J. Biochem. **136**, 343–350 (2004)
DOI: 10.1093/jb/mvh122

Differential Contributions of ATF6 and XBP1 to the Activation of Endoplasmic Reticulum Stress-Responsive *cis*-Acting Elements ERSE, UPRE and ERSE-II

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Received May 3, 2004; accepted June 10, 2004

ATF6 and XBP1 are transcription factors activated specifically in response to endoplasmic reticulum (ER) stress. Three *cis*-acting elements capable of binding to ATF6, XBP1 or both have been identified to date, namely ER stress–response element (ERSE), unfolded protein response element (UPRE) and ERSE-II. ERSE controls the expression of ER-localized molecular chaperones such as BiP that can refold unfolded proteins in the ER; transcription from ERSE is fully activated by ATF6 even in the absence of XBP1. In contrast, transcription from UPRE depends solely on XBP1 and it has been suggested that UPRE may control the expression of components of the ER-associated degradation system that can degrade unfolded proteins in the ER. The Herp gene, one of the most highly inducible genes under ER stress, encodes an ER membrane protein containing a ubiquitin-like domain with unknown functions, and carries ERSE-II in addition to ERSE in its promoter. In this report, we show that ERSE-II allows the NF-Y–dependent binding of ATF6 as in the case of ERSE and NF-Y–independent binding of XBP1 as in the case of UPRE, and that transcription from ERSE-II is mitigated in the absence of XBP1. Accordingly, the induction of Herp mRNA was diminished in the absence of XBP1 whereas that of BiP mRNA was not affected. These results may help in understanding the role of Herp in the quality control system in the ER.

Key words: *cis*-element, ER, intracellular signaling, protein folding, transcription factor.

Abbreviations: EMSA, electrophoretic mobility shift assay; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERSE, ER stress response element; MEF, mouse embryonic fibroblast; UPR, unfolded protein response; UPRE, UPR element.

Newly synthesized secretory and transmembrane proteins gain their native conformation in the endoplasmic reticulum (ER), the first organelle they encounter after their synthesis on membrane-bound ribosomes. An efficient quality control system operates in the ER so that only correctly folded molecules are able to exit the ER and reach their final destinations (*1*, *2*). However, the productive folding process is hampered under so-called ER stress, resulting in an accumulation of unfolded proteins in the ER. Eukaryotic cells counteract protein unfolding in the ER by activating a homeostatic response termed the unfolded protein response (UPR), which consists of transcriptional and translational controls in metazoan cells (*3–6*). Translational control allows cells to cease producing protein to decrease the burden on the ER, whereas transcriptional control allows cells to increase the expression of various gene products. The induction of ER-localized molecular chaperones and folding enzymes (collectively termed ER chaperones hereafter) is considered to be most important as they can directly cope with unfolded proteins in the ER (*3–6*).

The transcriptional induction of mammalian ER chaperone genes is mediated by the *cis*-acting ER stress response element (ERSE) present in their promoter regions, the consensus sequence of which is CCAAT-N9-CCACG (*7*, *8*). Two mammalian basic leucine zipper proteins, ATF6 and XBP1, have been identified as ERSE-binding proteins (*7*). Notably, ATF6 and XBP1 are activated in response to ER stress by highly characteristic mechanisms (*9*). ATF6 is constitutively synthesized as a type-II transmembrane protein in the ER (*10*). This membrane-bound precursor form, designated pATF6(P), is transported to the Golgi apparatus in response to ER stress where it is cleaved by the sequential actions of Site-1 and Site-2 proteases (*11–14*). The cytoplasmic region of ATF6 thus liberated from the membrane is translocated into the nucleus, where it functions as an

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active transcription factor designated pATF6(N) via direct binding to the CCACG part of the ERSE when the general transcription factor NF-Y binds to the CCAAT part of the ERSE (*15*). This post-translational mechanism for activating ATF6 is categorized as regulated intramembrane proteolysis (*16*). Mammalian ER expresses two closely related ATF6 proteins referred to as ATF6α and ATF6β (*17*, *18*); ATF6α appears to be a much stronger transcriptional activator than ATF6β (*19*).

XBP1 is activated by a unique post-transcriptional mechanism in which the evolutionally conserved protein IRE1 plays a key role; IRE1 from yeast to human is a type-I transmembrane protein kinase and endoribonuclease in the ER (*20–24*). IRE1 activated by ER stress–induced oligomerization and autophosphorylation initiates spliceosome-independent, unconventional splicing of XBP1 mRNA in metazoan cells (*25–28*). As a result, the open reading frame encoded by the XBP1 mRNA is switched (and, therefore has been proposed to be called frame switch splicing (*9*)), leading to a joining of the DNA-binding domain with the activation domain (*25*). Thus, pXBP1(S) translated from the spliced XBP1 mRNA functions as a potent transcriptional activator. Importantly, pXBP1(S) activates the transcription of ER chaperone genes as they can bind to the CCACG part of the ERSE when the general transcription factor NF-Y binds to the CCAAT part of the ERSE as in the case of pATF6(N) (*25*). Thus, the ATF6 pathway and IRE1-XBP1 pathway serve to activate the transcription of ER chaperone genes in response to ER stress in mammalian cells.

