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Cloning of mammalian Ire1 reveals diversity in the ER stress responses

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Cells modify their gene expression pattern in response to stress signals emanating from the endoplasmic reticulum (ER). The well-characterized aspect of this response consists of the activation of genes that encode protein chaperones and other ER resident proteins, and is conserved between mammals and yeast. In mammalian cells, however, ER stress also activates other pathways, including the expression of the transcription factor CHOP/GADD153 and its downstream target genes. ER stress is also linked to the development of programmed cell death, a phenomenon in which CHOP plays an important role. Here we report on the cloning of a murine homolog of yeast *IRE1*, an essential upstream component of the ER stress-response in yeast. The mammalian Ire1 is located in the ER membrane and its over-expression in mammalian cells activates both the endogenous ER chaperone GRP78/BiP and CHOP-encoding genes. Over-expression of a dominant-negative form of Ire1 blocks the induction of GRP78/BiP and CHOP in response to the ER stress induced by tunicamycin treatment. Over-expression of murine Ire1 also leads to the development of programmed cell death in transfected cells. These results indicate that a single upstream component, Ire1, plays a role in multiple facets of the ER stress-response in mammalian cells.

Keywords: chaperones/endoplasmic reticulum/membrane protein/protein folding/protein kinase

Introduction

A significant number of noxious circumstances lead to accumulation of unfolded intermediates in the endoplasmic reticulum (ER) and trigger a stress response (Lee, 1987; Gething and Sambrook, 1992). This response, referred to as the unfolded protein response (UPR), was initially characterized in terms of the transcriptional induction of a set of mRNAs, all of which have in common the fact that they encode ER resident proteins that are thought to participate in the folding of newly synthesized proteins. These include the genes encoding the ER chaperones GRP78/BiP and GRP94, protein disulfide isomerase (PDI) and the PDI-like ERp72 (Kozutsumi *et al*., 1988; Dorner *et al*., 1990; Little *et al*., 1994). Defined in such terms, the UPR can be easily rationalized in terms of the need to rectify a perturbation in protein folding in the ER by increasing the synthesis of components that promote the process of proper folding in that compartment.

In the yeast *Saccharomyces cerevisiae*, the UPR is effected by a signaling cascade in which the upstream-most component known to date is an ER-associated type I transmembrane protein kinase, Ire1p/Ern1p (Cox *et al*., 1993; Mori *et al*., 1993), a protein that also bears considerable homology to the regulated endoribonuclease RNaseL (Bork and Sander, 1993; Sidrauski and Walter, 1997). Ire1p senses the perturbed environment in the ER, presumably through its lumenal domain. This leads to activation of downstream signaling by a process that is thought to depend on oligomerization and *trans*-phosphorylation of the kinase domain (Shamu and Walter, 1996; Welihinda and Kaufman, 1996). This leads to activation of a sequence-specific endoribonucleolytic activity, resident in the C-terminal non-lumenal side of Ire1p (Sidrauski and Walter, 1997). The latter cleaves a pre-formed precursor mRNA that encodes the transcription factor Hac1p at two sites, excising a small intron (Cox and Walter, 1996; Nikawa *et al*., 1996; Kawahara *et al*., 1998). The exons of Hac1 are joined together by a non-canonical splicing reaction that is brought about by tRNA ligase (Sidrauski *et al*., 1996). This leads to enhanced translation of the spliced Hac1 mRNA (Chapman and Walter, 1997; Kawahara *et al*., 1997). The product of this translation, Hac1p, then binds to a UPR response elements in the promoters of the yeast homolog of *GRP78* (a gene known as *KAR2*) and two yeast PDI-like genes (Cox and Walter, 1996; Mori *et al*., 1998). Deficiencies in components of this pathway lead to increased sensitivity of the yeast to agents that induce the UPR, supporting the hypothesis that the UPR is geared to attenuating ER stress. In mammalian cells also, there is evidence favoring such a role. Over-expression of GRP78/BiP attenuates the cell-death-promoting effects of calcium ionophores that induce the UPR (Morris *et al*., 1997), and interfering with the induction of GRP78/BiP, by means of antisense constructs, renders the cells more sensitive to conditions that trigger a UPR (Little and Lee, 1995).

In mammalian cells, ER stress may also activate genes and pathways that are not directly linked to the expression of resident ER proteins. For example, treatment of cells with the glycosylation inhibitor tunicamycin, a potent inducer of the UPR, leads to the induction of growth arrest and programmed cell death in many cell types (Larsson *et al*., 1993; Pérez-Sala and Mollinedo, 1995; Dricu *et al*., 1996). A similar response is observed in cells that harbor temperature-sensitive mutations in components of the protein glycosylation pathway (Nakashima *et al*., 1993; Silberstein *et al*., 1995): switch to the non-permissive temperature leads to the induction of the UPR and triggers

growth arrest and programmed cell death. These events could be due to distant downstream consequences of the perturbation in ER function. However, recent evidence suggests that this outcome may be due to an active process directly triggered by the UPR: the gene encoding the transcription factor CHOP (also known as GADD153) is not normally expressed in cells but is potently induced by stress. Initially thought to be responsive to DNA-damage and growth arrest (Fornace *et al*., 1989), subsequent studies have uncovered a strong correlation between the development of ER stress and induction of *CHOP*. Known inducers of the UPR, such as tunicamycin, thapsigargin, the calcium ionophore A23187 and reducing agents such as dithiothreitol, all induce *CHOP* (Price and Calderwood, 1992; Halleck *et al*., 1997). *CHOP* is also induced by depriving cells of nutrients such as glucose and oxygen, a situation that is also associated with the induction of a UPR (Price and Calderwood, 1992; Carlson *et al*., 1993).

More direct evidence favoring a link between a primary ER signal and *CHOP* induction was provided by the observation that GRP78/BiP over-expression attenuates *CHOP* induction in response to stress (Wang *et al*., 1996). At the physiological level, over-expression of CHOP attenuates the process of adipocytic differentiation in 3T3-L1 cells (Batchvarova *et al*., 1995) and a gain of function mutation in *CHOP* is invariably associated with human myxoid liposarcoma (Crozat *et al*., 1993; Rabbitts *et al*., 1993). Under some circumstances, CHOP over-expression can lead to cell-cycle arrest (Barone *et al*., 1994), and can promote programmed cell death (Friedman, 1996). Knock-out of the *CHOP* gene in mouse cells and tissues leads to decreased programmed cell death in response to agents that induce the UPR (Zinszner *et al*., 1998). Collectively, these observations suggest a role for CHOP in modulating cellular phenotypes in response to ER stress. Therefore, it is of considerable interest to study the link between *CHOP* induction and other, more classical facets of the UPR. Here we describe an analysis of the role of one newly identified component of that signaling cascade, mammalian Ire1. Our results suggest that in mammalian cells, the UPR is not limited to the induction of ER resident proteins and it may exert pleiotrophic effects on cellular phenotypes through the induction of components such as CHOP.

