457/LUC, and the mutant CRE site in the CRE-mut/LUC reporter constructs are shown. C, NIH3T3 cells were transfected with 0.5 μg of β-actin/LacZ and 1 μg of −457/LUC or CRE-mut/LUC. Twenty-four hours after transfection the cells were non-treated or treated with Tg for 16 h. The cells were harvested and assayed for luciferase and β-gal activity. The luciferase activity in each sample was normalized to the β-gal activity and plotted with the non-treated −457/LUC activity set as 1. The standard deviations are shown. D, NIH3T3 cells were transfected with either 0.5 μg of −457/LUC or CRE-mut/LUC with increasing amounts (0, 0.5 and 1 μg) of pMyc-ATF4. The empty vector pCMVmyc was used to adjust to the same total amount of plasmid DNA in each transfection mixture. Twenty-four hours after transfection, the cells were harvested and the luciferase activity in each sample was measured. The relative luciferase activities are plotted with −457/LUC cotransfected with pCMVmyc set as 1. The standard deviations are shown.

**ER-stress Induced Complex Formation Between Endogenous ATF4 and ATF1**—Western blot analysis of whole cell lysates showed that ATF1 and CREB1 were constitutively expressed and their levels were relatively constant in control and Tg-treated cells (Fig. 5C). This is in contrast with the dramatic up-regulation of ATF4 by either Tg (Fig. 4B) or Tu treatment (Fig. 7A, lanes 5 and 6). Immunoprecipitation assays followed by immunoblot analysis showed in non-stressed cells, we did not detect ATF4 in complex with ATF1/CREB1 (Fig. 7A, lane 3). Upon Tu or Tg stress, ATF4 was readily detected in complex with ATF1/CREB1 (Fig. 7A, lanes 4 and 7). We note that the anti-ATF1/CREB1 antibody immunoprecipitated ATF1 more efficiently than CREB1 (Fig. 7A, lanes 3, 4, and 7), as compared with immunoblots of whole cell extracts using the same antibody (Fig. 7A, lanes 5, 6, and 8). Collectively, these results show that ER stress induces the complex formation of endogenous ATF4 with ATF1/CREB1 in vivo, thus providing a possible mechanism for ER-stress induced ATF4 binding to the Grp78 promoter through complex formation with ATF1 and CREB1.

**Dominant Negative Mutant of ATF4 Suppresses ER-stress Induction of Grp78 mRNA**—Since ATF4 belongs to a multiprotein family and can form heterodimers with partner proteins with compensatory function, we tested the requirement of ATF4 in ER-stress induction of endogenous Grp78 by overexpressing a dominant negative mutant ATF4ΔRK. This mutant protein has been previously shown to sequester ATF4 as well
as its binding partners into nonfunctional heteromeric complexes and effectively block their function as transcription activators (19, 33). As a control, we also overexpressed the wild-type ATF4 protein that will sequester the same binding partners into functional complexes. However, like the mutant type ATF4 protein that will sequester the same binding activators (19, 33). As a control, we also overexpressed the wild-type and mutant proteins will block expression.

We first determined through green fluorescence protein expression that over 90% of the cells were infected (data not shown). Immunoblot of whole cell extracts confirmed expression of ATF4 wild-type and mutant proteins in the infected cells (Fig. 7B). The infected cells were either non-treated or subjected to Tg and Tu treatment and RNA was extracted for determination of Grp78 mRNA level. Our results revealed a 30- and 40-fold induction of Grp78 mRNA by Tg and Tu, respectively, in vitro translated ATF4. The positions of complex I, II, and III are indicated. E. Tg-treated HeLa nuclear extract was used in the gel shift assays in the presence of different competitors. Lane 1, probe alone; lane 2, no competitor, lanes 3 and 4, 10× and 20×, respectively, of excess of unlabeled –210/–171 oligonucleotide; lanes 5 and 6, 10× and 20×, respectively, of excess of CRE-mutant competitor with the CRE site mutation as shown in Fig. 3B. Complex II A denotes the faster migrating band of the complex II doublet and IIB denotes the slower migrating band.

