Regulation of ERGIC-53 Gene Transcription in Response to Endoplasmic Reticulum Stress*\(5\)

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Accumulation of unfolded proteins within the endoplasmic reticulum (ER) activates the unfolded protein response, also known as the ER stress response. We previously demonstrated that ER stress induces transcription of the ER Golgi intermediate compartment protein ERGIC-53. To investigate the molecular events that regulate unfolded protein response-mediated induction of the gene, we have analyzed the transcriptional regulation of ERGIC-53. We found that the ERGIC-53 promoter contains a single cis-acting element that mediates induction of the gene by thapsigargin and other ER stress-causing agents. This ER stress response element proved to retain a novel structure and to be highly conserved in mammalian ERGIC-53 genes. The ER stress response element identified contains a 5'-CCAATGGCCATC-3' that is equally important for ER stress-mediated induction of the gene. This sequence is the binding site for endogenous YY1 at the 5'-CCAATGGCCATC-3' and is also bound to the ER stress response element of the translational initiation factor eIF2α, and intensifies the proteasomal degradation pathway (4, 5). In mammalian cells, UPR stimulates the expression of proteins ERGIC-53, MCFD2, and VIP36, which exert functions in the post-ER compartments of the secretory pathway (6–8). ERGIC-53 is a highly conserved calcium-dependent lectin that shares structural and functional homology with VIP36 (9). ERGIC-53 is mainly localized within the Golgi; it cycles continuously between the ER and the Golgi complex and exports a defined number of glycoproteins from the ER (9–16). The adaptor protein MCFD2 forms a complex with ERGIC-53 to transport newly synthesized clotting factors V and VIII to the Golgi complex (17–19). ERGIC-53 and MCFD2 accumulate in response to distinct signaling pathways of the cell stress response. In particular, heat shock stimulates preferential transcription of ERGIC-53 and MCFD2 mRNAs (7), whereas UPR induced by either thapsigargin or nitric oxide activates the transcription of both genes (7, 8).

Transcriptional activation by the UPR requires the presence of cis-acting ER stress response elements (ERSE) on the promoter region of target genes. Three types of ERSE have been identified: ERSE-I and ERSE-II (20–22, 23) and the mammalian unfolded protein response element (UPRE) (23–25). Multiple copies of the ERSE-I control the UPR-mediated induction of the ER-resident proteins Grp78/Bip, Grp94, calreticulin, and protein-disulfide isomersases (20, 21). ERSE-I contains a CCAAT site at its 5'-end for the constitutive transcription factor NFY/CBF (20, 26, 27), a 9-bp spacer containing the CGG triplet that is the TFII-I binding site (28), and a CCACG motif at the 3'-end that is required for ATF6α (activating transcription factor 6α) recruitment (20, 26, 28–31). ATF6α is an ER transmembrane protein that, during ER stress, can be recruited by transport vesicles destined for the Golgi complex, where, consequent to intramembrane proteolysis, a 50-kDa cytosolic form is generated that activates transcription of UPR genes (26–32). A single copy of the ERSE-II type regulates the ER stress response of the Herp gene, which encodes an ER integral membrane protein that is involved in the ER-associated degradation pathway (22, 33, 34). ERSE-II (5'-ATTGNNCCAC(G/A)-3') retains a reversed NFY/CBF binding site at its 5'-end and a flanking ATF6α site (22). The UPRE, which controls the expression of a subset of ER-resident chaperones (35, 36), contains the ATF6α binding site on its complementary strand and is also the preferred binding site for the transcription factor XBP1 (X-box-binding protein 1). XBP1 is under the control of
The ERGIC-53 ERSE

the ER membrane nuclease IRE1α (inositol-requiring enzyme 1α), which in response to protein misfolding triggers the processing of XBP1 mRNA, thereby leading to the synthesis of the transcription factor (35, 37, 38). Interestingly, XBP1 binds both the UPRE and ERSE sequences of target genes (23, 37) independently of NFY/CBF, whereas ATF6α binds UPRE albeit with a lower affinity compared with ERSE (23, 37).

We have studied the regulation of the ERGIC-53 promoter in an attempt to shed light on the transcriptional mechanisms that control ERGIC-53 expression. The transcriptional regulation of ERGIC-53 by the UPR requires a single cis-acting element (ERGIC-53 ERSE), which is highly conserved in mammals and is different from the other types of ERSE identified.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The following antibodies were used in EMSA and ChIP assays: mouse monoclonal anti-ATF6α (Active Motif), mouse monoclonal anti-YY1 and anti-HA epitope, and rabbit polyclonal anti-NFY-CBF/A, anti-XBP1, anti-Sp1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Cell Culture and Induction of ER Stress**—HeLa cells, mouse embryonic fibroblasts (MEFs) derived from wild type or IRE1α knock-out mice (38), were grown at 37 °C in a humidified 5% CO2, 95% atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 4.5 g/liter glucose, and 2 mM glutamine. To induce ER stress, we incubated actively growing cells for 8 h (unless otherwise indicated) with 300 nM thapsigargin (TG), 2 mM DTT, or 10 μM MG132 (Sigma) before harvesting.