Results

Search of the sequence databases with the yeast Ire1p sequence revealed a predicted *Caenorhabditis elegans* homolog (Q09499). Based on their similarity to that protein, we identified two different ESTs that encode potential mammalian homologs of Ire1p, one murine and one human. The murine EST was used to isolate a full-length coding region clone from a murine colon cDNA library. The encoded protein of 911 amino acids has an N-terminal hydrophobic stretch of 18 residues followed by a 403 amino acid sequence that exhibits 33.5% identity to the lumenal domain of *C.elegans* Ire1p and 19.9% identity to the yeast protein. A non-conserved 19 residue hydrophobic stretch (aa 429–447), predicted to serve as a transmembrane domain, precedes the C-terminal region. The latter is 40% identical to the kinase/endonuclease effector domain of yeast and *C.elegans* Ire1p (Figure 1A and B), and 20% identical to human RNaseL (not shown). The primary amino acid sequence of the clone is consistent with it being a mammalian homolog of yeast Ire1p. In both man and mouse, the gene encoding Ire1 is immediately adjacent to that of polo-like kinase (plk), and thus maps to the mouse locus at 59 cM on chromosome seven. The human gene was independently mapped on a radiation hybrid panel to the syntenic region on chromosome 16 (Figure 1C).

The domain organization of mIre1 is consistent with that of a type I transmembrane protein. An expression plasmid that encodes for the full-length protein, tagged at its C-terminus with a Myc epitope, was transfected into COS1 cells. Immunostaining of permeabilized cells with the tag-specific monoclonal antibody, 9E10, revealed a fine reticular cytoplasmic pattern that overlapped closely with the ER marker, ribophorin 2 (Figure 1D). This finding is consistent with the encoded protein being an ER resident protein, as proposed for yeast Ire1p. Our assay does not detect the endogenous protein, and therefore we can not formally exclude the possibility that retention in the ER is an artifact due to over-expression of a protein that is normally targeted to another exocytic compartment. However, we note similar expression patterns in different cells types (see Figure 5B) and across varying levels of expression including very low levels, supporting but not proving the physiological relevance of this localization.

Yeast Ire1p, when over-expressed in yeast cells, triggers the activation of the UPR and increases the expression of Kar2, the yeast homolog of GRP78/BiP (Cox *et al*., 1993; Mori *et al*., 1993). We therefore tested the ability of mIre1 over-expression to trigger similar increase in mammalian *GRP78/BiP* expression. 293T cells transfected with an expression plasmid for Ire1 exhibit a substantial increase in GRP78/BiP mRNA (Figure 1E). This increase is still significantly less than that observed in response to tunicamycin, a potent inducer of the UPR. This assay, together with the subcellular localization and the primary amino-acid sequence homology strongly supports a functional homology between the mouse protein and yeast Ire1p.

To determine whether over-expression of mIre1 can induce CHOP mRNA, the aforementioned mIre1 expression plasmid was transfected into COS1 cells and the cells were stained with a CHOP-specific antiserum. mIre1-expressing cells expressed high levels of CHOP protein, whereas adjacent non-expressing cells stained negative for CHOP (Figure 2A). The ability of mIre1-overexpression to activate endogenous *CHOP* was confirmed in two other assays. A Northern blot of RNA from 293T cells over-expressing mIre1 revealed strong induction of endogenous CHOP mRNA (Figure 2B, lane 3). Over-expression of immunoglobulin μ heavy chain, that is retained in the ER in the absence of its corresponding light chain and can even induce the UPR under some circumstances (Lenny and Green, 1991), does not induce CHOP expression in our system (Figures 2A, panels h, i and j, and 2B, lane 4). The immunoglobulin μ heavy chain and mIre1 closely co-localize when expressed in the same cell, supporting the validity of this control (Figure 2A, panels f and g). A membrane-spanning derivative of Ire1, truncated at the C-terminus and lacking the effector domain, when expressed at similar levels, is likewise incapable of