**Fig. 4.** ATF4 is up-regulated upon Tg stress and binds to the ATF/CRE-like element in the Grp78 promoter. A. in vitro translated ATF4 was detected by [35S]methionine labeling (lane 1) and by Western blot (lane 3), in vitro translation reaction without KS-ATF4 plasmid was included as mock control (lane 2). B. NIH3T3 cells were treated with Tg for the time (in hours) indicated on top and subjected to Western blot for ATF4 (upper panel). In vitro translated (IVT) ATF4 was included as a positive control. Immunoblotting the same membrane with β-actin antibody showed that the protein loading for each lane was even (lower panel). C. control (ctrl) and Tg-treated HeLa nuclear extracts (NE) were separated by 12% SDS-PAGE and subjected to Western blot with anti-ATF4 antibody to detect the level of ATF4. D. the synthetic oligonucleotides spanning –210 to –171 were labeled and used as probe in the gel shift assays. The addition of nuclear extracts or in vitro translated ATF4 to the reaction mixture are indicated on top. Lane 1, probe alone; lane 2, 5 μl of in vitro translated ATF4; lane 3, 10 μg of control HeLa nuclear extract; lane 4, 10 μg of control HeLa nuclear extract and 5 μl of in vitro translated ATF4; lane 5, 10 μg of Tg-treated HeLa nuclear extract; and lane 6, 2 μg of control HeLa nuclear extract and 10 μl of in vitro translated ATF4. The positions of complex I, II, and III are indicated. E. Tg-treated HeLa nuclear extract was used in the gel shift assays in the presence of different competitors. Lane 1, probe alone; lane 2, no competitor, lanes 3 and 4, 10× and 20×, respectively, of excess of unlabeled –210/–171 oligonucleotide; lanes 5 and 6, 10× and 20×, respectively, of excess of CRE-mutant competitor with the CRE site mutation as shown in Fig. 3B. Complex II A denotes the faster migrating band of the complex II doublet and IIB denotes the slower migrating band.
we confirm here as important cis-elements for Grp78 stress induction. However, these pathways cannot explain why transcription of Grp78 induced by Tu is substantially suppressed in cells with homozgyous mutation at an eIF2α phosphorylation site that resulted in blockage of PERK-mediated translation arrest in response to ER stress. We made several novel observations while resolving this puzzle.

Our discovery of the evolutionarily conserved ATF4 target site on the Grp78 promoter and the ability of exogenously expressed ATF4 to transactivate the Grp78 promoter provide evidence that ATF4 directly interacts with the Grp78 promoter and is a new activator for Grp78, which is a major target of the UPR. The ATF4 pathway is independent of ATF6 processing and does not require the ERSE on the UPR. The ATF4 pathway is independent of ATF6 processing and is a new activator for Grp78, which is a major target of the ATF/CRE family as indicated on top (lanes 3–8) with bovine serum albumin (2 μg) as a negative control (lane 2). Lanes 3 and 4, 1 and 2 μg of anti-ATF4 antibody was added, respectively; lanes 5 and 6, 1 and 2 μg of anti-ATF1/CREB1 antibody was added, respectively; lanes 6 and 7, 2 μg of anti-c-Fos or anti-c-Jun antibody was added respectively. The positions of complex I, IIA, IIB, and III are indicated. Addition of anti-ATF4 antibody resulted in partial suppression of complex II formation and addition of anti-ATF1/CREB antibody eliminated complex IIA and IIB and resulted in a supershifted complex. B, gel shift assays identical to A, lane 1, no addition; lane 2, bovine serum albumin, lanes 3–5, 2 μg of anti-ATF1/CREB1, anti-C/EBP(C19), and anti-C/EBP(Δ198) were added as indicated on top. C, NIH3T3 cells were treated with Tg for the time (in hours) as indicated on top and subjected to Western blot with anti-ATF1 antibody. The same membrane was stripped and blotted with anti-β-actin antibody to show the equal loading of each lane. The positions of ATF1, CREB1 and β-actin are indicated. D, Tg-induced phosphorylation of CREB1. NIH3T3 cells were treated with Tg for the time indicated on top (lanes 1–4) or with UV light for 5 min (lane 5). The cell lysates were subjected to Western blot to detect the phosphorylated form of CREB1 and ATF1 (upper panel) and the respective protein levels with β-actin as the loading control (lower panels).