Another ER stress-responsive *cis*-acting element distinct from ERSE was identified in mammals and is now designated the UPR element (UPRE); it contains the consensus sequence TGACGTGG/A (*29*, *30*). The presence of the CCACG sequence in UPRE (lower strand of the underlined sequence) suggests that both ATF6 and XBP1 can bind to UPRE. Unexpectedly, however, it was found that UPRE is the preferential binding site for XBP1; XBP1 binds to UPRE as a homodimer without assistance from NF-Y in contrast to the case of ERSE, probably because the UPRE sequence is longer and more palindromic than CCACG in ERSE (*25*). In contrast, ATF6 exhibits much lower affinity for UPRE than ERSE; ATF6 appears to prefer NF-Y–dependent binding of ERSE to NF-Y–independent binding of UPRE, although the structural basis for these findings is unclear (*25*).

Previous analysis of the Herp gene, one of the most highly inducible genes during the UPR, revealed that the Herp promoter contains not only ERSE but also a *cis*-acting element different from either ERSE or UPRE (*31*). The consensus sequence of this new element was determined to be ATTGG-N-CCACG by extensive mutational analysis. Evidently, this element contains CCAAT and CCACG as in the case of ERSE, although they are separated by a spacer of only one nucleotide and placed in the opposite orientation as compared with ERSE, leading to the designation of this new element as ERSE-II (*31*). It was also shown that overexpression of an active form of ATF6, pATF6(N), by transfection can activate transcription from ERSE-II. In this study, we characterized the properties of ERSE-II comprehensively by comparing its binding activity toward ATF6 with that toward XBP1 and determining its transcriptional activity in cells unable to produce pXBP1(S).

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection and Luciferase Assay—IRE1α+/+ and IRE1α–/– MEFs (*28*) and HeLa cells were grown at 37°C in a humidified 5% CO_2/95% air atmosphere in Dulbecco's modified Eagle's medium (glucose at 4.5 g/liter) supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics (penicillin at 100 U/ml and streptomycin at 100 μg/ml). Transfection was carried out by the standard calcium phosphate method (*32*) essentially as described (*7*). The luciferase assay was performed according to our published procedures (*15*). pGL3-GRP78P(–132)-luc (*7*) is called the ERSE reporter. p5xUPRE-GL3 is identical to p5xATF6GL3 (*29*) and is called the UPRE reporter.

Construction of Plasmids—Recombinant DNA techniques were performed according to standard procedures (*32*). An ERSE-II–containing double-stranded oligonucleotide corresponding to the Herp promoter region –127 to –104 (transcription start site is set as +1) flanked by 5′ *Bam*HI and 3′ *Bgl*II sites (5′-GATCCGCCGATTGGGCCACGTTGGGAGAA-3′ plus 5′-GATCTTCTCCCAACGTGGCCCAATCGGCG-3′; the ERSE-II consensus is underlined) was inserted into the *Bgl*II site of pGL3-Promoter vector (Promega) to create p1xERSE-II-GL3; pGL3-Promoter vector carries the firefly luciferase gene under the control of the SV40 promoter downstream of the *Bgl*II site. The oligonucleotide was also subjected to self-ligation followed by simultaneous digestion with *Bam*HI and *Bgl*II. Three tandem copies of the oligonucleotide were selected and then inserted into the *Bgl*II site of pGL3-Promoter vector to create p3xERSE-II-GL3, which is called the ERSE-II reporter.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as described previously (*15*). ATF6α(1–373) corresponding to pATF6α(N) as well as pXBP1(S) was translated *in vitro* using the TNT T7 quick-coupled transcription/translation system (Promega). The NF-Y trimer (NF-YA, NF-YB, and NF-YC) was reconstituted from recombinant subunit proteins as described previously (*15*). Anti-NF-YA antiserum was obtained from Rockland. The sequences of the synthetic double-stranded oligonucleotide probes ERSE, UPRE and ERSE-II are 5′-GGAGGGCCTTCACCAATCGGCGGCCTCCACGACGGGGCTGG-3′, 5′-GGTCGAGACAGGTGCTGACGTGGCGATTCCCC-3′, and 5′-GGGGATCCGGACGCCGATTGGGCCACGTTGGGAGAGTGCCT-3′, respectively (underlined sequences match the consensus sequence of ERSE, UPRE or ERSE-II). The sequences of mutant ERSE-II probes are 5′-GGGGATCCGGACGCCGATTGGGCCACGTTtGGAGAGTGCCT-3′ (m1), 5′-GGGGATCCGGACGCCGATTGGGCCACGTgtGGAGAGTGCCT-3′ (m2), and 5′-GGGGATCCGGACGCCGATTGGGCCACGggtGGAGAGTGCCT-3′ (m3) (mutated nucleotides immediately 3′ to the ERSE-II consensus sequence are marked by lower case letters). Radioactive bands were visualized using a FLA-3000G FluoroImage analyzer (Fuji Film).