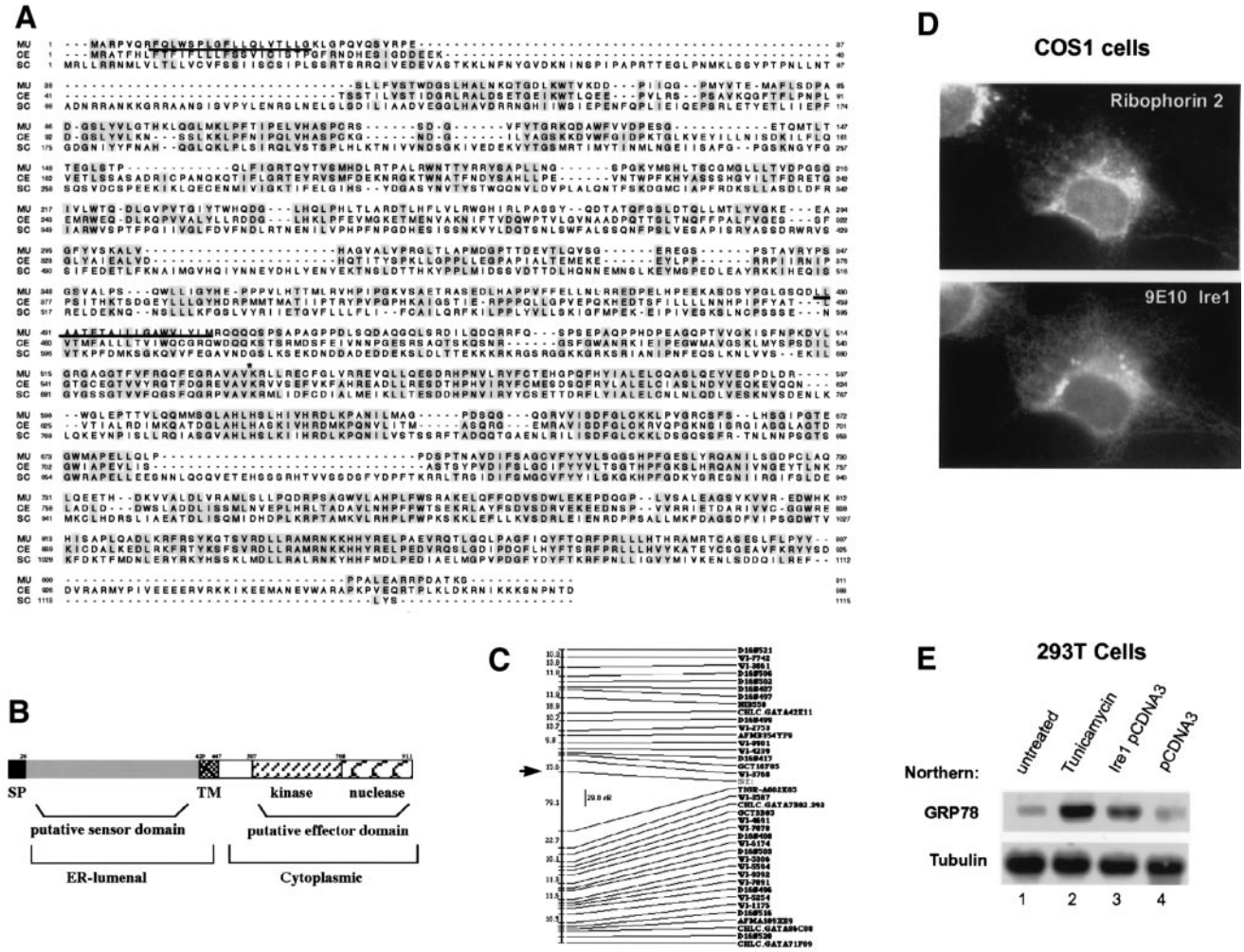

Fig. 1. Protein sequence of a murine homolog of yeast Ire1. (**A**) Global alignment of the murine (MU), *C.elegans* (CE) and yeast (SC) Ire1 protein sequence. Identical residues are shaded. The signal peptide and predicted transmembrane domain of the murine protein are underlined. The conserved lysine in kinase domain II, K536, is marked by the asterisks. (**B**) The predicted domain structure of mouse Ire1 is depicted in cartoon form. SP and TM refer to the signal peptide and transmembrane domains, respectively. (**C**) A radiation hybrid map of human chromosome 16 with the position of human *IRE1* indicated. The numbers to the left of the vertical bar reflect the distance between markers expressed in CentiRays. (**D**) Sub-cellular localization of mouse Ire1 expressed in COS1 cells. Cells transfected with an expression plasmid for Ire1 that had been tagged at its C-terminus with a Myc-epitope tag were fixed and stained with a polyclonal rabbit antiserum to the endogenous ribophorin 2, (an ER marker) and the anti-Myc epitope-tag MoAb 9E10 that stains the recombinant Ire1. (**E**) Northern blot analysis of GRP78/BiP expression in 293T cells that had been left untreated, transfected with the mouse Ire1 expression plasmid (Ire1 pcDNA3) the empty vector (pcDNA3) or treated with tunicamycin (1 μg/ml, 3 h) as a positive control.

activating *CHOP* (Figure 3A; data not shown). These results indicate that the induction of *CHOP* by Ire1 is not simply a consequence of the over-expression of an ER lumenal protein. Next, mIre1 was over-expressed in a Chinese hamster ovary (CHO) cell-line that contains an integrated CHOP–green fluorescent protein (GFP) reporter transgene; activity of GFP increased significantly (Figure 2C). This suggests that the effect of mIre1 on *CHOP* expression is mediated at a transcriptional level.

In yeast, over-expression of a truncated form of Ire1p that lacks a functional effector domain has been shown to interfere with the propagation of the signal leading to the induction of *KAR2* (Mori *et al*., 1993; Shamu and Walter, 1996). To determine if the lumenal domain of mIre1, when severed from the C-terminal putative effector domain, could exhibit similar dominant-negative effects on the mammalian UPR, an expression plasmid encoding a derivative of mIre1 that was truncated and tagged immediately C-terminal of the *trans*-membrane domain (Ire1-ΔC) was transfected into COS1 cells. The truncated protein is expressed at high level and is retained in the ER. Treatment of transfected cells with tunicamycin followed by double-staining for CHOP and Ire1-ΔC, consistently revealed lower levels of CHOP in cells that express Ire1-ΔC (Figure 3A, compare cells marked by arrows with adjacent unmarked cells). Similar results were observed in 293T cells transfected with Ire1-ΔC and treated with tunicamycin; levels of CHOP and GRP78/BiP induction in these cells are ~50% lower than in cells transfected with empty plasmid (Figure 3B). These experiments indicate that the lumenal domain of mIre1, when over-expressed in cells, can interfere with the activity of the endogenous inducer(s) of the UPR.

The results presented so far do not permit one to distinguish between a situation in which mIre1 over-expression activates the UPR by triggering a lumenal