**RNA Extraction and Real Time RT-PCR Analyses**—Serial dilutions of total RNA obtained from HeLa, wild type, and IRE1α−/− MEF cells (RNaseasy kit; Qiagen) were reverse transcribed (Invitrogen) and subjected to real time RT-PCR using the iCycler Apparatus (Bio-Rad) and analyzed by the SybrGreen method. The following primers were used to amplify the corresponding transcripts in human cells: ERGIC-53-forward, 5′-GGG CAG CAT GGG CAG ATT AC-3′; ERGIC-53-reverse, 5′-CAT AGA GGT GAA GGT CGG AGT C-3′; GAPDH-forward, 5′-GAA GGT GAA GGT CGG AGT C-3′; GAPDH-reverse, 5′-GAA GGT GAT GGT GAT GGG ATT TC-3′. The following primers were designed to amplify the corresponding transcripts in murine cells: ERGIC-53-forward, 5′-GGGA CAG CCT GGG CAG CTC TC-3′; ERGIC-53-reverse, 5′-GGG TGC TGG ATG CCA CTC A-3′; GAPDH-forward, 5′-GGG TGC TGG ATG CCA CTC A-3′; c-ABL-forward, 5′-GGG TGC TGG ATG CCA CTC A-3′; c-ABL-reverse, 5′-CAC CTT TCG GTC TCA CAA CT-3′. The following primers were used to amplify the Grp94 transcripts from both human and murine cells: Grp94-forward, 5′-TCC GGC CTT CTT GTA GCA CAG A-3′; Grp94-reverse, 5′-TGT TGC CTT CTT GTA GCA CAG A-3′. Expression level was calculated according to the 2ΔΔCT method (39) by using either GAPDH or c-ABL mRNA as a control gene.

**Construction of Plasmids**—A 1084-bp fragment spanning from nt −838 to −16 was PCR-amplified with synthetic oligonucleotides having Kpn1-Xhol flanking restriction sites and subcloned in the pGL3Basic vector (Promega) to drive the expression of firefly luciferase reporter gene. Similarly, the ERGIC-53 promoter region (nt −1000 to −1) was ligated to the Kpn1-HindIII site of the pGL3Basic vector (Promega) upstream from the luciferase reporter gene. Progressive deletion of the 5′-flanking region of the ERGIC-53 promoter was obtained by PCR amplification using forward primers containing the 5′-Kpn1 site and common reverse primers with the 3′-HindIII site. The −149 to −1 and −149 to −63 fragments were obtained by PCR, digested, and inserted in the Kpn1-BglII site of the pGL3 promoter vector (Promega), which contains the SV40 minimal promoter upstream from the luciferase coding sequence (SV40Luc). Synthetic oligonucleotides corresponding to the ERSE-1 consensus sequence, the −32 to −1 and the −65 to −31 region of the ERGIC-53 ERSE, and cassette and point mutations of ERGIC-53 ERSE were synthesized, in vitro annealed, and inserted in the Kpn1-BglII site of the pGL3 promoter vector and controlled by sequencing. The pCGN-ATF6-(1–373) and pCGN-ATF6-(1–273) expression vectors (31, 41) were kindly provided by A. S. Lee.

**Transfection Experiments for Transient Expression of Reporter Plasmids**—Actively growing HeLa and MEF cells were seeded onto 60-mm plates at −20 to −30% of confluence and fragments were gel-purified and cloned by using the pGEM-T Easy Vector system (Promega). The Bip/Gpr78 promoter region spanning from nt −92 to −16 was PCR-amplified with synthetic oligonucleotides having Kpn1-Xhol flanking restriction sites and subcloned in the pGL3Basic vector (Promega) to drive the expression of firefly luciferase reporter gene. Similarly, the ERGIC-53 promoter region (nt −1000 to −1) was ligated to the Kpn1-HindIII site of the pGL3Basic vector (Promega) upstream from the luciferase reporter gene. Progressive deletion of the 5′-flanking region of the ERGIC-53 promoter was obtained by PCR amplification using forward primers containing the 5′-Kpn1 site and common reverse primers with the 3′-HindIII site. The −149 to −1 and −149 to −63 fragments were obtained by PCR, digested, and inserted in the Kpn1-BglII site of the pGL3 promoter vector (Promega), which contains the SV40 minimal promoter upstream from the luciferase coding sequence (SV40Luc). Synthetic oligonucleotides corresponding to the ERSE-1 consensus sequence, the −32 to −1 and the −65 to −31 region of the ERGIC-53 ERSE, and cassette and point mutations of ERGIC-53 ERSE were synthesized, in vitro annealed, and inserted in the Kpn1-BglII site of the pGL3 promoter vector and controlled by sequencing. The pCGN-ATF6-(1–373) and pCGN-ATF6-(1–273) expression vectors (31, 41) were kindly provided by A. S. Lee. The pCGN-ATF6-(1–373)m1 was already described (25).
transfected with 1 μg of luciferase reporter plasmids and 0.5 μg of the RSV-β-Gal reporter control plasmid (Promega) by using the FuGene Transfection Reagent (Roche Applied Science). Cells were incubated with the Fugene-DNA complex for 16 h at 37 °C, washed with cold phosphate-buffered saline, and lysed in reporter lysis buffer (Promega) 48 h after transfection. To
measure LacZ reporter gene activity (β-galactosidase), we incubated cell extracts for 1 h at 37 °C in β-galactosidase assay buffer (200 mM sodium phosphate buffer, pH 7.3, 2 mM MgCl₂, 100 mM β-mercaptoethanol, 1.33 mg/ml o-nitrophenyl-β-D-galactopyranoside). The reaction was blocked by adding 1 M sodium carbonate, and absorbance was measured at 420 nm.