Northern Blot Hybridization—Total RNA was extracted by the acid guanidinium-phenol-chloroform method using ISOGEN (Nippon Gene), and analyzed by standard

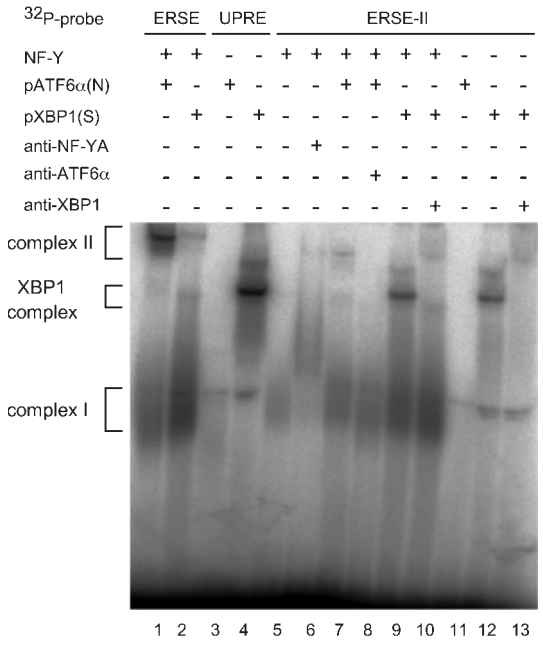

Fig. 1. **Direct binding of pATF6α (N) and pXBP1(S) to ERSE-II.** Zero point one pmol each of ^{32}P-labeled ERSE (lanes 1 and 2), UPRE (lanes 3 and 4), and ERSE-II (lanes 5–13) was incubated with (+) or without (–) 1 μl of *in vitro* translated pATF6α(N) or pXBP1(S) in the presence (+) or absence (–) of recombinant NF-Y (5 fmol) as indicated. Protein-DNA complexes formed were separated from the free DNA probe by electrophoresis in a nondenaturing gel. For supershift experiments, the protein-DNA complexes formed were treated with (+) or without (–) the various antibodies indicated prior to electrophoresis. The migration positions of complex I, complex II, and XBP1 complex are indicated.

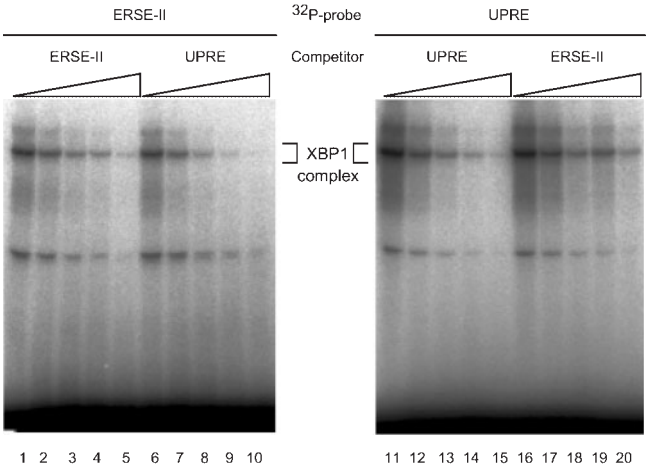

Fig. 2. **Competition between ERSE-II and UPRE in binding pXBP1(S).** Zero point one pmol of ^{32}P-labeled ERSE-II (lanes 1–10) or ^{32}P-labeled UPRE (lanes 11–20) was incubated with 1 μl of *in vitro* translated pXBP1(S) in the absence (lanes 1, 6, 11 and 16) or presence of increasing amounts (10-, 30-, 100-, and 300-fold molar ratio) of unlabeled ERSE-II (lanes 2–5 and 17–20) or unlabeled UPRE (lanes 7–10 and 12–15). The protein-DNA complexes formed were analyzed as in Fig. 1. The migration position of the XBP1 complex is indicated.

Northern blotting (*32*) using an AlkPhos Direct Labeling kit (Amersham Biosciences). Chemiluminescence was visualized using an LAS-1000plus LuminoImage analyzer (Fuji Film).

RESULTS

Direct Binding of ATF6 and XBP1 to ERSE-II—We carried out EMSA to determine whether ATF6 and XBP1 can bind to ERSE-II. pATF6α(N) and pXBP1(S) were translated *in vitro* and recombinant NF-Y (a heterotrimer of NF-YA, NF-YB and NF-YC subunits) was prepared as described in Experimental Procedures. ERSE and UPRE were employed as positive controls. Both pATF6α(N) and pXBP1(S) bound to ^{32}P-ERSE in the presence of NF-Y as reported previously (*15*, *25*) and shown in Fig. 1; complex I corresponds to a binary complex consisting of NF-Y and ^{32}P-ERSE, whereas complex II corresponds to a ternary complex consisting of ^{32}P-ERSE, NF-Y, and pATF6α(N) or pXBP1(S) (Fig. 1, lanes 1 and 2). On the other hand, pXBP1(S) bound to ^{32}P-UPRE even in the absence of NF-Y, giving rise to the formation of the XBP1 complex (lane 4), while pATF6α(N) failed to bind to ^{32}P-UPRE (lane 3). These findings are consistent with our previously published results (*25*).