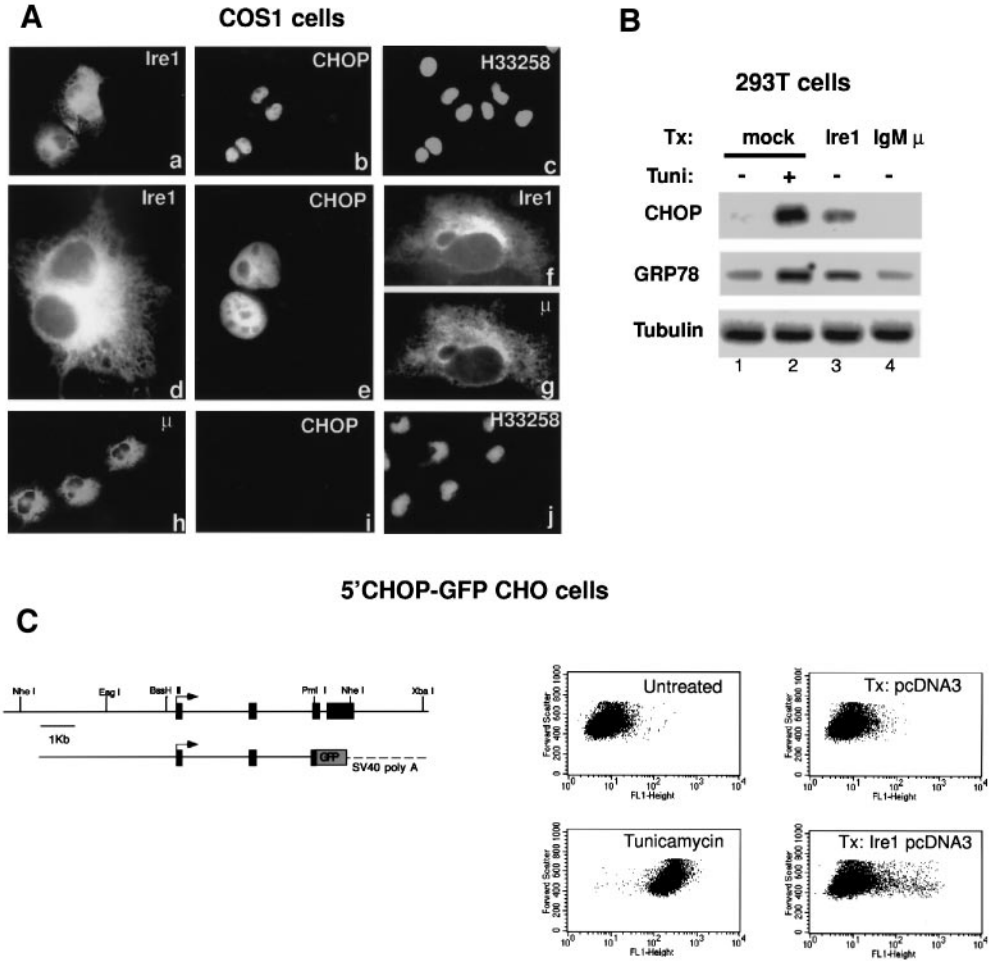

Fig. 2. Activation of endogenous CHOP by over-expression of mouse Ire1. (**A**) Immunocytochemical detection of the recombinant Ire1, the endogenous CHOP, the recombinant immunoglobulin μ heavy chain and nuclear staining with the karyophilic dye H33258 in COS1 cells transfected with expression plasmids expressing either Ire1 or μ heavy chain. Panels a–c, d and e, f and g, and h–j are photomicrographs of the same cells stained with different markers. Ire1 but not μ heavy chain induce endogenous CHOP expression. (**B**) Northern blot of mRNA derived from 293T cells transfected with expression plasmids for mouse Ire1 or immunoglobulin μ heavy chain hybridized sequentially to CHOP, BiP and α-tubulin probes. (**C**) FACScan of GFP activity in a CHO cell line carrying a CHOP–GFP reporter transgene. The GFP signal is on the horizontal axis and the forward scatter, a reflection of cell size, is on the vertical axis. The cells were untreated, treated with tunicamycin (0.5 μg/ml for 16 h), transfected with Ire1 or mock transfected. The structure of the murine CHOP gene and the derivative CHOP–GFP reporter transgene is depicted in cartoon form to the left of the FACScan scatter plots. Note the smaller size of the GFP positive cells in the Ire1-transfected experimental point.

event that is then transduced through the endogenous signaling cascade, and a situation in which the effector domain of the over-expressed mIre1 protein participates in propagating the signal. To address this issue, we over-expressed the isolated C-terminal domain of mIre1 in COS1 cells and measured its ability to induce endogenous *CHOP* expression. Two derivatives of the protein were prepared: Gal4–Ire^c, in which the C-terminus was fused to the DNA-binding domain of the yeast DNA-binding protein GAL4 (a domain that is known to impart nuclear localization; Silver *et al*., 1984), and GST–Ire^c in which the same domain of mIre1 was fused to *Schistosoma japonica* glutathione *S*-transferase (a cytoplasmic protein). Both fusion proteins were expressed at high levels in the transfected COS1 and 293T cells. Surprisingly, both were retained in the cytoplasm and both were able to potently activate the endogenous *CHOP* and, to a lesser degree, the endogenous *GRP78/BiP* genes (Figure 4A and B). The level of induction by the two proteins differed between COS1 and 293T cells, most probably reflecting differences in their expression level in these two cell types. While it is impossible to exclude that a small portion of GST–Ire^c or Gal4–Ire^c make their way into the nucleus, this result suggests that mIre1 can execute its role as a transducer of ER stress through a cytoplasmic signaling node. This conclusion holds true regardless of the mechanism by which these truncated proteins activate *CHOP* expression.

A mutant form of GST–Ire^c containing the K536A mutation was fully active in this assay (data not shown). However, a truncation that removes the C-terminal endonuclease homology domain of mIre1 significantly attenuated but did not entirely abolish the induction of CHOP (Figure 4C). In yeast, a similar deletion of the C-terminus of Ire1p resulted in a protein that was unable to rescue Kar2 induction in a Δire strain (Shamu and Walter, 1996). All other GST fusion proteins tested, including GST alone, fusions between GST and the cytosolic stress-activated kinases SEK and SAPKβ as well as a fusion between the stress-activated and apoptosis-inducing transcription factor ATF3 were expressed at similar or higher levels than

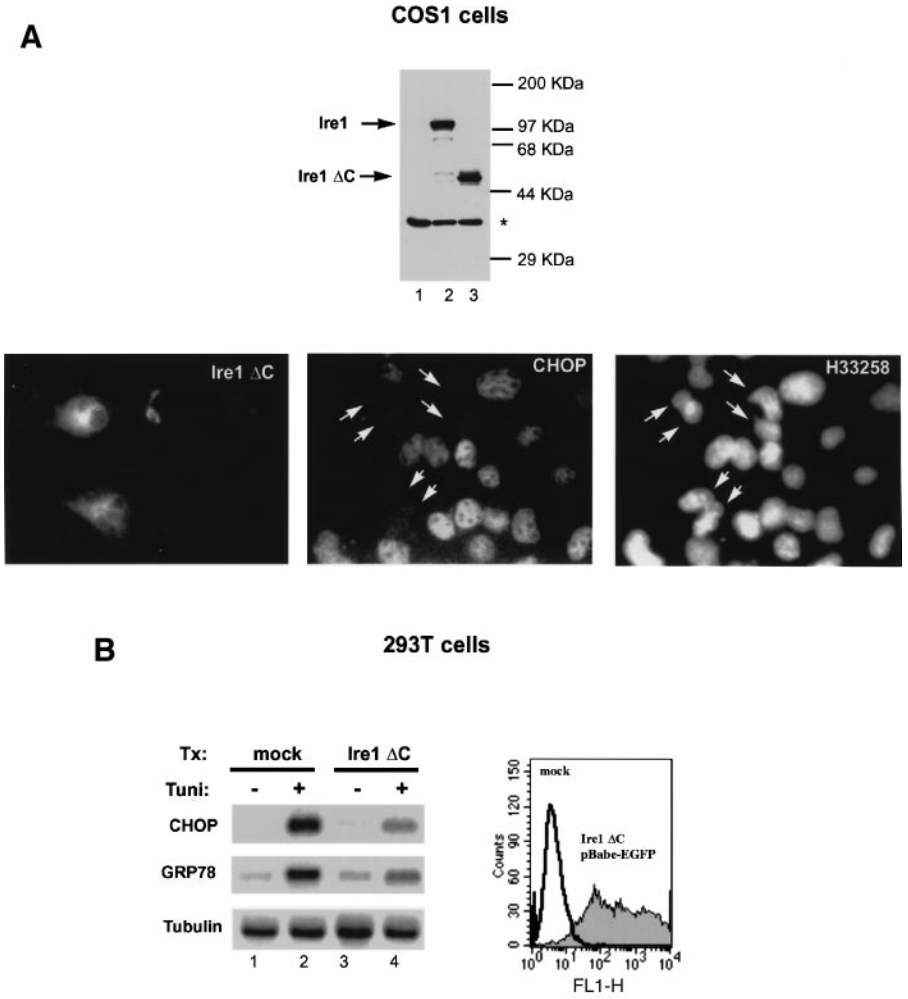

Fig. 3. Attenuated expression of CHOP in cells expressing a dominant negative form of Ire1. (**A**) Staining of the endogenous CHOP and tagged dominant-negative Ire1 derivative in tunicamycin treated cells that had been transfected with an plasmid expressing only the lumenal domain of mouse Ire1 (Ire1 ΔC). The arrows draw attention to the Ire1 expressing cells in both fields. The 9E10 Western blot above the photomicrograph reveals the apparent size of the tagged wild-type and ΔC forms of Ire1. The asterisks denotes a non-specific band detected by the 9E10 antibody. (**B**) Northern blot analysis of endogenous CHOP and GRP78/BiP in untreated and tunicamycin-treated 293T cells transiently expressing the ΔC derivative of Ire1. The FACScan to the right of the autoradiogram reveals that nearly 90% of the cells had been transfected in this experiment.

