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A Time-Dependent Phase Shift in the Mammalian Unfolded Protein Response

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Summary

Unfolded or misfolded proteins in the endoplasmic reticulum (ER) must be refolded or degraded to maintain homeostasis of the ER. The ATF6 and IRE1-XBP1 pathways are important for the refolding process in mammalian cells; activation of these transcriptional programs culminates in induction of ER-localized molecular chaperones and folding enzymes. We show here that degradation of misfolded glycoprotein substrates requires transcriptional induction of EDEM (ER degradation-enhancing α-mannosidase-like protein), and that this is mediated specifically by IRE1-XBP1 and not by ATF6. As XBP1 is produced after ATF6 activation, our results reveal a time-dependent transition in the mammalian unfolded protein response: an ATF6-mediated unidirectional phase (refolding only) is followed by an XBP1-mediated bidirectional phase (refolding plus degradation) as the response progresses.

Introduction

The endoplasmic reticulum (ER) is the place for the folding and assembly of newly synthesized secretory and transmembrane proteins. Unfolded or misfolded proteins accumulated in the ER must be refolded or degraded to maintain the homeostasis of the ER. Cellular mechanisms of refolding incorrectly folded molecules include inducing ER-localized molecular chaperones and folding enzymes (called ER chaperones collectively here) at the level of transcription to augment the folding capacity in the ER as well as attenuating translation to decrease the load in the ER; these processes are collectively termed the unfolded protein response (UPR; Kaufman, 1999; Mori, 2000; Urano et al., 2000a; Patil and Walter, 2001). On the other hand, proteins misfolded in the ER may also be retrotranslocated through the translocon to the cytoplasm, where they are usually ubiquitinated and degraded by the proteasome; this process is called ER-associated degradation (ERAD; Kopito, 1997; Plemper and Wolf, 1999). However, it is poorly understood how the quality control system in the ER discriminates proteins to be refolded from proteins to be degraded.

Recent extensive studies of the UPR mechanism in mammalian cells have identified three types of transmembrane proteins localized in the ER, namely ATF6α and ATF6β, IRE1α and IRE1β, and PERK, which sense the presence of unfolded proteins in the ER and transmit signals across the ER membrane to induce transcription or attenuate translation (see below). In addition, a *cis*-acting element necessary and sufficient for transcriptional induction of ER chaperone genes has been identified and termed the ER stress response element (ERSE), the consensus sequence of which is CCAAT-N9-CCACG (Yoshida et al., 1998).

ATF6α and ATF6β are ER membrane-bound transcription factors that are expressed ubiquitously and activated by regulated intramembrane proteolysis (Haze et al., 1999, 2001; Ye et al., 2000; Shen et al., 2002). In response to ER stress, their membrane-bound precursor forms, designated pATF6α(P) and pATF6β(P), are converted to soluble, nuclear forms designated pATF6α(N) and pATF6β(N), which activate transcription of ER chaperone genes in the nucleus via direct binding to the ERSE in collaboration with the general transcription factor NF-Y; pATF6α(N) and pATF6β(N) can bind to the CCACG part of the ERSE as a homo- or heterodimer only when NF-Y is bound to the CCAAT part (Yoshida et al., 2000, 2001b).

IRE1α (expressed ubiquitously) and IRE1β (expressed mainly in the gut) are ER membrane-bound endoribonucleases that are conserved from yeast to man and that initiate spliceosome-independent mRNA splicing in response to ER stress (Patil and Walter, 2001). XBP1 mRNA encoding a bZIP transcription factor is the only substrate for this unconventional splicing system that has been identified in mammalian cells (Shen et al., 2001; Yoshida et al., 2001a; Calfon et al., 2002; Lee et al., 2002). As the open reading frames encoded by XBP1 mRNA are switched at the point of splicing and consequently the C-terminal region of XBP1 protein synthesized differs depending on the presence or absence of splicing, we call this novel type of regulation "frame switch splicing." Most importantly, only the spliced form of XBP1, pXBP1(S), functions as a potent transcription factor, as this frame switch splicing joins the bZIP and transactivation domains (Yoshida et al., 2001a). pXBP1(S) binds directly to the ERSE in collaboration with NF-Y, as in the case of ATF6, and thus can activate transcription of ER chaperone genes (Yoshida et al., 2001a).