Incubation of ^{32}P-ERSE-II with NF-Y produced complex I (lane 5), which was supershifted by the addition of anti-NF-YA antibody (lane 6), revealing that NF-Y can bind to ERSE-II by itself as expected given that ERSE-II contains a CCAAT sequence as does ERSE. Although pATF6α(N) alone did not bind to ^{32}P-ERSE-II (lane 11), complex II was formed when pATF6α(N) was incubated with ^{32}P-ERSE-II together with NF-Y (lane 7); complex II but not complex I was supershifted by the addition of anti-ATF6α antibody (lane 8, supershifted complex did not enter the gel). Thus, pATF6α(N) can bind to ERSE-II only in the presence of NF-Y as in the case of ERSE. It should be noted that the amount of complex II formed with ^{32}P-ERSE-II, NF-Y and pATF6α(N) was much smaller than the amount of complex II formed with ^{32}P-ERSE, NF-Y and pATF6α(N) (compare lane 1 with 7), suggesting that pATF6α(N) possesses much less affinity for ERSE-II than for ERSE. In contrast, pXBP1(S) was found to bind to ^{32}P-ERSE-II regardless of the absence (lane 12) or presence (lane 9) of NF-Y, and the complex formed between pXBP1(S) and ^{32}P-ERSE-II was supershifted by the addition of anti-XBP1 antibody (lanes 10 and 13). These results clearly indicate that ERSE-II is distinct from ERSE or UPRE in that it allows both NF-Y–dependent binding of pATF6α(N) and NF-Y-independent binding of pXBP1(S).

Since pXBP1(S) can bind to both UPRE and ERSE-II without assistance from NF-Y, we carried out EMSA to determine whether ERSE-II competes with UPRE to bind pXBP1(S). As shown in Fig. 2, unlabeled ERSE-II competed with both ^{32}P-ERSE-II and ^{32}P-UPRE to bind pXBP1(S) in a dose-dependent manner, and unlabeled UPRE competed with both ^{32}P-ERSE-II and ^{32}P-UPRE to bind pXBP1(S) in a dose-dependent manner. It appeared that pXBP1(S) possesses a slightly higher affinity for UPRE than for ERSE-II as evidenced by the findings that the amount of complex formed between pXBP1(S) and ^{32}P-UPRE was more than that formed between pXBP1(S) and ^{32}P-ERSE-II (Fig. 1, compare lane 4 with lane 12),

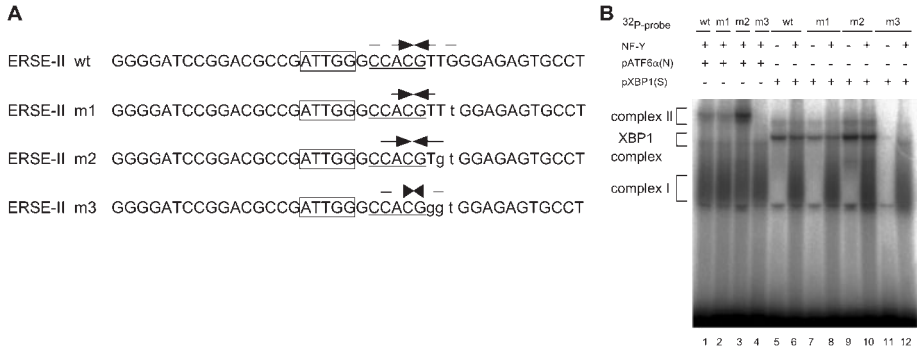

Fig. 3. **Effects of mutations of nucleotides outside the ERSE-II consensus sequence on the binding of pATF6α(N) and pXBP1(S).** (A) The nucleotide sequences of the wild-type (wt) ERSE-II probe and its mutants (m1, m2 and m3) are shown. The sequence complementary to CCAAT is boxed; the sequence CCACG is underlined. Mutated nucleotides are marked by small letters. Palindromic sequences are indicated by arrows. (B) Zero point one pmol each of ^{32}P-labeled wt ERSE-II and its mutants (m1, m2 and m3) was incubated with (+) or without (–) 1 μl of *in vitro* translated pATF6α(N) or pXBP1(S) in the presence (+) or absence (–) of recombinant NF-Y (5 fmol). The protein-DNA complexes formed were analyzed as in Fig. 1. The migration positions of complex I, complex II, and XBP1 complex are indicated.

and that UPRE was better than ERSE-II as a competitor for the binding of both ^{32}P-ERSE-II and ^{32}P-UPRE to pXBP1(S) (Fig. 2).