We measured luciferase activity with a Berthold luminometer in 40 μl of cell lysate supplemented with 100 μl of luciferase assay reagent (Promega). “Relative luciferase activity” is defined as the luciferase-to-β-galactosidase activity ratio and normalized for the protein concentration of each sample. “-Fold induction” is defined as the ratio between the enzyme activities of treated cells with respect to that of untreated cells. In all experiments, values are reported as the average and S.D. of at least three independent experiments carried out in duplicate. Statistical analysis was performed using Student’s t test (n = 6–8).

Chromatin Immunoprecipitation Assays—1 × 10⁶ HeLa cells were treated or not for 2–4 h with 300 nM TG for the UPR induction. For the ChIP analysis of overexpressed ATF6α, HeLa cells were transfected with 6 μg of the pCGN-ATF6-(1–373) and pCGN-ATF6-(1–273) expression vectors (31, 41) and harvested 24 h after transfection. Experiments were performed according to the instructions of the manufacturer (Upstate Biotechnology, Inc.). Briefly, cells were exposed to 1% formaldehyde for 10 min at 37 °C to obtain protein-DNA cross-linking. The nuclear fraction was sonicated to obtain chromatin fragments of 200–1000 bp; an aliquot (5% of the total volume) was removed from each sample and used as the input fraction. Chromatin was precleared by preincubation with a DNA salmon sperm/protein A-agarose 50% slurry (Upstate Biotechnology) for 1 h at 4 °C. The agarose was centrifuged, and the precleared chromatin supernatant was then incubated with the indicated antibodies overnight at 4 °C. The protein-DNA-antibody complexes were collected by the addition of the salmon sperm DNA-protein A-agarose (2 h at 4 °C) and washed, and protein-DNA cross-linking was reversed (4 h at 65 °C). DNA was purified by phenol/chloroform extraction and ethanol precipitation, and aliquots (25%) of the purified materials underwent PCR (5 min at 94 °C; 1 min at 94 °C, 1 min at 52 °C, 1 min at 72 °C for 40 cycles; 5 min at 72 °C). The following primers were used in the PCR: 5'-TGAACCAATGGGACCAGC-3' and 5'-CTCACCGTGCCCTACTCG-3' to amplify a 254-bp fragment of the human Bip/Grp78 promoter spanning from nt -267 to nt -13; 5'-AAGCGAAGGTTGGAGTCC-3' and 5'-CGCCATCTTGGATTCTCC-3' to amplify a 271-bp fragment of the LMAN1/ERGIC-53 promoter extending from nt -226 to nt +45.

Preparation of Nuclear Extracts and Electrophoresis Mobility Shift Assays—5–6 × 10⁶ HeLa cells were either treated for 2 h with 300 nM TG to induce ER stress or transiently transfected
with 6 μg of the pCGN-ATF6-(1–373) expression vector and harvested 48 h after transfection. Cells were washed with cold phosphate-buffered saline and harvested by scraping. The cell pellet was resuspended in extraction buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, passed through a needle, kept on ice for 45 min, and centrifuged (15 min at 14,000 rpm at 4 °C). The nuclear extract supernatant was obtained by centrifugation (30 min at 14,000 rpm at 4 °C), protein concentration was determined, and 5-μg aliquots were stored at −80 °C until used. Double-stranded synthetic oligonucleotides were radiolabeled using [γ-32P]ATP (3000 Ci mmol⁻¹; Amersham Biosciences) and T4 polynucleotide kinase (Fermentas). The binding reaction was carried out for 20 min at room temperature with 5 μg of nuclear proteins in 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, pH 8.0, 0.5 mM DTT, 50 mM Tris-HCl, pH 7.5, 0.250 μg/μl poly(dI-dC) containing 30,000 cpm of radiolabeled probe and a 50–100-fold molar excess of unlabeled competitor oligonucleotide when indicated. For supershift experiments, 4 μg of specific anti-NFY/CBF-A and 10 μg of either anti-YY1- or anti-HA-specific antibody was added to the binding reaction and incubated for 30 min before the addition of the radiolabeled probe. DNA-protein complexes were separated by 5% nondeaturing polyacrylamide gel and revealed by PhosphorImager analysis (Typhoon 8600; Amersham Biosciences).