GST–Ire^c and did not induce endogenous *CHOP* (Figure 4C). These experiments artificially uncouple *CHOP* induction from a primary ER event and render it highly unlikely that the gene is induced through the activity of an ER stress transducer distinct from Ire1. This conclusion is valid regardless of whether the over-expressed cytosolic derivatives described above effect Ire1 action directly or if they do so by activating the endogenous protein.

In the course of conducting the over-expression experiments described above we noted the presence of many floating and crenated cells in the plates transfected with the mIre1 expression plasmid, but not in the control plates. This phenomenon was more pronounced in the CHO cells than in the COS1 or 293T cells. We also noted on the FACScan that the mIre1-expressing cells were smaller than average (compare the forward scatter with the level of GFP-expressing cells in Figure 2C), suggesting that they may be undergoing programmed cell death. In addition, we were unable to recover clones of cells stably over-expressing mIre1, but were able to recover clones over-expressing the truncated form of the protein (data not shown). Noting that conditions known to trigger the UPR are also associated with the induction of programmed cell death (see Introduction), we examined the potential role of mIre1 in that process. To this end, a CHO cell-line expressing a LacZ reporter gene under the control of the CHOP regulatory sequences was used. Incorporation of the mIre1 expression plasmid leads to induction of LacZ and blue staining of the fixed cells with X-gal. When we compared the morphology of the blue cells in a plate transfected 48 h earlier with mIre1 with that of the same cells expressing LacZ from a transfected LacZ-expression plasmid (CMV–LacZ), we noted that the blue cells in the Ire1-transfected plate were smaller and rounded-up, protruding from the plane of the cellular monolayer. The CMV–LacZ transfected cells, on the other hand, were flat and otherwise indistinguishable from the neighboring non-transfected cells (Figure 5A). To examine the nuclear morphology associated with these changes we fixed the transfected CHO cells, and stained them simultaneously with the Myc-tag antibody to reveal the mIre1-expressing cells and the karyophilic dye H33258

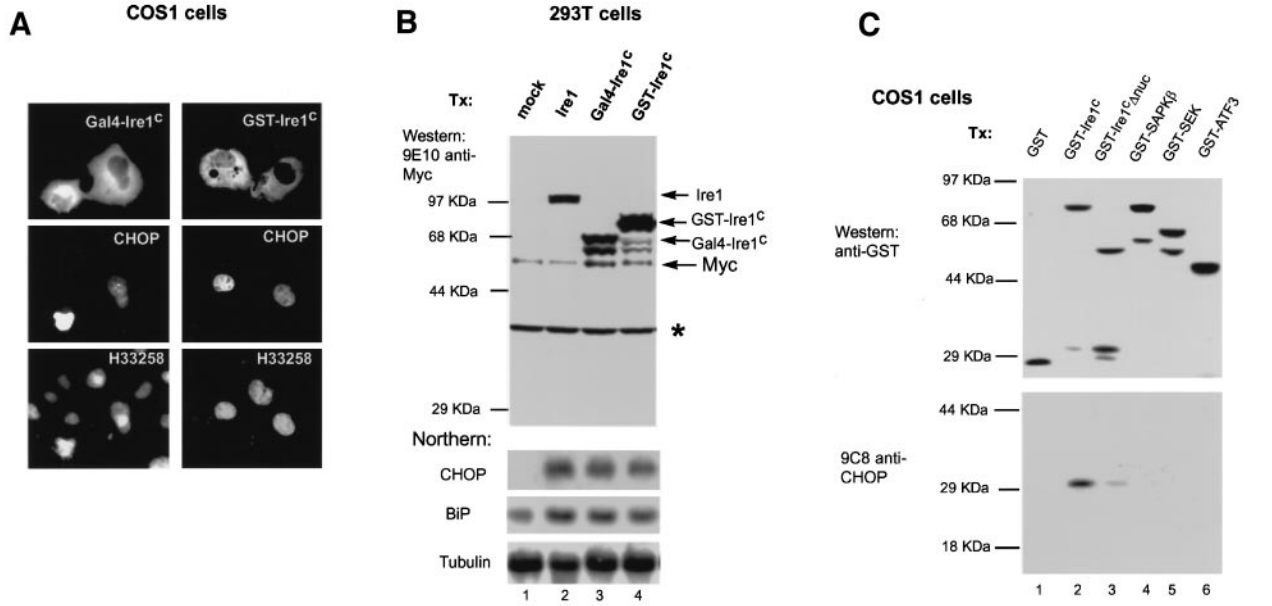

Fig. 4. Activation of CHOP by over-expression of the effector domain of Ire1 in the cytoplasm. (**A**) Immunostaining of endogenous CHOP, recombinant Ire1 derivative and chromatin in COS1 cells over-expressing fusion proteins between the effector domain of mouse Ire1 and the DNA-binding domain of Gal4 (Gal4–Ire1^c) or GST (GST–Ire1^c). Note that the normally-nuclear Gal4 is retained in the cytoplasm by fusion to the C-terminus of Ire1. (**B**) Northern and Western blot analysis of 293T cells transfected with the indicated Myc-tagged Ire1 derivatives. The asterisks on the Western blot denotes a non-specific band detected by the 9E10 antibody. (**C**) Anti-GST (upper panel) and Anti-CHOP (lower panel) Western blots of COS1 cells expressing the GST–Ire1c fusion protein (lane 2) and a derivative in which the endonuclease-specific portion of the effector had been removed, GST–Ire1^c Δnuc (lane 3). The other GST fusion proteins containing the kinases SEK1 and SAPKβ and the transcription factor ATF3 serve as negative controls for the induction of the endogenous CHOP by the mouse Ire1 derivatives.

to stain the chromatin. mIre1-expressing cells were reproducibly smaller and exhibited condensed chromatin, a feature of programmed cell death. Cells transfected with the control μ-chain expression plasmid had normal morphology and chromatin pattern (Figure 5B).

To validate these results, the Ire1 cDNA was co-expressed with a membrane-targeted form of green fluorescent protein, farnesylated enhanced fluorescence protein (GFP^{farn}). CHO cells transfected with this plasmid were stained with propidium iodide, and analyzed for DNA content and expression of GFP^{farn} by dual channel FACScan. As controls, the CHO cells were left untransfected or transfected with the empty GFP^{farn} vector. The cells co-expressing GFP^{farn} and Ire1 contained a significant sub-population with reduced DNA content ('hypo-diploid' cells), consistent with cells undergoing programmed cell death. A small increase in the proportion of such dying cells is also observed in the population transfected with the empty GFP^{farn} vector and is presumably due to the toxicity of the Lipofectamine transfection procedure (Figure 5C). These results support a role for mIre1 in the induction of programmed cell death in response to conditions that trigger the UPR.