The CCACG part of ERSE constitutes half of CANNTG, a consensus sequence to which many basic region-containing transcription factors bind (*33*, *34*), whereas UPRE contains a more palindromic sequence. We then took a closer look at the ERSE-II sequence. As a result, we found that the CCACG part of Herp ERSE-II is quite palindromic if the 3 nucleotides 3′ to the CCACG are considered (Fig. 3A). Therefore, we introduced mutations into these adjacent nucleotides and examined their effects. The mutation (m1) of the most 3′ nucleotide from G to T (transversion) affected neither NF-Y–dependent binding of pATF6α(N) (Fig. 3B, compare lane 2 with lane 1) nor NF-Y–independent binding of pXBP1(S) (compare lanes 7 and 8 with lanes 5 and 6), indicating that the palindrome comprising the 4 nucleotides (ACGT) is sufficient for the binding activity of ERSE-II toward both pATF6α(N) and pXBP1(S). The simultaneous mutation (m2) of 2 nucleotides on the 3′ side from TG to GT created a palindrome of 6 nucleotides (CACGTG). Interestingly, both pATF6α(N) (compare lane 3 with lane 1) and pXBP1(S) (compare lanes 9 and 10 with lanes 5 and 6) showed stronger binding to ERSE-II (m2) than to ERSE-II (wt) revealing that pATF6α(N) and pXBP1(S) bind to ERSE-II by recognizing the palindrome. The simultaneous mutation (m3) of the 3 adjacent nucleotides from TTG to GGT abolished the palindrome, and the binding of both pATF6α(N) (compare lane 4 with lane 1) and pXBP1(S) (compare lanes 11 and 12 with lanes 5 and 6) was lost. We conclude that the palindromic nature is critical to the function of ERSE-II.

Transcriptional Activity of ERSE-II—It was previously shown that ERSE-II–containing fragments of the Herp promoter respond to ER stress, and that mutations in

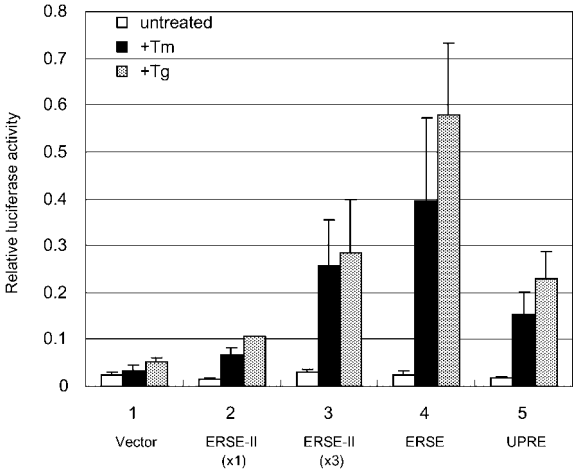

Fig. 4. **Transcriptional activity of ERSE-II in comparison with those of ERSE and UPRE.** HeLa cells were transiently transfected with pGL3-Promoter vector (vector), p1xERSE-II-GL3 [ERSE-II(x1)], p3xERSE-II-GL3 [ERSE-II(x3)], pGL3-GRP78P(–132)-luc (ERSE) or p5xUPRE-GL3 (UPRE) together with the reference plasmid pRL-SV40. Transfected cells were incubated with or without 10 μg/ml tunicamycin (Tm) or 1 μM thapsigargin (Tg) for 16 h. The relative luciferase activity was determined and the averages of triplicate determinations in three independent experiments are presented with standard deviations (error bars).

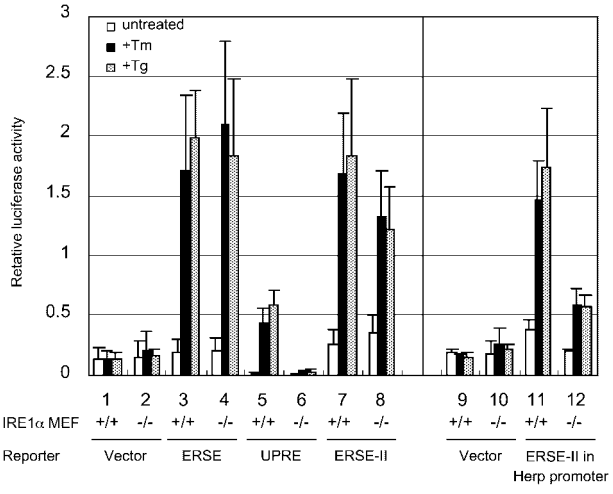

Fig. 5. **Transcriptional activity of ERSE, UPRE and ERSE-II in the presence or absence of IRE1α.** IRE1α+/+ and IRE1α–/– MEFs were transiently transfected with pGL3-Promoter vector (vector), pGL3-GRP78P(–132)-luc (ERSE), p5xUPRE-GL3 (UPRE), or p3xERSE-II-GL3 (ERSE-II) together with the reference plasmid pRL-SV40. IRE1α+/+ and IRE1α–/– MEFs were also transiently transfected with the Herp promoter (–200 to +98)–luciferase fusion gene constructed on the basis of pGL3 in which ERSE-II (–122 to –112) is functional whereas ERSE (–88 to –70) is mutated (*31*). Reporter assays were carried out and the relative luciferase activity is presented as in Fig. 4.