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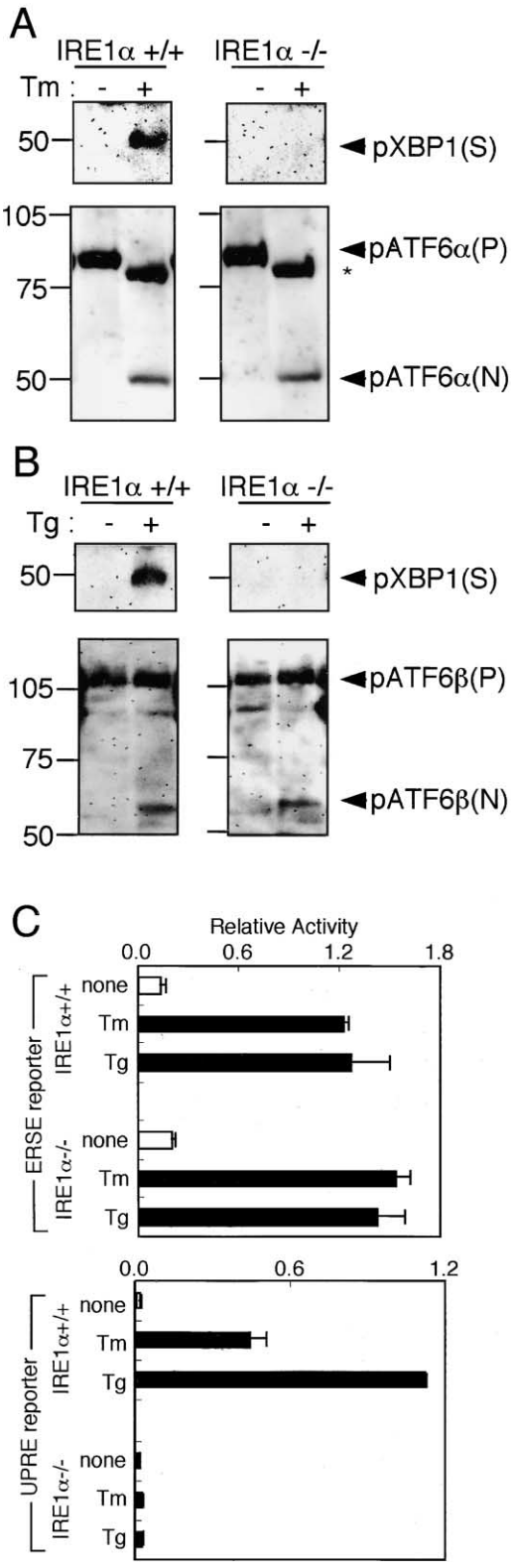

Figure 1. Characterization of *IRE1*$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs

(A and B) *IRE1*$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs were treated with (+) or without (−) 2 μg/ml tunicamycin (Tm) for 8 hr (A) or 300 nM thapsigargin (Tg) for 4 hr (B). Cell lysates were prepared and analyzed by immunoblotting. The migration positions of full-range rainbow molecular weight markers (Amersham Bioscience) as well as pXBP1(S), pATF6α(P), pATF6α(N), pATF6β(P), and pATF6β(N) are indicated. The asterisk denotes the nonglycosylated form of pATF6α(P).

(C) *IRE1*$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs transfected with reporter plasmid pGL3-GRP78P(-132)-luc (ERSE reporter, upper panel) or p5xUPRE-GL3 (UPRE reporter, lower panel) together with reference plasmid pRL-SV40 were incubated with or without 2 μg/ml Tm or 300 nM Tg for 16 hr. The relative luciferase activity was determined and the averages from four independent experiments are presented with standard deviations (error bars).

PERK is a ubiquitously expressed, ER membrane-bound protein kinase that phosphorylates the α subunit of eukaryotic initiation factor 2 in response to ER stress (Harding et al., 1999), which not only attenuates translation but also stimulates the transcription of certain genes by inducing the transcription factor ATF4 at the level of translation (Harding et al., 2000). The PERK pathway is also implicated in the induction of ER chaperone genes (Scheuner et al., 2001; Calfon et al., 2002); however, this effect may result from crosstalk with other pathways because ATF4 binding sites important for the induction have not been identified in the promoter regions of ER chaperone genes.

The above results indicate that activation of both the ATF6 and IRE1-XBP1 pathways culminates in enhanced transcription at the ERSE site, thus upregulating the levels of ER chaperones. This situation is in marked contrast to that in yeast cells, which utilize a single program, the Ire1p-Hac1p pathway, for transcriptional induction of all UPR target genes (Travers et al., 2000). We attempted to identify a specific role of the IRE1-XBP1 pathway in the mammalian ER quality control system in this study.