Discussion

The mIRE1 cDNA described here is a good candidate for encoding a mammalian ortholog of yeast Ire1p: beyond the similarity in primary amino-acid sequence, the similar predicted domain structure and membrane topology, the experimental results indicate that mIre1 is an ER-resident protein that is capable of activating *GRP78/BiP* expression in the absence of other signals when over-expressed in mammalian cells. Its ability to also activate transcription of the *CHOP* gene provides evidence for the existence of a common signaling node that coordinates at least two different read-outs for ER stress: one that culminates in the induction of ER chaperones and another that induces non-ER resident proteins such as the transcription factor CHOP. The *CHOP* knock-out mice have normal inducibility of *GRP78/BiP* (Zinszner *et al*., 1998), indicating that the two responses represent a bifurcation of one signaling pathway downstream of IRE1, as opposed to one pathway that operates in a linear fashion. The fact that *CHOP* is itself required for the activation of a set of genes that are induced by the UPR (Wang *et al*., 1998), implies that in mammalian cells Ire1 controls a rather diverse and arborized stress response. Based on the yeast model, this dual regulation could come about if both the GRPs and CHOP are co-regulated by the mammalian Hac1 equivalent(s). However, the data presented here by no means exclude a more complex relationship between Ire1 and *CHOP*. For example, Ire1 may induce a secondary modification in ER structure or function which then leads to *CHOP* activation through a parallel signaling pathway initiating from the ER. The hypothesized role of yeast Ire1p in regulating endomembrane biogenesis (Cox *et al*., 1997) implies that signaling downstream of Ire1 may modulate aspects of ER function that go beyond the mere production of ER chaperones. These might include modifications that impact on *CHOP* induction. The identification of the hypothesized mammalian Hac1-equivalent(s) will hopefully resolve the details of the epistatic relationship between *IRE1* and *CHOP*.

One facet of the complexity of the UPR, demonstrated here, is the association between activated Ire1 signaling

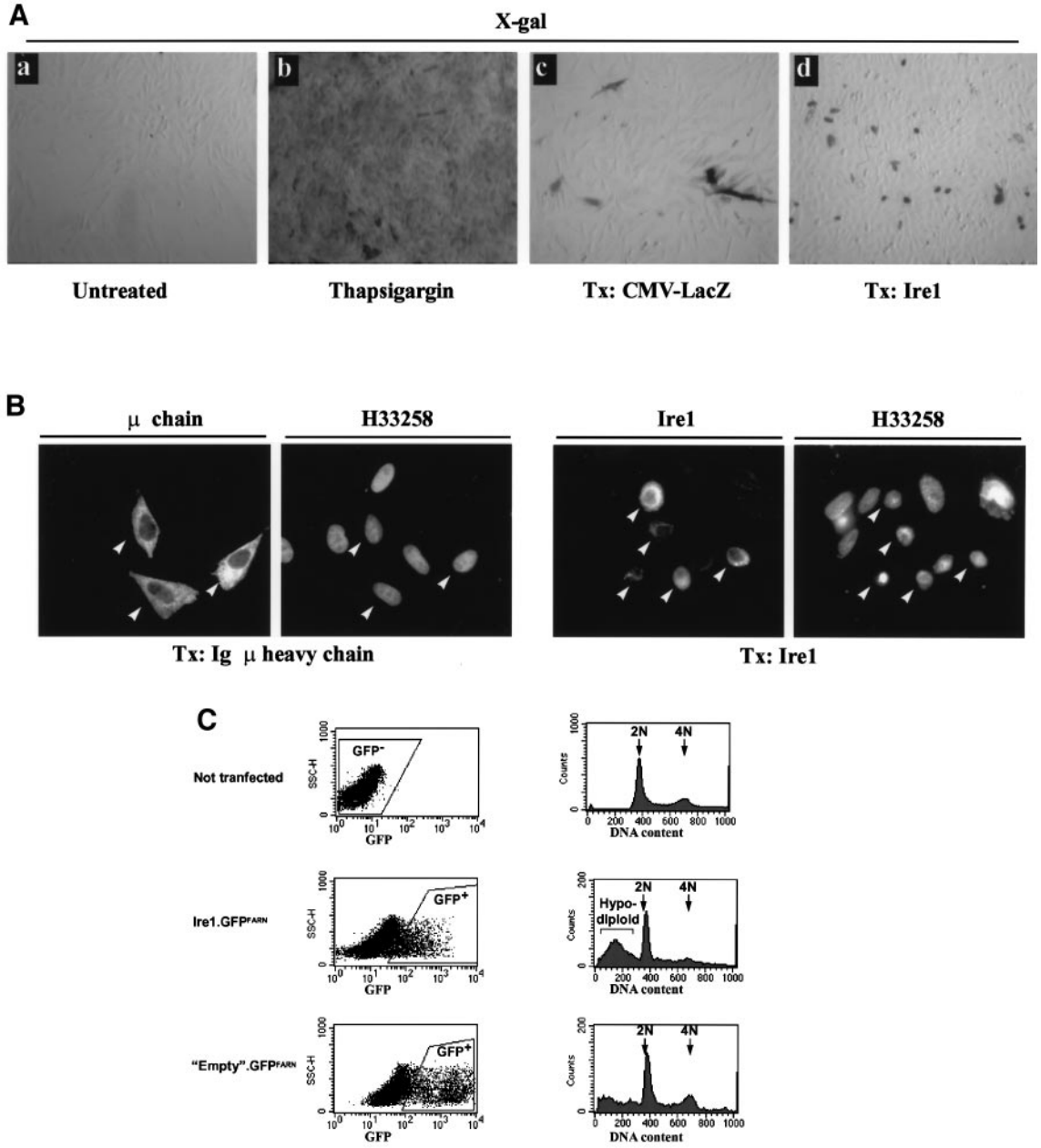

Fig. 5. Ire1 over-expression promotes cell death. (**A**) CHOP–LacZ reporter CHO cells were stained with X-gal following treatment with thapsigargin (2 μM, 2 h), transfection with a LacZ expression plasmid and transfection with the mouse Ire1 expression plasmid. Note that many of the LacZ expressing cells in the plate transfected with the Ire1-expression plasmid (plate d) are smaller and are in a different focal plane than the monolayer of untransfected cells or the LacZ-expressing cells in the adjacent CMV–LacZ transfected control panel (plate c). (**B**) Nuclear morphology of CHO cells expressing mouse Ire1 and immunoglobulin μ heavy chain. The arrows denote the transfected cells. Note the condensed appearance of the chromatin in the Ire1 expressing cells but not in the μ heavy chain expressing cells. (**C**) Dual channel FACScan of CHO cells transfected with a plasmid expressing Ire1 and GFP^{farn}, GFP^{farn} alone or non-transfected cells stained with propidium iodide. The left panels show the distribution of green fluorescence in the total population and the gating parameters used to define the transfected (GFP^+) cells. The right panels show the distribution of DNA content in the GFP^+ population. The position of the 2N and 4N peaks and the hypo-diploid population of dying cells is indicated.

and the induction of cell death. This is a feature that is likely to be unique to metazoans and can be rationalized in terms of the fact that ER stress is often associated with conditions that are severely damaging to cells. Therefore, linking the ER stress response to programmed cell death through Ire1 may be beneficial under certain circumstances. The observation that CHOP plays a role in promoting programmed cell death, suggests that it may serve as link between Ire1 signaling and the induction of that response (Zinszner *et al*., 1998).