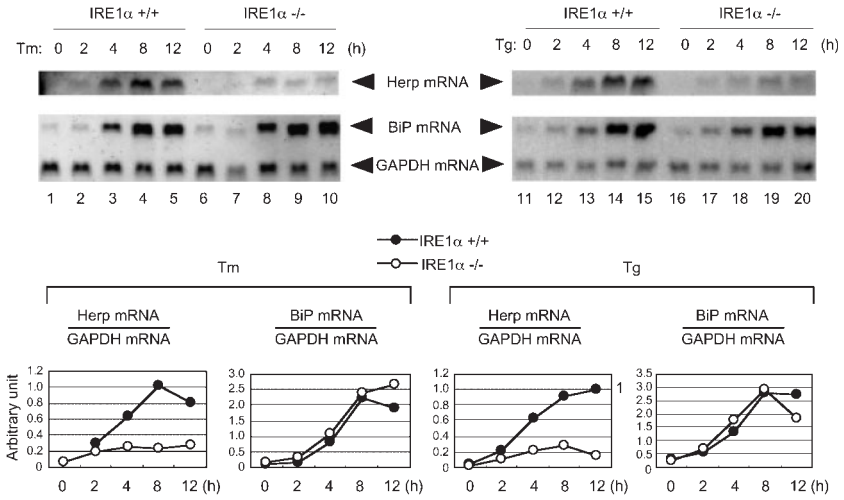

Fig. 6. **Effects of the presence or absence of the IRE1-XBP1 pathway on the induction of Herp mRNA and BiP mRNA.** IRE1α+/+ and IRE1α–/– MEFs were treated with 10 μg/ml tunicamycin (Tm) or 1 μM thapsigargin (Tg) for the periods indicated. Total RNA was extracted and analyzed by Northern blot hybridization using a DNA probe specific for Herp, BiP or GAPDH. The chemiluminescence intensities of each band (Herp mRNA and BiP mRNA) were determined using an LAS-1000plus LuminoImage analyzer, corrected for GAPDH mRNA values, and plotted as arbitrary units against incubation time.

ERSE-II abolish the induction (*31*); however, it has not been determined whether ERSE-II alone is sufficient to confer inducibility on an unresponsive promoter. To address this issue, we placed ERSE-II upstream of the minimum SV40 promoter fused to the firefly luciferase gene. Plasmid constructs were then introduced into HeLa cells by transfection and reporter luciferase assays were carried out. The minimum SV40 promoter did not respond to treatment with ER stress inducers such as tunicamycin and thapsigargin (Fig. 4, lane 1); tunicamycin and thapsigargin cause ER stress by inhibiting protein N-glycosylation and ER Ca^{2+}-ATPase, respectively (*3*). The insertion of a single copy of ERSE-II made the SV40 promoter responsive to ER stress, albeit slightly (lane 2), while the insertion of three tandem copies of ERSE-II conferred strong ER stress-inducibility on the SV40 promoter (lane 3). The extent of the induction from 3xERSE-II was comparable to that from ERSE (lane 4) and from UPRE (lane 5); the ERSE reporter used in this analysis is the firefly luciferase gene under the control of human BiP promoter (–132 to +7 where the transcriptional start site is set as +1), which contains three functional ERSE sequences. The UPRE reporter contains five tandem copies of UPRE upstream of the minimum SV40 promoter-firefly luciferase fusion gene. Thus, ERSE-II *per se* possesses an ability to induce transcription in response to ER stress.

Differential Contributions of ATF6 and XBP1 to Transactivation from ERSE, ERSE-II and UPRE—Next we assessed the relative contribution of XBP1 to transactivation from ERSE-II by measuring the transcriptional activity of ERSE-II in IRE1α+/+ and IRE1α–/– MEFs. It was shown previously that IRE1α–/– MEF can not produce pXBP1(S) because it can not carry out IRE1α-mediated splicing of XBP1 mRNA in response to ER stress (*28*, *30*). In contrast, ER stress-induced processing of ATF6α and the production of pATF6α(N) were not affected by the absence of IRE1α (*30*). Under such conditions, the ERSE reporter was activated by tunicamycin or thapsigargin treatment quite similarly in IRE1α+/+ and IRE1α–/– MEFs as shown in Fig. 5 (compare lane 4 with lane 3), consistent with our previously published results (*28*, *30*); this was explained by the fact that the absence of the IRE1-XBP1 pathway is fully compensated for by the ATF6 pathway as far as transactivation from ERSE is concerned. In contrast, the transactivation from UPRE observed in tunicamycin or thapsigargin-treated IRE1α+/+ MEF (lane 5) was completely lost in IRE1α–/– MEF (lane 6) because pATF6α(N) cannot bind to UPRE efficiently (see Fig. 1); pXBP1(S) is responsible for transactivation from UPRE (*28*, *30*). Interestingly, ERSE-II appeared to behave differently from ERSE or UPRE; the transactivation from ERSE-II observed in tunicamycin or thapsigargin-treated IRE1α+/+ MEF (lane 7) was mitigated in IRE1α–/– MEF (lane 8).