Fig. 5. ATF1 and CREB1 are components of the ATF4 binding complex to the Grp78 promoter. A, gel mobility shift assays were performed using Tg-treated HeLa nuclear extract and the probe spanning −210 to −170 in the absence (lane 1) or presence of antibodies against ATF protein family as indicated on top (lanes 3–8) with bovine serum albumin (2 μg) as a negative control (lane 2). Lanes 3 and 4, 1 and 2 μg of anti-ATF4 antibody was added, respectively; lanes 5 and 6, 1 and 2 μg of anti-ATF1/CREB1 antibody was added, respectively; lanes 6 and 7, 2 μg of anti-c-Fos or anti-c-Jun antibody was added respectively. The positions of complex I, IIA, IIB, and III are indicated. Addition of anti-ATF4 antibody resulted in partial suppression of complex II formation and addition of anti-ATF1/CREB antibody eliminated complex IIA and IIB and resulted in a supershifted complex. B, gel shift assays identical to A, lane 1, no addition; lane 2, bovine serum albumin, lanes 3–5, 2 μg of anti-ATF1/CREB1, anti-C/EBP(C19), and anti-C/EBP(Δ198) were added as indicated on top. C, NIH3T3 cells were treated with Tg for the time (in hours) as indicated on top and subjected to Western blot with anti-ATF1 antibody. The same membrane was stripped and blotted with anti-β-actin antibody to show the equal loading of each lane. The positions of ATF1, CREB1 and β-actin are indicated. D, Tg-induced phosphorylation of CREB1. NIH3T3 cells were treated with Tg for the time indicated on top (lanes 1–4) or with UV light for 5 min (lane 5). The cell lysates were subjected to Western blot to detect the phosphorylated form of CREB1 and ATF1 (upper panel) and the respective protein levels with β-actin as the loading control (lower panels).
ATF/CRE site and contribute toward Grp78 promoter activation. Thus, when the ATF/CRE site is mutated, ATF4 binding is lost, resulting in a drop of ER stress inducibility of the Grp78 promoter. Nonetheless, Grp78 promoter can still be ER-stress induced through the ERSEs utilizing ATF6, YY1, NF-Y, TFII-I and possibly XBP-1. This scenario is not unique to the Grp78 promoter since mutation of the composite C/EBP-ATF site of the chop promoter also suppresses both the basal and ER stress induced promoter activity (10). Both observations could be attributed to the ATF site being involved in the control of basal expression activity through binding of basal transcription factors while ER stress replaces or augments these factors with ATF4 and its nuclear co-factors. Further, our finding that in vitro-translated ATF4 by itself is unable to bind to the Grp78 promoter and that binding requires specific components from the nuclear extracts such as co-factors or post-translational modifiers provides a plausible explanation why ATF4 increase under specific stress conditions may be sufficient to induce other promoters but not Grp78 (19).

In search of ATF4 interactive partners relevant to the ER stress response, we discovered that ER stress induces complex formation between endogenous ATF1/CREB1 and ATF4, correlating with the appearance of novel ATF4 complex binding to the ATF/CRE site of the Grp78 promoter in gel shift assays. Using such assays, we were able to resolve two ATF4 containing complexes in Tg-treated nuclear extracts. According to the relative molecular size of ATF1 and CREB1, our results are consistent with the faster migrating complex containing ATF4 and ATF1, and the slower complex containing ATF4 and CREB1. Further, we have previously observed that recombinant CREB1 can bind the ATF/CRE site of the Grp78 promoter (28). While we were able to detect in vivo binding of ATF1/CREB1 to the Grp78 promoter, attempts to immunoprecipitate ATF4 using anti-ATF4 antibody were unsuccessful in both ChIP and immunoprecipitation assays (data not shown). This could be due to either a weak antibody titer or the unavailability of the ATF4 epitope in the complex.

In contrast to ATF4, which undergoes dramatic increase in protein level after ER stress, the level of both ATF1 and CREB1 are relatively constant. While ATF1 is not phosphorylated in NIH3T3 cells subjected to 300 nM Tg treatment, Tg induces strong phosphorylation of CREB with kinetics parallel to Grp78 induction (34). This new result provides the physiological relevance for our previous observation that the Grp78 promoter can transactivated by CREB that has been phosphorylated and therefore activated (28). Interestingly, in human melanoma cells a higher dose of Tg (1 μM) can cause transient phosphorylation of both CREB1 and ATF1 within 30 min of the drug treatment (43), and ATF1 is phosphorylated in response to EGF stimulation (44). Thus, in different cell types and under different conditions, post-translational modification of ATF1/CREB1 such as stress-induced phosphorylation can play an important roles in modifying the ability as transcription co-factors. Future studies are required to dissect whether such modifications are required for Grp78 activation.

Since ATF1 and CREB1 can compensate for each other functionally (45), it is likely that both ATF1 and CREB1 can both act as co-factor for ATF4. Likewise, it is also possible that other members of the ATF4 protein family can substitute for its function. To circumvent the complications due to functional redundancy, we utilized adenoviral vector to overexpress a dominant negative mutant of ATF4 that can sequester ATF4 and its binding partners. This mutant has been used previously to inhibit basal and cadmium-dependent activation of the mouse heme oxygenase-1 gene distal enhancer by blocking the formation of functional ATF4/Nrf-2 complex and to inhibit the activation of the human asparagine synthetase proximal promoter by blocking the formation of functional ATF4/C/EBP complex (19, 33). Using the asparagine synthetase system, we confirmed that the wild-type and the mutant ATF4 proteins expressed by the adenoviral vectors functioned as expected (data not shown). In the infected cells, we observed that only overexpression of the dominant negative mutant ATF4 but not the wild-type protein resulted in suppression of ER-stress induced Grp78 mRNA. This provides direct evidence that when ATF4 function is suppressed and its binding partners are not able to compensate for its function, Grp78 induction by ER stress is suppressed. Similarly, the dominant negative ATF4 mutant but not the wild-type protein was able to suppress endogenous Grp78 mRNA induction by homocysteine, glutamine-starvation and anoxia in retinal pigmented epithelial cells. Thus, the contribution of ATF4 to Grp78 induction applies to multiple forms of stress. While this manuscript is in