The mechanism of induction of *CHOP* and *GRP78/BiP* by mIre1 over-expression is not clear. In yeast it has been proposed that over-expression of Ire1p leads to a constitutive dimer/oligomer formation even in the absence of ER stress. This, in turn, promotes *trans*-phosphorylation of Ire1p and activates the endonucleolytic activity of the protein which then signals downstream. Supporting this model is the observation that a lysine to alanine mutation in the conserved ATP binding lobe of the kinase domain of Ire1p abolishes the ability of the over-expressed yeast

protein to constitutively activate the UPR (Cox *et al*., 1993; Mori *et al*., 1993; Shamu and Walter, 1996). The same mutation also abolishes the ability of the protein to rescue a null allele of yeast *IRE1* and the expressed protein may even have dominant-negative activity on the pathway (Mori *et al*., 1993), again pointing to a pivotal role for the kinase activity in yeast Ire1p function. Surprisingly, an mIre1 derivative, GST–Ire^c (Figure 4B and C) that bears a homologous mutation, K536A, is at least as active as the wild-type protein in terms of inducing *CHOP* and *GRP78/BiP* when over-expressed in mammalian cells (data not shown). Assuming that these truncated derivatives of mIre1 function through the activity of their effector domain, these findings suggest that over-expression in mammalian cells somehow abrogates the requirement for *trans*-phosphorylation.

The mammalian system described here also reveals an additional feature of Ire1 signaling, namely the possibility of uncoupling the C-terminal effector domain of the protein from its regulatory lumenal domain. The transient transfection paradigm is particularly well suited to this kind of experiment because it is not encumbered by the negative selection that would probably impact the stable expression of a constitutively active Ire1 fragment. Surprisingly, over-expression of the effector domain of mIre1 in the cytoplasm elicits high-level activation of *CHOP*. At the very least, the results presented here suggest that the signaling pathway can also be accessed from the cytoplasmic side. This conclusion holds true regardless of whether the GST–Ire^c fusion protein activates the UPR by mimicking activated Ire1 and splicing a mammalian Hac1-like substrate (as suggested by the diminished activity of the truncated protein that lacks the endonuclease portion of mIre1; Figure 4C, lane 3), or by competing for an inhibitory factor or de-localizing the endogenous mIre1 (as suggested by the residual activity of the endonuclease deleted GST–Ire^c Δnuc fusion protein; Figure 4C, lane 3). The recent identification of a phosphatase that can bind to and dephosphorylate yeast Ire1p and attenuate the UPR (Welihinda *et al*., 1998) provides one plausible mechanism by which such dominant negative effects of GST–Ire^c might take place. Either way, a component(s) of the signaling pathway is likely to be cytosolic. Even a model whereby the substrate mRNA resides in the cytoplasm and is cleaved there by Ire1 is not so heretical, given that in yeast, the ligase that effects the last step of the splicing reaction, tRNA-ligase, is localized to the nuclear pore (Clark and Abelson, 1987) and can presumably be accessed from both sides of that structure.

Our studies also hint at the possibility that the non-lumenal domain of mIre1 may positively interact with a cytoplasmic factor: it has previously been demonstrated that the Gal4 DNA-binding domain serves as a dominant nuclear localization domain that leads to the translocation of fusion proteins to the nucleus (Silver *et al*., 1984). The Gal4–Ire^c fusion was made in an effort to promote stronger signaling by localizing the effector domain of mIre1 to that compartment, and was based on the prevailing model that stipulates a nuclear site of action for the Ire1 endonuclease. Unexpectedly, that fusion protein is selectively retained in the cytoplasm and activates the downstream target genes no better than GST–Ire^c. It will be interesting to learn if the C-terminal domain of mIre1 is capable of leading to the cytoplasmic retention of other nuclear import substrates. Finally, it is important to point out that all the conclusions presented here have been drawn from genetic gain-of-function over-expression experiments. A more definitive understanding of the role of mIre1 in the mammalian UPR will come from studying deficiency mutations in that gene.

During the review of this manuscript, a paper describing the cloning of a human homolog of yIRE1 was published (Tirasophon *et al*., 1998). Analysis of the EST database and cloning of a partial cDNA of the mouse orthologue of that human gene informs us that mice and humans have at least two *IRE1* genes. The murine locus corresponding to the gene described in the aforementioned manuscript is now referred to as Ern1 and the locus containing the gene described here is Ern2.

Materials and methods

cDNA library construction, cDNA cloning and chromosomal mapping of Ire1

An oligo-dT primed murine colon cDNA library was constructed according to the manufacturer's instructions in a λ-ZapII vector (Stratagene). The unamplified library was screened by hybridizing to the insert of the mouse EST clone vo50h02, obtained from Genome Systems. Thirty-seven positive clones were identified among 4 million insert-containing recombinants screened (implying an mRNA abundance of ~1:100 000). The cDNA was sequenced from several overlapping clones to generate a full-length coding region sequence that has been deposited in the DDBJ/EMBL/GenBank (accession No. AF071777). To map the chromosomal localization of the human Ire1 gene, a pair of primers based on the sequence of the 3′ end of the human EST clones z189c06 and ng38c07 were designed (sense: 5′-CACAAG ACTGGC AGAGGC TGGGCA-3′; antisense: 5′-CTGTCC TTTCCT TGGCTT TATGCA CA-3′). When used under the following PCR conditions; 94°C (5′) → [94°C (1), 55°C (1), 72°C (1)] X30 → 72°C (10), these amplify the human but not the hamster allele. These were used to screen the GeneBridge 4 radiation hybrid panel (Research Genetics) and the following vector was obtained: 00000 00101 00000 01001 11000 00110 00100 01010 11100 10001 20000 01110 01101 00200 01100 00000 00111 11001 000, corresponding to the address presented in Figure 1C. The 3′ ends of the aforementioned human ESTs also contain a perfect match to the last 55 base pairs from the extreme 3′ end of the human PLK cDNA, suggesting a tail-to-tail orientation of *IRE1* and *PLK* on human chromosome 16. Three different genomic PACs containing the murine *Ire1* gene also specifically hybridized to the *plk* cDNA, supporting a similar arrangement in the mouse genome (data not shown). *plk* is mapped in the mouse to chromosome 7 at 59 cM (Clay *et al*., 1997), a region syntenic to the address obtained here for the human *IRE1*.