Because the ERSE-II reporter carries three tandem copies of ERSE-II, the above results prompted us to determine the effects of the absence of IRE1α on the transcriptional activity of a single ERSE-II. As the insertion of a single copy of ERSE-II upstream of the minimum SV40 promoter resulted in low inducibility (Fig. 4), we employed a mutant Herp promoter-luciferase fusion gene with functional ERSE-II but mutated ERSE, which was constructed previously and shown to be responsive to ER stress (*31*). As a result, the transcriptional activity of a single ERSE-II in the Herp promoter was found to be greatly reduced when IRE1α was absent (Fig. 5, compare lane 12 with lane 11). These results indicate that the contribution of the IRE1-XBP1 pathway to transactivation from ERSE-II is greater than that to transactivation from ERSE but smaller than that to transactivation from UPRE (see DISCUSSION for explanation).

Differential Induction of BiP mRNA and Herp mRNA in the Absence of the IRE1-XBP1 Pathway—We finally examined whether the absence of the IRE1-XBP1 pathway indeed affects the induction of Herp mRNA by ER stress as expected from the results of reporter assays. Northern blot hybridization analysis revealed that both BiP mRNA and Herp mRNA were induced with a similar time course in response to the treatment of IRE1α+/+ MEF with tunicamycin and thapsigargin (Fig. 6, lanes 1–5 and lanes 11–15, respectively). The extent of the induction of BiP mRNA in IRE1α–/– MEF was similar to that in IRE1α+/+ MEF, whereas the extent of the induction of Herp mRNA was diminished significantly (one-third to one-fourth) in IRE1α–/– MEF as compared with that in IRE1α+/+ MEF (Fig. 6, lanes 6–10 and lanes 16–20). Thus, the IRE1-XBP1 pathway contributes more to the

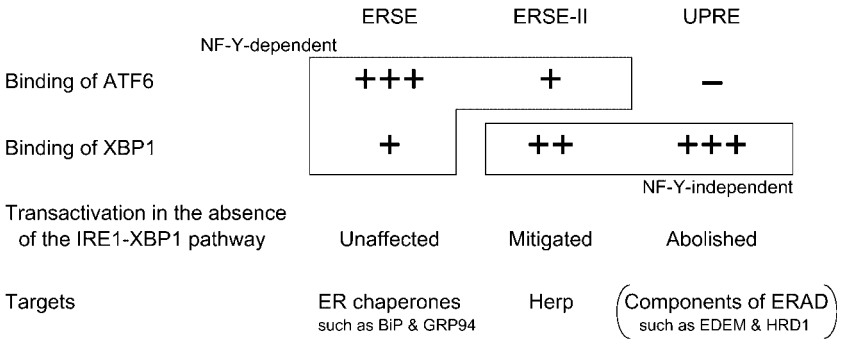

Fig. 7. **Summary (see text).**

induction of Herp mRNA than BiP mRNA basically because the induction of BiP mRNA is mediated by ERSE while that of Herp mRNA is mediated by ERSE and ERSE-II (see "DISCUSSION").

DISCUSSION

ATF6 and XBP1 are transcription factors activated by unique post-translational and post-transcriptional mechanisms, respectively, in response to ER stress (*9*). Activation of ATF6 is rapid as ER stress-induced cleavage of the preexisting ER-membrane bound precursor form of ATF6 produces an active nuclear form of ATF6. In contrast, activation of XBP1 takes more time because the XBP1 mRNA must undergo an IRE1-dependent splicing reaction in response to ER stress, and then the spliced XBP1 mRNA must be translated to produce an active form of XBP1. Once produced, however, the active XBP1 transactivates its own transcription via binding to ERSE present in the XBP1 promoter, allowing the production of active XBP1 as long as IRE1 is activated or as long as unfolded proteins accumulate in the ER. Based on these results, it is considered that the ATF6 pathway is in charge of a rapid response whereas the IRE1-XBP1 pathway is responsible for a sustained response (*9*, *25*).

To date, three ER stress–responsive *cis*-acting elements capable of binding to ATF6 or XBP1 or both have been identified, namely ERSE (*7*, *8*), UPRE (*25*, *29*), and ERSE-II (*31*). Previous findings and the results of this study have revealed different contributions of ATF6 and XBP1 to the transactivation from these elements as summarized in Fig. 7. ERSE is present in promoter regions of ER chaperone genes and is responsible for their induction in response to ER stress (*7*, *8*). Both ATF6 and XBP1 are able to bind to the CCACG part of the ERSE when NF-Y binds to the CCAAT part of the ERSE (*15*, *25*); however, the binding of ATF6 is much more extensive than that of XBP1 (see Fig. 1 and compare lane 1 with lane 2), which is why transcription from ERSE is fully activated in response to ER stress even in the absence of the IRE1-XBP1 pathway (see Fig. 5 and compare lane 3 with lane 4). In the case of ERSE, the absence of XBP1 is fully compensated for by ATF6. Indeed, the induction of BiP mRNA encoding a typical ER chaperone is not affected by the presence or absence of IRE1 (Fig. 6), a finding consistent with previously published results (*28*, *30*, *35*).