\[\text{2} \quad \text{C. N. Roybal, S. Yang, D. Hurtado, D. L. Vander Jagt, and S. F. Abcouwer, manuscript in preparation.}\]
preparation, it was reported that in MEF derived from ATF4/H11002 mouse, Grp78 induction by Tg treatment was largely intact (46). As discussed above, ATF4 belongs to a multiprotein family with functional redundancy. Thus, one possibility is that the ATF4 dominant negative mutant was able to block the functions of the compensating factors and that is why the repressive effect could be detected. Another possibility for this is that in the ATF4/H11002 MEF, there are some other ATF4 compensating factor(s) for the Grp78 stress induction, and these factor(s) are no longer available in stable cell lines like NIH3T3 or retinal cells. Since the unfolded protein response as exemplified by Grp78 induction is of central importance for maintenance of cellular homeostasis, it is not surprising that the cells have evolved multiple compensatory pathways to ensure stress induction of Grp78.

ATF1/CREB1 and its related protein family members are known to mediate transcriptional responses to various extracellular signals, including that of calcium efflux from the intracellular stores such as the ER and is important for cell survival (45). GRP78 exhibits anti-apoptotic properties and its up-regulation has also been reported in a variety of human cancers, correlating with tumorigenicity and malignancy (6). Specific overexpression of GRP78 can confer drug resistance in cancer cells through inhibition of caspase activation (47). Thus, GRP78 as a potential novel target gene for ATF1/CREB1 can mediate its survival effect and contribute to cancer growth and resistance to therapy. In support of the CRE regulatory site in Grp78 expression, preliminary data observed with Grp transgenic mouse models suggested that the in vivo Grp78 transcription may not be entirely dependent on the ERSE (48, and data not shown). Further, mouse models with disrupted PERK and ATF4 function results in increased tissue-specific apopto-

**Fig. 7.** ER stress-induced formation of endogenous ATF4 and ATF1/CREB1 complex and functional contribution of ATF4. A, endogenous ATF1/CREB1 and ATF4 associate with each other following ER stress. NIH3T3 cells were either non-treated or treated with Tu or Tg for 6 h. The cell lysates were subjected to immunoprecipitation with normal mouse IgG (lanes 1 and 2) or antibody against ATF1/CREB1 (lanes 3, 4, and 7). Whole cell extract (WCE) from the non-treated (−) or treated cells were loaded as a positive control for the Western blot (lanes 5, 6, and 8). The immunoprecipitated complexes were resolved by SDS-PAGE and subjected to Western blot against ATF4 (upper panel) or ATF1/CREB1 (lower panel). The positions of ATF1, CREB1, and β-actin are indicated. B, cell lysates prepared from NIH3T3 cells which were infected with empty vector (v) or infected with adenoviral vector expressing the dominant negative form (ATF4RK) (lane 2) or wild-type ATF4 (wt) (lane 3) were subjected to immunoblot with anti-ATF4 and anti-β-actin antibodies. C, RNA blot analysis of Grp78 mRNA levels in mock-infected cells (lanes 1–3), cells infected with adenoviral vector expressing wild-type ATF4 (Wt) (lanes 4–6) or the dominant negative form (ATF4RK) (lanes 7–9). The cells were either non-treated (−), or treated with Tu or Tg as indicated on top. The level of GAPDH mRNA was also determined and used as loading control for the RNA samples. The RNA band intensities were quantitated by phosphorimaging, normalized against the GAPDH levels and plotted below. The level of Grp78 mRNA in the non-treated, empty vector-infected cells was set as 1.
sis (49, 50). This raises the question of what are the relevant critical physiologic targets mediating this effect. Our studies suggest that in addition to general translational arrest, upon PERK activation the transcription induction of anti-apoptotic proteins such as GRP78 by ATF4 may in part contribute to the survival response in specific tissues. In conclusion, mammalian cells have evolved highly versatile mechanisms to respond to ER stress and ATF4 represents a novel pathway linking PERK/eIF2α to transcriptional activation of a major chaperone protein in the ER that may contribute to cell survival.

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