Various components of the ERAD machinery have been identified in both yeast and mammalian cells (Wilhovsky et al., 2000; Tsai et al., 2002). It is known that the capacity of ERAD is easily saturated in ER-stressed cells, and therefore some components of the ERAD machinery must be induced by the UPR in order to maintain degradation activity (Travers et al., 2000). Among ERAD components identified in mammals so far, the molecule designated EDEM (ER degradation-enhancing α-mannosidase-like protein) is particularly interesting to us because it may be directly involved in the recognition of substrates for degradation, and is inducible by ER stress (Hosokawa et al., 2001). EDEM is a type II transmembrane protein localized in the ER, and its lumenal domain shows significant homology to α1,2-mannosidase but lacks such enzymatic activity. Genetic (Jakob et al., 1998) and pharmacological (Liu et al., 1999) evidence has revealed that the number of mannose residues in N-linked oligosaccharides affects the fate of glycoproteins profoundly when they become misfolded. The results have predicted the presence of a lectin-like molecule in the ER that directs misfolded glycoproteins to the ERAD by recognizing the Man8 structure. EDEM is the most promising candidate for such a receptor-like molecule, as overexpression of EDEM enhances degradation of misfolded glycoproteins with the Man8 structure, and EDEM indeed binds to the substrate directly (Hosokawa et al., 2001). In this report, we show that transcriptional induction of EDEM is achieved by the

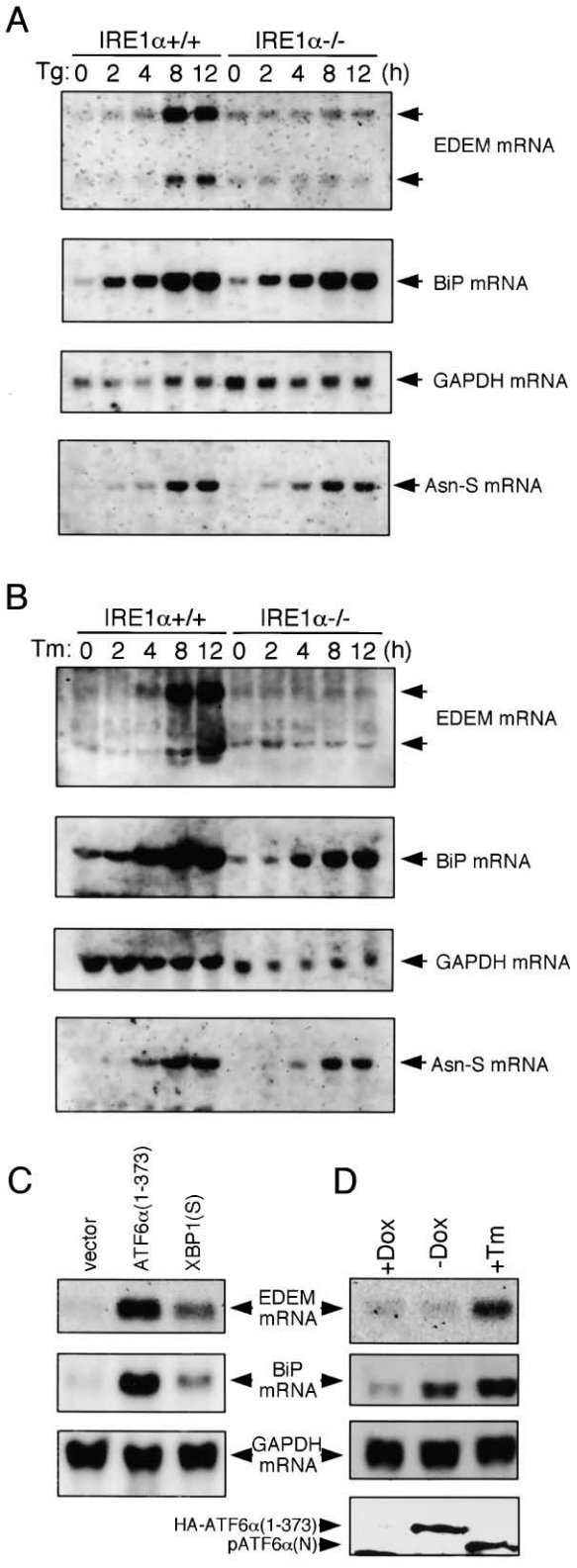

Figure 2. Induction of EDEM by the IRE1-XBP1 Pathway

(A and B) *IRE1*$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs were treated with 300 nM thapsigargin (Tg, [A]) or 2 μg/ml tunicamycin (Tm, [B]) for the indicated periods. Total RNA was isolated and analyzed by Northern blot hybridization using a cDNA probe specific to EDEM, BiP, Asn-S, or GAPDH.