RESULTS

Isolation of the ERGIC-53 Minimal Inducible Promoter—To identify ERSE elements in the ERGIC-53 promoter, we looked for transcription factor binding sites (TESS analysis) on about 100 bp of the 5′-region of the ERGIC-53 gene. Three

### TABLE 1

| Species            | Sequence | Position |
|--------------------|----------|----------|
| Homo sapiens       | CCAATGAGGAGCCCTGTTGGCCATCCACG-3′ | −62 to −32 |
| Pan troglodytes    | CCAATGAGGAGCCCTGTTGGCCATCCACG-3′ | −79 to −49 |
| Mus musculus       | CCAATGAGGAGCCCTGTTGGCCATCCACG-3′ | −68 to −38 |
| Rattus norvegicus  | CCAATGAGGAGCCCTGTTGGCCATCCACG-3′ | −61 to −31 |
| Canis familiaris   | CCAATGAGGAGCCCTGTTGGCCATCCACG-3′ | −62 to −42 |

**Conservation of the ERSE ERGIC-53 element**

The DNA sequence of the human ERGIC-53 ERSE (NT_025028) was used as a probe in BLAST analysis. The table shows the similar ERGIC-53/LMAN1 orthologue promoter sequences identified in monkeys (NW_119156.1), mice (NT_039674), rats (NW_047514), and dogs (NC_006583.2) as reported in the NCBI genomic data base of ERSE with respect to the transcription start site. No significant similarity has been found in Caeonrhoditis elegans, Drosophila melanogaster, or Gallus gallus.

**FIGURE 4.** Mutational analysis of the ERGIC-53 ERSE. A, effect of clusters of nucleotide substitutions on ERGIC-53 ERSE activity. Synthetic oligonucleotides corresponding to the ERGIC-53 ERSE or to ERGIC-53 ERSE mutants (mut 1–5) were inserted in the PGL3 promoter vector, and the basal and thapsigargin-dependent luciferase activities (n = 6–8; *p < 0.001) were measured as described in the legend to Fig. 2. Synthetic oligonucleotides corresponding to the consensus ERSE-I or to its inactive form served as control. Mutated nucleotide sequences are underlined. Numbers on the right are the relative positions of the 5′-CCAAT motifs in boldface type, and nonidentical nucleotides are underlined. Mutated fragments were inserted in the PGL3 promoter vector, and the corresponding reporter activity was assayed as reported in the legend to Fig. 2. The activity of a double substitution (mut 18) is also shown. The graphs represent the average ± S.D. of three independent experiments. Statistical analysis was performed using Student’s t test (n = 6; *p < 0.001; **p < 0.025; #p < 0.01, respectively).

The DNA sequence of the human ERGIC-53 ERSE (NT_025028) was used as a probe in BLAST analysis. The table shows the similar ERGIC-53/LMAN1 orthologue promoter sequences identified in monkeys (NW_119156.1), mice (NT_039674), rats (NW_047514), and dogs (NC_006583.2) as reported in the NCBI genomic data base of ERSE with respect to the transcription start site. No significant similarity has been found in Caeonrhoditis elegans, Drosophila melanogaster, or Gallus gallus.
5'-CCAAT motifs scored high as binding sites for the transcription factor NFY/CBF. This result suggested the presence of three putative ERSEs located at positions 334 to 316, 277 to 259, and 62 to 44 (Fig. 1A). Alignment of the three ERGIC-53 ERSE-like sequences with ERSE of human genes responsive to ER stress (Fig. 1B) revealed differences in the downstream region (excluding the 5'-CCAAT box). In addition, five sites had a high affinity for transcription factors of the SP family of proteins (Fig. 1A), and the ERGIC-53 promoter lacked known transcription initiation sequences (Fig. 1A).

Thus, we attempted to isolate the ERGIC-53 minimal inducible promoter by measuring the activity of the firefly luciferase reporter gene driven by deletion mutants lacking the three ERSE-like sequences we had identified (Fig. 2A). Expression vectors were transfected in HeLa cells treated or not with TG. The 1036-bp region containing all three putative ERSE enhanced luciferase expression 3.61 ± 0.98-fold in TG-treated cells (Fig. 2A, right). Deletions of the −335 to −317 and −278 to −259 ERSE-like sequences reduced the basal activity in untreated cells, but the fold induction was retained in the −149 to −1 region (Fig. 2A, right) that contains the −62 to −35 ERSE-like motif (Fig. 1A). With the three intact ERSE-Is, TG caused a 6.2 ± 1.12-fold increase in the expression of the control Grp78/BiP promoter. Thus, the minimal inducible region was retained in the −149 to −1 region. This region could initiate transcription in response to various ER stress inducers (Fig. 2B), giving a fold induction of the reporter gene of 4.2 ± 0.49 with TG, 3.24 ± 0.62 with the reducing agent DTT, and 2.14 ± 0.38 with proteasome inhibitor MG132. In contrast, the undeleted Grp78/BiP promoter enhanced luciferase activity in response to TG and DTT by 6.79 ± 0.72- and 6.72 ± 0.78-fold, respectively and by 4.98 ± 0.52-fold in response to MG132. Finally, the activity of the −149 to −1 ERGIC-53 promoter was consistent with the -fold ERGIC-53 mRNA induction by TG as measured by real time RT-PCR analysis (Fig. 2C), indicating that the −149 to −1 region (Fig. 2A, right) that contains a single ERSE-like is sufficient to ensure higher ERGIC-53 mRNA levels in response to TG treatment.