Expression vector construction

Mouse Ire1 was tagged with a Myc epitope (9E10) at its C-terminus by overlapping PCR (Squinto *et al*., 1990) using the following primers: 'mIre(c).9E10', 5′-GAGATC AGCTTC TGCTCG CCTCCG CTCTTT GTGGCA TCTG-3′; and '9E10. 3′U', 5′-GGGGCT CGAGTC ACA-GAT CCTCCT CAGAGA TCAGCT TCTGCT C-3′. The full-length coding region of mIre1 with the C-terminal 9E10 tag was cloned as *Eco*RI–*Xho*I fragment into pCDNA3 (Invitrogen). The same strategy was to used to generate a truncated form of mIre1, 'Ire1-ΔC'. The overlapping primer used in PCR; 'mIre(1574).9E10', 5′-GAGATC AGCTTC TGCTCC CCACGG CCCAGC ACA-3′, truncates the protein at Ala518, just 3′ of the transmembrane domain. The *Eco*RI–*Xho*I fragment encoding the mIre1 ER lumenal domain and transmembrane domain was then cloned into *Eco*RI–*Sal*I site of pBabe-puro or pBabe-EGFP. To generate the in-frame fusion of mIre 1 cytoplasmic domain with Gal4 DNA-binding domain or GST, *Xba*I (in-frame with the *Spe*I site of pEBG; Sánchez *et al*., 1994) and *Eco*RI sites (in-frame with *Eco*RI site of pSG424; Sadowski and Ptashne, 1989) were introduced by PCR into the mIre1 cDNA at a position corresponding to aminoacid Ser453 using the primer 'mIre.1380.*Xba*I–*Eco*RI', 5′-GAGGTC TA-GAGA ATTCAG CCCTTC AGCACC TGCT-3′ and the primer '9E10. 3′U', as described above. The corresponding fragment was then cloned

into the indicated recipient plasmids. The *Hin*dIII–*Xba*I fragment containing the entire protein coding region of Gal4–Ire1^c was then transferred into pCDNA3 to achieve higher level of expression. The GST–Ire1^c deleted of the nuclease domain, GST–Ire1^c Δnuc, was constructed by deleting the 3′ end of mIre1 at *Bam*HI^{2196}. This truncates the mIre1 protein at amino acid Asp725. The GST–SEK and GST–SAPKβ expression plasmids (Sánchez *et al*., 1994) were a gift of Len Zon (Harvard) and the GST–ATF3 plasmid has been previously described (Wang *et al*., 1998). The plasmid used for co-expressing Ire1 and GFP^{farn} was constructed in a pCDNA3 backbone. Briefly, because wild-type *Aequorea victoria* GFP is extracted from cells by the permeabilization step required for propidium iodide (PI) staining (Kalejta *et al*., 1997), we tagged the enhanced GFP cDNA (Clontech) with the 20 C-terminal residues of Ha-Ras which encode the farnesylation sequence and target the GFP^{farn} to the plasma membrane, leading to its retention during PI staining. The GFP^{farn} coding region was used to replace the *neo^r* gene in pCDNA3, creating pCDNA3.GFP^{farn}. The Ire1 coding region was introduced into this plasmid as an *Eco*RI–*Xho*I fragment, downstream of the CMV promoter.

RNA isolation and Northern blot analysis

Total mouse colon RNA for cDNA synthesis was prepared by GTC lysis and CsCl ultra centrifugation. Poly(A)$^+$ RNA was isolated by two passes over an oligo-dT cellulose column (Molecular Research Center, Inc.). For Northern blot analysis, total RNA (20 μg/lane) was fractionated on a 1.2% formaldehyde agarose gel and transferred onto HyBond-N nylon membrane (Amersham). The ^{32}P murine CHOP, BiP and α-tubulin cDNA fragments were hybridized to the blot as previously described (Wang *et al*., 1998).

Cell culture, transfection and treatment

COS-1, 293T and CHO K1 cells were originally obtained from ATCC. They were cultured in Dulbecco's modified Eagle's medium (DMEM) or DMEM:F12 (CHO cells) in the presence of 10% fetal calf serum (Atlanta Biological). The construction of the CHOP–GFP and CHOP–LacZ CHO reporter cells used in Figures 2C and 5A will be described in detail elsewhere (H.P.Harding and D.Ron, in preparation). The murine CHOP genomic fragment used was 8.5 kb in length and its 3′ end corresponds to the *Pml*I site in exon 3, nine nucleotides 5′ to the initiation methionine of CHOP. Cells were treated with tunicamycin (1 μg/ml) for 3 h. 5′ CHOP–LacZ reporter CHO K1 cells were treated with 2 μM thapsigargin for 2 h. Tunicamycin and thapsigargin were purchased from Sigma (St Louis, MO). 293T cells were transfected by the calcium phosphate precipitation method using 7 μg plasmid DNA for each 60 mm plate. COS-1 cells were transfected by DEAE-dextran method using 3 μg of plasmid DNA per 60 mm plate. CHO K1 cells carrying the GFP– or LacZ–CHOP-reporter genes were transfected with 1 μg plasmid DNA per 35 mm plate by the Lipofectamine *plus* method (Gibco-BRL). Cells were harvested at 48 h after transfection for either immunocytochemistry, Western blot, Northern blot or FACScan analysis as previously described (Wang *et al*., 1998). Transfection efficiency was monitored by FACScan analysis of the GFP^{farn} expression, either as a marker in bi-cistronic expressing vector or by co-transfection of CMV–EGFP marker. Propidium iodide analysis of DNA content was performed by FACScan, as previously described (Zinszner *et al*., 1998).

Antibodies, Western blot and immunocytochemistry

The various constructs of Ire1 were tagged with a Myc epitope at their C-terminus, replacing the stop codon by the tag. The expression of the recombinant protein was detected by the murine anti-human Myc monoclonal antibody 9E10 (Evan *et al*., 1985). To detect endogenous CHOP in COS-1 cells, rabbit anti-CHOP polyclonal antiserum (Ron and Habener, 1992) was used in all experiments except Figure 2A (bottom panel) and Figure 4A, where the murine anti-CHOP monoclonal antibody 9C8 (Batchvarova *et al*., 1995) was used to avoid either the cross-reactivity of the secondary antibodies (Figure 2A, bottom) or reactivity of the rabbit anti-CHOP with GST (Figure 4A and B). Rabbit anti-ribophorin 2 polyclonal antibody (a gift of Gert Kreibich, NYU, USA) was used at a dilution of 1:200 to visualize the ER staining pattern. To reveal the various GST fusion proteins in Figure 4C, rabbit GST-reactive antisera was used for Western blot analysis. To detect protein expression at the single-cell level, cells were fixed with 3.7% formaldehyde phosphate-buffered saline (PBS) for 5 min, and permeabilized with 0.25% Triton X-100 for 5 min and methanol for 5 min. The cells were then stained for the presence of protein with the indicated antibodies.

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