IRE1-XBP1 pathway and is required for efficient degradation of glycoproteins misfolded in the ER. Possible implications of these findings are discussed.

Results

IRE1$\alpha^{-/-}$ Cells Are Unable to Induce EDEM in Response to ER Stress

Mouse embryonic fibroblasts devoid of IRE1α (*IRE1*$\alpha^{-/-}$ MEF) have been established (Urano et al., 2000b; Lee et al., 2002), and their phenotypes with respect to the UPR have been characterized extensively (Lee et al., 2002). Upon ER stress, *IRE1*$\alpha^{-/-}$ MEF cannot splice XBP1 mRNA and thus cannot produce pXBP1(S), as shown in Figures 1A and 1B; tunicamycin and thapsigargin cause ER stress by inhibiting protein N-glycosylation and ER Ca^{2+} ATPase, respectively (Kaufman, 1999). In contrast, the absence of IRE1α has no effect on ER stress-induced conversion of pATF6α(P) and pATF6β(P) to pATF6α(N) and pATF6β(N), respectively (Figures 1A and 1B, and data not shown). Activation of ATF6α/β appears to be sufficient for ERSE-mediated transcriptional induction in response to ER stress (Figure 1C, upper panel).

An ER stress-responsive *cis*-acting element distinct from the ERSE was recently identified as a sequence to which ATF6 can bind without assistance from NF-Y (Wang et al., 2000), and designated as the ATF6 site (its consensus sequence is TGACGTGG/A). Importantly, however, we found that the ATF6 site is the binding site for XBP1 but not for ATF6 (Yoshida et al., 2001a). We thus proposed renaming the ATF6 site as the mammalian UPR element (UPRE), after yeast UPRE to which yeast UPR-specific transcription factor Hac1p binds, so that the name UPRE would then represent a binding site specific for a transcription factor regulated by frame switch splicing, such as Hac1p and XBP1. As already documented (Lee et al., 2002), *IRE1*$\alpha^{+/+}$ but not *IRE1*$\alpha^{-/-}$ MEF could activate transcription through the UPRE in response to ER stress (Figure 1C, lower panel), indicating that the IRE1-XBP1 pathway is solely responsible for UPRE-mediated transcriptional induction and that XBP1 can activate transcription of certain genes that cannot be activated by ATF6.

Transcription of EDEM is inducible by ER stress and, interestingly, the induction time course of EDEM is different from that of BiP/GRP78 (a major ER chaperone)—namely, it is slower (Hosokawa et al., 2001). These differences in time course were clearly demonstrated by Northern blot hybridization analysis of *IRE1*$\alpha^{+/+}$ MEF after treatment with thapsigargin (Figure 2A) or tunicamycin (Figure 2B). It should be noted that the mouse

(C) HeLa cells were transfected with pcDNA (vector), pcDNA-ATF6α(1–373), or pcDNA-XBP1(S). Total RNA was isolated and analyzed as in (A).

(D) HeLa-pATF6α(N) cells were cultured in the presence (+) or absence (−) of doxycyclin (Dox) in the medium for 1 day, or incubated with 2 μg/ml Tm for 8 hr in the presence of doxycyclin. Total RNA was isolated and analyzed as in (A). Cell lysates were also prepared and analyzed by immunoblotting using anti-ATF6α antibody. The migration positions of endogenous pATF6α(N) and exogenous HA-ATF6α(1–373) are indicated.