**UPR Regulates ERGIC-53 Transcription by Means of a Single ERSE**—To identify the ERSE within the −149 to −1 region, we first compared the luciferase activity of this region with that of the −149 to −63 deletion mutant that lacks the putative ERSE (Fig. 3A). Deletion mutants were placed upstream from the SV40Luc vector to drive the expression of the luciferase reporter gene. As expected, reporter assays confirmed that the −149 to −1 region conferred thapsigargin-mediated activation of the SV40Luc vector and enhanced the activity.
of several mammalian ERGIC-53 genes as shown by BLAST analysis (42) carried out on the NCBI genomic data base (available on the World Wide Web). This suggests that ERGIC-53 could be similarly regulated by ER stress in different species (Table 1).

**Mutational Analysis Reveals the Novel Structure of the ERGIC-53 ERSE**—To define the sequence required for the ER stress response of the ERGIC-53 ERSE (Fig. 3C), we inserted oligonucleotides bearing the substitutions shown in Fig. 4A in the SV40Luc reporter plasmid and determined their capacity to confer the ER stress response to the minimal SV40 promoter. We also performed these experiments using reporter vectors bearing a single copy of the consensus or a mutated ERSE-I (20, 21). As expected, mutation of the entire 5′-CCAAT box (mut 1) impaired ERSE activity. Interestingly, nucleotide replacement of the −48 to −35 segment (mut 2–4) inhibited the response to TG. Inhibition was greater when we replaced the segment between nucleotides −44 and −35 (mut 2 and 4), which indicates that this sequence is crucial for TG-dependent activation of ERGIC-53. Mutation in the segment spanning nucleotides −55 to −49 (mut 5) did not result in a similar reduction (Fig. 4A). To verify these findings, we introduced single point mutations into each nucleotide of the −44 to −35 sequence (Fig. 4B). Replacement of any of the nucleotides within this sequence impaired -fold induction, albeit to different degrees (Fig. 4B, mut 7–16). In particular, the −44 G-to-C transition (mut 7) resulted in a -fold induction decrease comparable with that of the −48 to −44 cassette mutant (mut 3), whereas the −35 C-to-T transversion (mut 16) almost completely abolished induction. Replacement of the outer nucleotides (mut 6 and 17) had little effect on the ER stress-dependent activity of the ERGIC-53 ERSE.

Therefore, the sequence of the ERGIC-53 ERSE crucial for UPR-mediated gene induction is constituted by a leader 5′-CCAT-3′ sequence followed by a novel −48 to −35 sequence (5′-CCCTGTTGGCCATC-3′), both of which are required to confer full inducibility in response to ER stress. The ERGIC-53 ERSE is a general sensor of ER stress. The induction of the ERGIC-53 ERSE reporter gene transected in HeLa cells was 2.61 ± 0.32-fold with TG, 2.42 ± 0.35 with DTT, and 1.88 ± 0.12 with MG132 (Fig. 5). These values were comparable...
with those obtained with the ERGIC-53 deletion mutant −149 to −1 despite the different reporter vector (Fig. 2B). mut 4 did not respond to any of the ER stress inducers, thus confirming the regulatory role of the 3′ region (5′-CCATC-3′) of the ERGIC-53 ERSE.

NFY/CFB and ATF6α-Y1Y1 Interact in Vivo with the ERGIC-53 Promoter—We next asked whether transcription factors involved in the ER stress response could interact with the ERGIC-53 promoter. Accordingly, we examined DNA-protein complexes from untreated and TG-stressed cells using chromatin immunoprecipitation. The presence of endogenous factors NFY, Sp1, ATF6α, YY1, or XBPI in the immunoprecipitates was revealed by PCR amplification of a 271-bp fragment of the ERGIC-53 promoter extending from nucleotide −226 to nucleotide +45, containing a single ERGIC-53 ERSE (Fig. 6A). As a control, we examined the 254-bp fragment of the human Bip/Grp78 promoter spanning from nucleotide −267 to nucleotide −13, which harbors the three ERSEs (Fig. 6A). Untreated cells retained endogenous NFY, YY1, and Sp1 bound on ERGIC-53, and NFY binding was more evident in untreated cells on the control Bip/Grp78 promoter, which contains multiple copies of ERSE (Fig. 6B). Consequent to TG-induced ER stress, and on both promoters, NFY binding was more pronounced, whereas Sp1 and YY1 binding was slightly higher than in uninduced cells. In TG-stressed cells, there was endogenous ATF6α binding on the ERGIC-53 promoter and on the control gene Bip/Grp78. In contrast, induction of ER stress raises the interaction of endogenous XBPI with Bip/Grp78 but not with the proximal region of the ERGIC-53 gene (Fig. 6B, bottom). To verify ATF6α binding to the ERGIC-53 promoter, we examined DNA-protein complexes from HeLa cells transiently transfected with plasmid vectors expressing two distinct HA-tagged ATF6α nuclear forms (Fig. 6C): the active HA-tagged form ATF6(373) and the deletion mutant ATF6(273) that lacked the B-ZIP domain (31). Chromatin immunoprecipitation with anti-HA antibody revealed binding of the ATF6(373) form to the ERGIC-53 promoter and to the control gene Bip/Grp78 (Fig. 6C). Instead, immunoprecipitation with anti-HA antibody did not reveal binding of the HA-tagged ATF6(273)-deleted form, which confirms recruitment of active ATF6α at the ERGIC-53 promoter (Fig. 6C).