UPRE was originally identified as a DNA sequence to which bacterially expressed ATF6 can bind (*29*), and the overexpression of ATF6 has been shown to activate transcription from UPRE (*28–30*); however, *in vitro* translated ATF6 fails to bind to UPRE at a concentration capable of efficient binding to ERSE (see Fig. 1 and compare lane 1 with lane 3). In contrast, XBP1 binds to UPRE in a NF-Y–independent manner, and the extent of binding is much more than that of NF-Y–dependent binding to ERSE when compared at the same concentration (see Fig. 1 and compare lane 4 with lane 2). Importantly, transcription from UPRE is abolished completely in the absence of the IRE1-XBP1 pathway (see Fig. 5 and compare lane 5 with lane 6) as reported previously (*28*, *30*, *35*), indicating that ATF6 has very low affinity for UPRE and is unable to activate transcription from UPRE at a physiological concentration. It should be noted that UPRE has not been found in natural promoters yet and, therefore, target genes under the control of UPRE remain unknown. The most promising candidates for such genes are components of the ER-associated degradation (ERAD) machinery such as EDEM (*30*) and HRD1 (*36*) because the induction of the EDEM mRNA and the HRD1 mRNA by ER stress solely depends on the IRE1-XBP1 pathway, although the characterization of their promoter regions is definitely required to test this notion (see Fig. 7). The induction of the EDEM mRNA is significantly delayed as compared with that of the BiP mRNA in ER-stressed cells (*30*), reflecting the difference in the activation time course between ATF6 and XBP1 mentioned above. Based on these results, it is proposed that mammalian cells determine the fate of unfolded proteins accumulated in the ER utilizing differential properties between the ATF6 and IRE1-XBP1 pathways. Thus, mammalian cells execute a time-dependent phase transition from the ATF6-mediated unidirectional phase (refolding only) to the XBP1-mediated bi-directional phase (refolding plus degradation) depending on the quality or quantity or both of unfolded proteins accumulated in the ER (*30*).

The results described here show that ERSE-II differs from ERSE and UPRE in that it allows both NF-Y–dependent binding of ATF6 and NF-Y–independent binding of XBP1 (Fig. 1, lanes 7 and 12, respectively). Most importantly, transactivation from ERSE-II is mitigated in the absence of the IRE1-XBP1 pathway as evidenced not only by an artificial reporter system (Fig. 5 and compare lane 7 with lane 8) but also by a system using the Herp promoter (Fig. 5, compare lane 11 with lane 12), indicating that both ATF6 and XBP1 contribute to the activation of ERSE-II in response to ER stress at physiological concentrations. Herp is the only gene whose promoter is known to carry a functional ERSE-II (*31*). The observation that the Herp mRNA is induced as early as

the BiP mRNA in ER-stressed IRE1α+/+ cells (Fig. 6) firmly supports the notion that ATF6 is actively involved in the induction of the Herp mRNA. Another observation that induction of the Herp mRNA becomes much less efficient in IRE1α–/– cells (Fig. 6) demonstrates the involvement of XBP1. Nonetheless, we notice that the decrease in the level of Herp mRNA caused by the absence of IRE1α (Fig. 6) was significantly more extensive than that expected from the results of the reporter assays (Fig. 5), given that the Herp promoter carries both ERSE and ERSE-II. We currently do not know the exact reasons for the difference. The absence of IRE1α may affect other signaling pathways involved in the induction of the Herp mRNA by ER stress, such as the PERK-ATF4 pathway (see below); there might be cross-talk to maximally induce Herp in response to ER stress.

Herp is an ER membrane protein originally identified as a homocysteine-inducible protein (*37*). Herp is unstable with a half-life of approximately 2.5 h. A ubiquitin-like domain present in the N-terminus facing the cytoplasm appears to be involved in such instability as its deletion stabilizes the residual protein (*38*). Although Herp has been shown to interact with presenilin and its overexpression enhances the production of amyloid β-protein (*39*), its function remains unknown. Given that target genes under the control of ERSE-II can constitute a group with novel functionality, distinct from the group of ER chaperones under the control of ERSE or from components of the ERAD machinery possibly under the control of UPRE, the involvement of both ATF6 and XBP1 in induction of Herp mRNA may indicate a role for Herp as a linker between the chaperone system and the degradation system.

In addition to the ATF6 and IRE1-XBP1 pathways, a third signaling pathway is activated in response to ER stress, the PERK-ATF4 pathway, culminating in the transcriptional induction of genes encoding the transcription factor CHOP, proteins involved in amino acid biosynthesis and metabolism, and proteins counteracting oxidative stress (*6*, *40*). Interestingly, it was found recently that the Herp promoter carries a *cis*-acting element recognized by ATF4 in addition to ERSE and ERSE-II, and that the PERK-ATF4 pathway participates in the induction of the Herp mRNA in response to ER stress (*41*). Thus, all three signaling pathways activated during the mammalian UPR are directly involved in the induction of Herp, further suggesting the importance of Herp in the homeostasis of the ER. Understanding the role of Herp will provide new insight into the quality control system operating in the ER.

We thank Ms. Kaoru Miyagawa for technical and secretarial assistance. This work was supported, in part, by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (14037233 and 15GS0310 to K.M.).

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