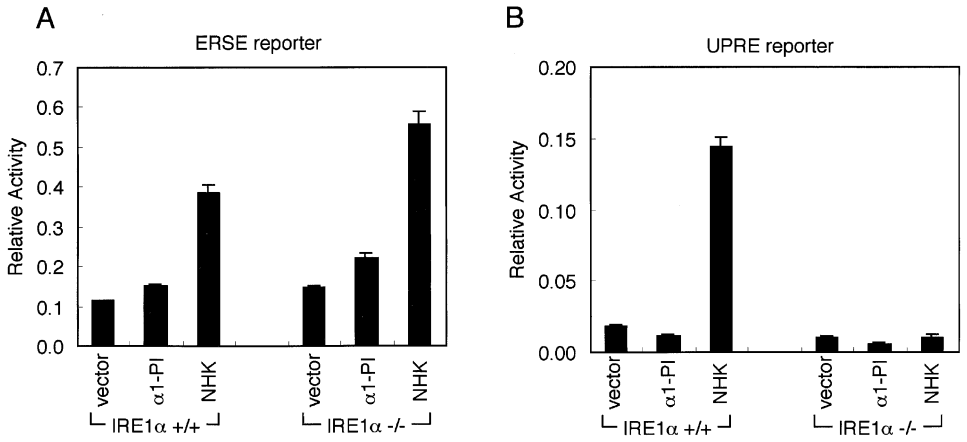

Figure 3. Effects of Overexpression of Wild-Type and NHK Variant of α1-PI on the UPR

IRE1$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs were transfected with the vector alone, pREP9-α1-PI, or pREP9-NHK to overexpress wild-type α1-PI or its NHK variant, respectively, in addition to reporter plasmid pGL3-GRP78P(-132)-luc (ERSE reporter, [A]) or p5xUPRE-GL3 (UPRE reporter, [B]) as well as reference plasmid pRL-SV40. The relative luciferase activity constitutively expressed in transfected cells was determined and presented as in Figure 1C.

EDEM gene was transcribed into two (6 kb and 2.4 kb) mRNA species, depending on the polyadenylation site, and both were induced by ER stress (Hosokawa et al., 2001). Considering the fact that pXBP1(S) is produced after detection of pATF6α(N) and pATF6β(N) in ER-stressed cells (see Discussion), and assuming that there is no significant difference in stability between BiP mRNA and EDEM mRNA, the above results suggested that EDEM might be a target specific to the IRE1-XBP1 pathway. We therefore examined the induction of EDEM transcription by ER stress in *IRE1*$\alpha^{-/-}$ MEF. As a result, we indeed found that EDEM mRNA was not induced at all in *IRE1*$\alpha^{-/-}$ MEF treated with thapsigargin or tunicamycin (Figures 2A and 2B). In contrast, transcription of BiP was highly inducible in both *IRE1*$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs, consistent with previously published results (Lee et al., 2002). We also examined the induction of asparagine synthetase (Asn-S) mRNA. Transcription of Asn-S was shown previously to be inducible by ER stress in an ERSE-independent manner (Barbosa-Tessmann et al., 1999), and we recently provided evidence suggesting that Asn-S might be regulated specifically by the PERK pathway (Okada et al., 2002). Northern blot hybridization analysis revealed that Asn-S mRNA was induced similarly in both *IRE1*$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs (Figures 2A and 2B), suggesting that not only the ATF6 but also the PERK pathway is operating normally in *IRE1*$\alpha^{-/-}$ MEF. Thus, to our knowledge, EDEM is the first UPR target gene whose induction has been shown to be selectively eliminated by the absence of the IRE1 pathway.

Transcriptional Induction of EDEM Is Mediated by the IRE1-XBP1 Pathway, Not by the ATF6 Pathway

We next examined whether transcription of EDEM is directly regulated by XBP1. Because the transfection efficiency of MEFs is quite low, transient transfection experiments to determine the levels of endogenous mRNAs were carried out in HeLa cells. Overexpression of ATF6α(1–373), corresponding to pATF6α(N), and of pXBP1(S) enhanced the levels of BiP mRNA (Figure 2C) as reported previously (Yoshida et al., 2001a). In contrast to our expectations, however, the level of EDEM mRNA was also enhanced by overexpression of not only pXBP1(S) but also ATF6α(1–373) (Figure 2C). Nonetheless, this upregulation was consistent with the results of reporter assays showing that UPRE reporter expression was markedly enhanced by overexpression of both pXBP1(S) and ATF6α(1–373) in *IRE1*$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs (data not shown). Thus, apparently contradictory results were obtained as reported previously (Lee et al., 2002): the UPRE reporter was not activated at all by pATF6α/β(N) produced from endogenous ATF6α/β in response to ER stress treatment of *IRE1*$\alpha^{-/-}$ MEF (see Figure 1C), but was markedly activated by pATF6α(N) introduced into the cells exogenously by transfection. Given that association and dissociation between pATF6α(N) and the UPRE are at equilibrium in the nucleus, we hypothesized that pATF6α(N) produced at a physiological concentration is insufficient for transactivation of the UPRE reporter due to its low affinity to the UPRE, but pATF6α(N) expressed at an extremely high concentration in transfected cells forces the equilibrium to move toward the association side, which allows exogenous pATF6α(N) to transactivate the UPRE reporter markedly.