ATF6α but Not XBPI Stimulates ERGIC-53 ERSE Activity—In transiently transfected HeLa cells, the overexpression of the HA-tagged ATF6α nuclear form was able to stimulate, in the absence of ER stress induction, the activity of the ERGIC-53 minimal promoter (−149 to −1) by 5.97 ± 0.59-fold and the control plasmid Grp78/Bip-driven reporter gene by 15.28 ± 1.35-fold (Fig. 7A). To define the sequence required for the ATF6α-dependent transactivation, we compared the effect of the HA-tagged ATF6(373) expression on the wild-type and mutant (mut 1–5) ERGIC-53 ERSE. HeLa cells were co-transfected with the pGL3 promoter vector bearing the indicated oligonucleotide sequences and 0.5 μg of the HA-ATF6(373) expression vector. The consensus ERSE-I and its inactive form served as control. Mutated nucleotide sequences are underlined. The histograms represent the fold-induction calculated as the ratio between the luciferase activity of ATF6(373) and mock-transfected cells. Values are the average ± S.D. of three independent experiments performed in duplicate. Statistical analysis was performed using Student’s t test (n = 6; *, p < 0.001 relative to the mock-transfected control). B, effect of caspase mutation on the ATF6α-driven trans-activation of the ERGIC-53 ERSE. HeLa cells were co-transfected with the pGL3 promoter vector bearing the indicated oligonucleotide sequences and 0.5 μg of the HA-ATF6(373) expression vector. The consensus ERSE-I and its inactive form served as control. Mutated nucleotide sequences are underlined. The histograms represent the fold-induction calculated as the ratio between the luciferase activity of ATF6(373) and mock-transfected cells. Values are the average ± S.D. of three independent experiments performed in duplicate. Statistical analysis was performed using Student’s t test (n = 6; *, p < 0.001; **, p < 0.005, respectively). C, HeLa cells were transfected with 1 μg of dominant negative ATF6-(1–373)m1 and the indicated reporter vectors, treated or not for 8 h with 300 nM TG, and then assayed for luciferase activities. Values are the average ± S.D. of three independent experiments performed in duplicate; statistical analysis was performed using Student’s t test (n = 6; *, p < 0.001 relative to control cells).

FIGURE 7. Requirement of ATF6α for full induction of ERGIC-53 in response to ER stress. A, HeLa cells were co-transfected with either Bip/Grp78 or ERGIC-53 reporter plasmids in combination or not with HA-ATF6(373) expression vector and then assayed for luciferase activities. Values reported represent the average ± S.D. of at least three independent experiments performed in duplicate. Statistical analysis was performed using Student’s t test (n = 6; *, p < 0.001 relative to the mock-transfected control). B, effect of caspase mutation on the ATF6α-driven trans-activation of the ERGIC-53 ERSE. HeLa cells were co-transfected with the pGL3 promoter vector bearing the indicated oligonucleotide sequences and 0.5 μg of the HA-ATF6(373) expression vector. The consensus ERSE-I and its inactive form served as control. Mutated nucleotide sequences are underlined. The histograms represent the fold-induction calculated as the ratio between the luciferase activity of ATF6(373) and mock-transfected cells. Values are the average ± S.D. of three independent experiments performed in duplicate. Statistical analysis was performed using Student’s t test (n = 6; *, p < 0.001; **, p < 0.005, respectively). C, HeLa cells were transfected with 1 μg of dominant negative ATF6-(1–373)m1 and the indicated reporter vectors, treated or not for 8 h with 300 nM TG, and then assayed for luciferase activities. Values are the average ± S.D. of three independent experiments performed in duplicate; statistical analysis was performed using Student’s t test (n = 6; *, p < 0.001 relative to control cells).
The ERGIC-53 ERSE

A

Relative Luciferase Activity

|                | IRE1+/+ | IRE1−/− |
|----------------|---------|---------|
| Bip/Grp78      | ![Graph](image1) | ![Graph](image2) |
| ERGIC-53 -149-1| ![Graph](image3) | ![Graph](image4) |
| ERSE-I         | ![Graph](image5) | ![Graph](image6) |
| ERGIC-53 -65-31| ![Graph](image7) | ![Graph](image8) |