We therefore examined the effects of physiological level expression of pATF6α(N) on the transcription of BiP and EDEM. Using the so-called "Tet-Off" system, we previously constructed HeLa-pATF6α(N) cells, in which exogenous ATF6α(1–373) tagged with influenza virus hemagglutinin (HA) epitope is synthesized upon removal of tetracycline from the culture medium (Okada et al., 2002). As shown in Figure 2D, HA-ATF6α(1–373) migrating more slowly than endogenous pATF6α(N) was detected in HeLa-pATF6α(N) cells cultured in the absence of doxycyclin, a derivative of tetracycline, and the level of HA-ATF6α(1–373) was comparable to that of pATF6α(N) produced in response to 8 hr treatment of HeLa-pATF6α(N) cells with tunicamycin. Under these conditions, the level of BiP mRNA was markedly enhanced

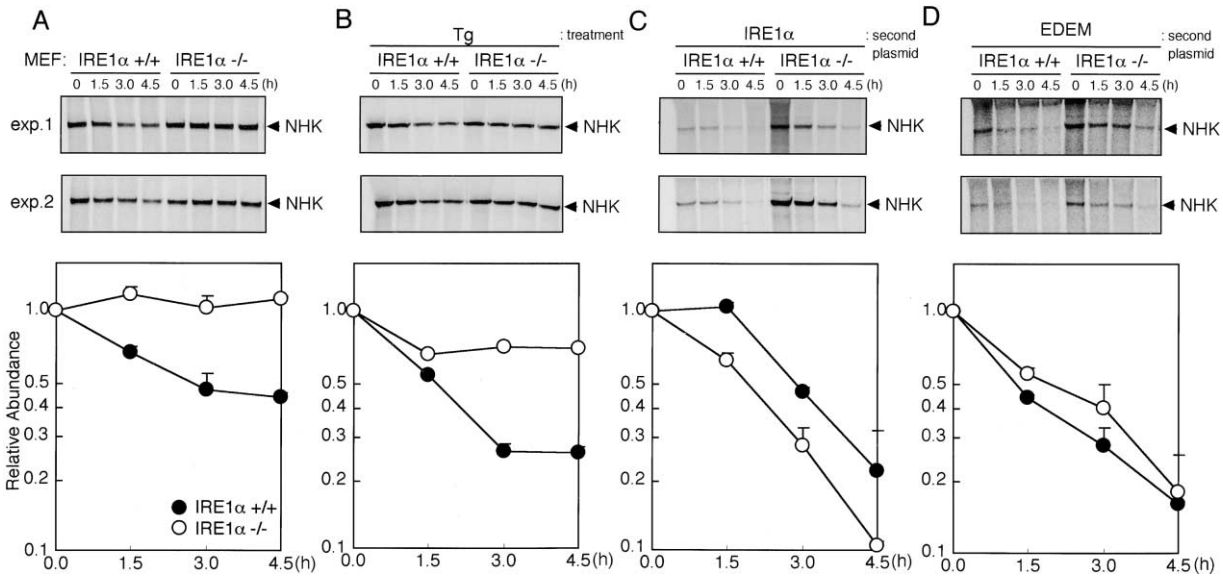

Figure 4. Degradation of the NHK Variant of α1-PI in *IRE1*$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs

IRE1$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs transfected with pREP9-NHK were pulse labeled for 1 hr and then chased for the indicated periods. NHK immunoprecipitated using anti-α1-PI antibody was subjected to SDS-PAGE. The results of two independent experiments are shown. The migration position of NHK is indicated. The radioactivity of each band was determined and the data (averages from the two experiments with standard deviations) are plotted at the bottom of each panel as relative abundance (arbitrary units) versus chase time.

(A) No treatment.

(B) Thapsigargin (Tg; 300 nM) was included from 12 hr before the pulse labeling until the end of the chase period.

(C) Cells were cotransfected with pcDNA-IRE1α.

(D) Cells were cotransfected with pSPORT-HA-EDEM.

by inducible expression of HA-ATF6α(1–373), but the level of EDEM mRNA remained unaffected. As a positive control, tunicamycin treatment was shown to cause induction of both BiP and EDEM mRNAs. These results clearly demonstrate that pATF6α(N) expressed at a physiological concentration can activate transcription of BiP but cannot activate that of EDEM due to its high affinity to the ERSE but low affinity to the UPRE. Based on all these results, we concluded that transcriptional induction of EDEM is achieved by the IRE1-XBP1 pathway, not by the ATF6 pathway.

IRE1$\alpha^{-/-}$ Cells Are Unable to Degrade Glycoproteins Misfolded in the ER Efficiently

We then examined whether the inability of *IRE1*$\alpha^{-/-}$ MEF to transcribe EDEM at elevated levels in response to ER stress affects the rate and/or extent of degradation of glycoproteins misfolded in the ER. A mutant form of α1-proteinase inhibitor (α1-PI, also called α1-antitrypsin) designated the null Hong Kong (NHK) variant (Sifers et al., 1988) was used as a model substrate to monitor cellular ERAD activity. The genetic NHK mutation hampers the maturation of α1-PI into a serum glycoprotein and causes the variant to be misfolded in the ER and degraded via ERAD (Liu et al., 1999). As shown in Figure 3, overexpression of the NHK but not the wild-type α1-PI markedly activated cellular UPR activity; both ERSE- and UPRE-mediated transcription was elevated in *IRE1*$\alpha^{+/+}$ MEF. It should be noted that transactivation of the UPRE reporter by NHK was eliminated in *IRE1*$\alpha^{-/-}$ MEF (Figure 3B), consistent with the results shown in Figure 1C. These results indicated that NHK was indeed recognized by the cell as a misfolded protein in the ER.

IRE1$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs were transfected with plasmid to overexpress NHK, pulse labeled with [^{35}S]methionine and [^{35}S]cysteine for 1 hr, and then chased. The NHK variant was immunoprecipitated and subjected to SDS-PAGE. As shown in Figure 4A, NHK was degraded in *IRE1*$\alpha^{+/+}$ MEF. In marked contrast, NHK was stable in *IRE1*$\alpha^{-/-}$ MEF (Figure 4A). Degradation of NHK in *IRE1*$\alpha^{+/+}$ MEF was accelerated by the addition of thapsigargin from 12 hr before the pulse labeling until the end of the chase period (Figure 4B) to evoke ER stress and thus to activate the UPR; perhaps, cellular ERAD activity was enhanced by transcriptional induction of various ERAD components in *IRE1*$\alpha^{+/+}$ MEF. In contrast, NHK was not degraded efficiently in *IRE1*$\alpha^{-/-}$ MEF even under the condition of ER stress (Figure 4B). Importantly, this defect in degradation was corrected by introducing IRE1α (Figure 4C) or EDEM (Figure 4D) into *IRE1*$\alpha^{-/-}$ MEF by cotransfection. These results clearly indicate that *IRE1*$\alpha^{-/-}$ MEF are unable to degrade a misfolded glycoprotein efficiently due to their inability to transcribe EDEM at elevated levels in response to accumulation of unfolded or misfolded proteins in the ER.

Discussion

Mammalian cells have evolved two transcriptional induction programs, the ATF6 and IRE1-XBP1 pathways, to adjust the levels of ER chaperones according to the

needs within the ER. Comparison of their activation mechanisms has revealed an important difference between the two. Activation of ATF6 is rapid as it is achieved by cleavage of a preexisting protein, whereas activation of XBP1 requires multiple steps: XBP1 mRNA must be induced, spliced, and then translated to produce an active form of XBP1. This difference in kinetics is well reflected by the timing of their appearance in ER-stressed cells: detection of pATF6(N) precedes that of pXBP1(S) (Yoshida et al., 2001a). On the other hand, XBP1 but not ATF6 carries a functional ERSE sequence in its promoter (Yoshida et al., 2000), allowing only XBP1 to transactivate its own transcription. Thus, once produced, XBP1 can function in a more sustained fashion than ATF6, as the XBP1 activation cycle [induction of XBP1 mRNA, splicing of XBP1 mRNA, and production of pXBP1(S)] continues as long as IRE1 is activated or as long as unfolded proteins are present in the ER. Another important difference between the two programs is the mode of recognition of the target DNA by the respective transcription factor; ATF6 shows stronger NF-Y-dependent binding to the ERSE than NF-Y-independent binding to the UPRE, whereas XBP1 binds to both ERSE and UPRE (Yoshida et al., 2001a). These results taken together imply that mammalian cells activate ATF6 to induce transcription through the ERSE promptly in response to ER stress, and subsequently activate XBP1 to induce not only ERSE-mediated but also UPRE-mediated transcription.