Induction Fold

|                | C | TG |
|----------------|---|----|
| IRE1+/+        | ![Graph](image9) | ![Graph](image10) |
| IRE1−/−        | ![Graph](image11) | ![Graph](image12) |

B

![Graph](image13)
out mobility shift assays using wild-type or mutated forms of ERGIC-53 ERSE as probes (Fig. 9A). In both uninduced and TG-induced cell extracts (data not shown), there were two distinct ERSE-binding complexes (EBC-I and EBC-II) that specifically interacted with the ERGIC-53 ERSE (Fig. 9B and supplemental materials). Preincubation with anti-NFY/CFB antibody resulted in the supershift of EBC-II in all samples, suggesting that NFY/CFB is the major component of the slower migrating complex (Fig. 9B). Preincubation with anti YY1 antibody resulted in inhibition of the faster migrating complex, suggesting that YY1 is the major component of EBC-I (Fig. 9C). The ERGIC-53 ERSE mut 1, in which the 5'-CCAAT-3' motif was mutated, retained the EBC-II interaction, which indicates that NFY/CFB could interact with the inner NFY/CFB-like. Mutation of the 5'-CCCTGT-TGG-3' sequence (mut 2 and mut 3) impaired EBC-I activity, which suggests that YY1 interacts with the 5'-CCCTGT-TGG-3' sequence. Furthermore, EBC-II binding to mut 4 (Fig. 9B) was severely reduced, thereby confirming that the pentameric 5'-CCATC-3' motif plays a critical role within the 5'-CCCTGTGGCCATC-3' of the ERSE. In conclusion, the results of in vitro binding assays strongly support the concept that the ERGIC-53 ERSE is constituted by the 5'-CCAAT box and two distinct motifs located 9 nt downstream, the 5'-CCCTGTGG and the CATC-3' part, both of which are important for the ERSE induction.

**DISCUSSION**

In an attempt to identify the transcription mechanisms that control the ERGIC-53 gene, we have studied the transcription regulation of the ERGIC-53 promoter. Here we show that a single cis-acting element, the ERGIC-53 ERSE, which has a novel ERSE organization, enhances the UPR-dependent expression of the gene, and favors the formation of a transcriptional complex constituted by ER stress factors.

The Novel ERSE Structure of the ERGIC-53 ERSE—The ERSE found in the ERGIC-53 promoter region is distinct from all other ERSEs and UPREs so far identified in the promoter regions of UPR-regulated genes (20–25). In the newly identified ERGIC-53 ERSE, the NFB/CFB CCAAT site, which is a feature of both ERSE-I and -II elements, is functionally coupled to the CCCTGTGGCCATC ER stress regulatory sequence, located 9 nucleotides downstream from the CCAAT site and equally important for UPR-mediated regulation of the gene.

The CCAAT site of the ERGIC-53 ERSE constitutively binds NFB/CFB that in all ERSE types previously reported serves as the "foundation" upon which the UPR-induced ER stress factors are assembled (20, 26–30). In our study, replacement of the CCAAT domain impaired the functional activity of the ERGIC-53 ERSE, which suggests that NFB/CFB exerts a function in the ER stress-induced activation of ERGIC-53 similar to that exerted in other ERSE types. Interestingly, we showed that the downstream CCCTGTGGC-CCATC sequence is equally important for the UPR induction. TESS analysis of the sequence revealed, in the reverse orientation of the DNA sequence, a low affinity binding site for the basal transcription factor NFB/CFB (GTGG). We found that NFB/CFB could interact with the inner NFB/CFB-like. Interestingly, a double substitution mutant that reconstituted the NFB/CFB consensus binding site (CCCAATGC) did not enhance ERGIC-53 ERSE activity in response to ER stress (Fig. 4B, mut 18), which suggests that the NFB/CFB protein is not involved in the UPR regulation of that sequence. The downstream sequence could be divided into two parts: the 5'-end part CCCTGTGG that is required for the interaction of YY1 and the 3'-end part CCATC that possibly represents the binding site for accessory and as yet unidentified regulatory protein(s).

The ERGIC-53 ERSE Is the Binding Site for Basic Factors NFB/CFB and YY1 and Is Activated by the ATF6 Pathway of the UPR—The results of our ChIP assays showed that ERGIC-53 is a target gene for nuclear factors commonly involved in UPR-mediated activation of gene expression. Moreover, we found that not only NFB/CFB but also YY1 is recruited by the ERGIC-53 promoter, and both proteins are immunoprecipitated particularly well in the nuclei of ER-stressed cells. YY1 is a co-activator of the ER stress response in mammalian cells (28–31), and our results suggest that it plays a similar role during UPR-mediated activation of ERGIC-53. We also found that SP proteins constitutively interact with the ER stress-responsive region of the ERGIC-53 gene, presumably by recognizing a high affinity site in the promoter region analyzed. This finding is compatible with the report that nuclear factors of the SP family are essential for the stress-induced response of Grp78 in which they constitutively bind ERSE sequences (43).