In this report, we show that, to our knowledge, EDEM is the first UPR target gene whose induction has been shown to depend completely and solely on the IRE1-XBP1 pathway. Importantly, the lack of EDEM induction produced a very interesting phenotype in *IRE1*$\alpha^{-/-}$ cells: *IRE1*$\alpha^{-/-}$ cells were unable to degrade the NHK variant of α1-PI, a glycoprotein misfolded in the ER, efficiently. As we analyzed the function of endogenous (but not overexpressed) EDEM here, our results further substantiated the notion that EDEM is critically involved in the quality control of proteins in the ER by targeting misfolded glycoproteins to ERAD. Nonetheless, we cannot rule out the possibility that *IRE1*$\alpha^{-/-}$ cells fail to degrade NHK because they cannot upregulate, not only EDEM, but also other component(s) of the ERAD machinery in response to NHK expression; overexpression of EDEM in *IRE1*$\alpha^{-/-}$ cells by transfection may have corrected the defect by bypassing the requirement of such non-EDEM component(s).

Our results described here clarify one mechanism by which mammalian cells determine the fate of proteins unfolded or misfolded in the ER, that is, a mechanism involving a time-dependent phase transition. Our recent microarray analysis indicated that a majority of ATF6 target genes encode ER chaperones (Okada et al., 2002). Therefore, when unfolded proteins are accumulated in the ER, mammalian cells undertake to refold them first by activating ATF6 and thus inducing ER chaperones via ERSE-mediated transcription. If the unfolded proteins are refolded by the action of the induced ER chaperones, the UPR is finished. In this sense, ATF6-mediated phase is unidirectional (refolding only). However, if unfolded proteins are still present in the ER, mammalian cells begin to synthesize pXBP1(S). XBP1-mediated phase is bidirectional (refolding plus degradation), as it induces not only ER chaperones via ERSE-mediated transcription but also EDEM via UPRE-mediated transcription to degrade unfolded proteins. Thus, the involvement of two transcriptional induction programs certainly gives versatility to mammalian cells; they have developed multiple phases to cope with unfolded proteins and are able to execute phase transition, depending on the quality and/or quantity of unfolded proteins accumulated in the ER. Further identification of XBP1-specific target genes will definitely provide us with better understanding of the quality control system in the ER.

Experimental Procedures

Cell Culture, Transfection, and Luciferase Assay

IRE1$\alpha^{+/+}$ and *IRE1*$\alpha^{-/-}$ MEFs (Lee et al., 2002) as well as 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 4.5g/L) supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Transfection was carried out by the standard calcium phosphate method essentially as described (Yoshida et al., 1998). The luciferase assay was performed according to our published procedures (Yoshida et al., 2000). pGL3-GRP78P(-132)-luc (Yoshida et al., 1998) is called the ERSE reporter. p5xUPRE-GL3 is identical to p5xATF6GL3 (Wang et al., 2000) and is called the UPRE reporter.

Immunoblotting and Northern Blot Hybridization

Cell lysates were prepared as described (Okada et al., 2002). Each antigen was detected with anti-ATF6α (Haze et al., 1999), anti-ATF6β (Haze et al., 2001), or anti-XBP1-A (Yoshida et al., 2001a) antibody using an enhanced chemiluminescence Western blotting detection system kit (Amersham Bioscience). Total RNA was extracted by the acid guanidinium-phenol-chloroform method using ISOGEN (Nippon Gene) and analyzed by standard Northern blotting using an Alkaphos direct labeling kit (Amersham Bioscience). Chemiluminescence was visualized using an LAS-1000plus LuminoImage analyzer (Fuji film).

Pulse-Chase Experiments

IRE1$\alpha^{+/+}$ or *IRE1*$\alpha^{-/-}$ MEF cultured in 30 mm dishes was transfected with 2.5 μg of pREP9-NHK (Hosokawa et al., 2001). Twenty-four hours later, cells were starved for methionine and cysteine for 1 hr, pulse labeled with [^{35}S]methionine and [^{35}S]cysteine for 1 hr using 0.05 mCi (1.85 MBq)/dish EXPRE^{35}S^{35}S protein labeling mix (DuPont), and then chased with fresh complete medium. The NHK variant was immunoprecipitated from cell lysates as described (Haze et al., 1999) with the following modifications: anti-α1-PI IgG (Cappel) covalently linked to agarose resin (AminoLink Plus Coupling gel, Pierce) was used to immunoprecipitate the antigen without addition of a secondary antibody. The immunoprecipitates were subjected to SDS-PAGE (4%–20% gradient gel) and radioactive bands were analyzed using an FLA-3000G FluoroImage analyzer (Fuji film).

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