Our experiments show that, differently from the Grp78 promoter, ER stress-induced XBP1 factor does not enter the ERGIC-53 regulatory region. This result, together with our previous findings that, in response to nitric oxide-induced ER stress, ERGIC-53 mRNA accumulated independently of XBP1 activation (8), suggested that XBP1 is not required for ERGIC-53 activation during ER stress. Results of ERGIC-53 expression analyses performed in IRE1 knocked-out cells clearly show that ERGIC-53 induction does not rely on the IRE1α-XBP1 pathway, suggesting that the UPR modulates ERGIC-53 expression selectively during the ER stress response.

Earlier evidence showed that induction of ERGIC-53 during the UPR was dependent on the activation of the ERSE binding factor ATF6α (6). Now we show that ATF6α is
engaged at the ERGIC-53 promoter of ER-stressed cells. The nuclear form of ATF6α is actively involved in the transcriptional regulation of ERGIC-53 and regulates ERGIC-53 ERSE activity in a sequence-specific manner. These results together with the finding that dominant negative ATF6α exerts an inhibitory effect on UPR induction of ERGIC-53 conclusively demonstrated that ERGIC-53 expression is linked to the UPR by the ATF6 pathway.

A Model for the UPR Regulation of the ERGIC-53 Gene—In a model proposed for UPR-mediated activation of Grp78, activation of ATF6α by ER stress requires, in addition to NFY/CBF binding to the CCAAT box (26), the presence of the CCACG motif at the 3′-end of the ERSE-I and interaction between the two co-activators YY1 and TFII-I (28, 30). YY1 and ATF6α interact through the b-Zip domain of ATF6α and the region flanking the zinc finger domain of YY1. The integrity of these domains is crucial for activation of the Bip/Grp78 promoter (30, 31). Despite the different organization of the ERGIC-53 ERSE, this model is probably applicable to ERGIC-53 transcriptional activation. Fig. 10 illustrates how the transcription factors we identified could converge to form the transcriptional complex required for UPR-mediated control of the gene. We propose that, in response to ER stress, ATF6α is rapidly activated and associates with YY1. The ATF6α-YY1 complex could bind the ERGIC-53 promoter to the CCCTGTTGG part of the ERSE. The nuclear proteins that form the EBC-I complex in association with ATF6α-YY1 could act as coactivators for NFY/CBF present in the EBC-II complex. In this context, Sπ1 proteins could contribute to the formation of the complex by recognizing the high affinity site in the regulatory region.

Chromatin remodeling has been demonstrated to be critical for the ER stress-mediated transcriptional activation of Bip/Grp78 (31). In our

FIGURE 9. In vitro binding analysis of proteins interacting with ERGIC-53 ERSE. A, sequence of the oligonucleotides used in the EMSA. Mutated nucleotides are underlined. B, 5 μg of nuclear extracts obtained from control HeLa cells were incubated with 32P-labeled ERGIC-53 ERSE oligonucleotide and with either specific or nonspecific unlabeled oligonucleotides, as indicated. EBCs were analyzed by EMSA and revealed by autoradiography. In supershift experiments, binding reactions were incubated 20 min before the addition of the radiolabeled probe with 4 μg of anti-NFY/CBF-A antibody. The positions of EBC are indicated on the left. The asterisk on the right indicates migration of the antibody-supershifted complex. C, EMSA of nuclear extracts obtained from untreated HeLa cells incubated with the indicated 32P-labeled ERGIC-53 ERSE oligonucleotides. In supershift, binding reactions were incubated with 10 μg of anti-YY1 antibody 20 min before the addition of the radiolabeled probe. EBCs are indicated on the left.
context, we found that ER stress rapidly stimulated similar modifications of the histone acetylation pattern at both the ERGIC-53 and Bip/Gip78 promoter. In particular, we observed a rapid increase of H3 acetylation and decrease of the H4 acetylation on both promoters. Therefore, we believe that the observed modifications of histone acetylation patterns could favor the formation of the ER transactivation complex at the ERGIC-53 promoter during UPR. In addition, the recruitment of co-activators that either interact with other transcription factors or modify histones represents a critical event for the achievement of an open chromatin state that has been well described for, among other functions, the regulation of gene expression mediated by YY1 (44, 45), which we show to play a pivotal role in the ERGIC-53 ERSE complex. Because the ERSE sequence is highly conserved, ERGIC-53 could be regulated by the same mechanism in diverse species (Table 1). Our model is consistent with the requirements for conservation of the ER stress response and could explain how the newly identified ERGIC-53 ERSE is a general sensor for ER stress.

A putative ERSE sequence is present in the promoter of MCDF2, a protein that is functionally related to ERGIC-53. To gain further insight into the role of UPR in the control of genes involved in post-ER functions, it would be interesting to establish whether UPR regulates MCDF2 transcription by a similar mechanism. A crucial question is the function of ERGIC-53 and MCDF2 during the UPR. We shall address this issue studying the effect of the ER stress on the intracellular trafficking and interaction properties of the two proteins.

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