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Review Series

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Introduction

The ER fulfills multiple cellular functions (reviewed in refs. 1–4). The lumen of the ER is a unique environment, containing the highest concentration of Ca^{2+} within the cell because of active transport of calcium ions by Ca^{2+} ATPases. The lumen is an oxidative environment, critical for formation of disulfide bonds and proper folding of proteins destined for secretion or display on the cell surface. Because of its role in protein folding and transport, the ER is also rich in Ca^{2+}-dependent molecular chaperones, such as Grp78, Grp94, and calreticulin, which stabilize protein folding intermediates (reviewed in refs. 1, 5–7).

Many disturbances, including those of cellular redox regulation, cause accumulation of unfolded proteins in the ER, triggering an evolutionarily conserved response, termed the unfolded protein response (UPR). Glucose deprivation also leads to ER stress, by interfering with N-linked protein glycosylation. Aberrant Ca^{2+} regulation in the ER causes protein unfolding, because of the Ca^{2+}-dependent nature of Grp78, Grp94, and calreticulin (6). Viral infection may also trigger the UPR, representing one of the ancient evolutionary pressures for linking ER stress to cell suicide in order to avoid spread of viruses. Further, because a certain amount of basal protein misfolding occurs in the ER, normally ameliorated by retrograde transport of misfolded proteins into the cytosol for proteasome-dependent degradation, situations that impair proteasome function can create a veritable protein traffic jam and can even cause inclusion body diseases associated with neurodegeneration.

The initial intent of the UPR is to adapt to the changing environment, and reestablish normal ER function. These adaptive mechanisms involve transcriptional programs that induce expression of genes that enhance the protein folding capacity of the ER, and promote ER-associated protein degradation to remove misfolded proteins. Translation of mRNAs is also initially inhibited, reducing the influx of new proteins into the ER for hours until mRNAs encoding UPR proteins are produced. When adaptation fails, ER-initiated pathways signal alarm by activating NF-κB, a transcription factor that induces expression of genes encoding mediators of host defense. Excessive and prolonged ER stress triggers cell suicide, usually in the form of apoptosis, representing a last resort of multicellular organisms to dispense of dysfunctional cells. Progress in understanding the mechanisms underlying these 3 phases of adaptation, alarm, and apoptosis has improved our knowledge of ER stress, and its role in disease.

Adaptation to ER stress: mechanisms to restore homeostasis

When unfolded proteins accumulate in the ER, resident chaperones become occupied, releasing transmembrane ER proteins involved in inducing the UPR. These proteins straddle ER membranes, with their N-terminus in the lumen of the ER and their C-terminus in the cytosol, providing a bridge that connects these 2 compartments. Normally, the N-termini of these transmembrane ER proteins are held by ER chaperone Grp78 (BiP), preventing their aggregation. But when misfolded proteins accumulate, Grp78 releases, allowing aggregation of these transmembrane signaling proteins, and launching the UPR. Among the critical transmembrane ER signaling proteins are PERK, Ire1, and ATF6 (Figure 1) (reviewed in refs. 1, 2, 8).

PERK (PKR-like ER kinase) is a Ser/Thr protein kinase, the catalytic domain of which shares substantial homology to other kinases of the eukaryotic initiation factor 2α (eIF2α) family (9, 10). Upon removal of Grp78, PERK oligomerizes in ER membranes, inducing its autophosphorylation and activating the kinase domain. PERK phosphorylates and inactivates eIF2α, thereby globally shutting off mRNA translation and reducing the protein load on the ER. However, certain mRNAs gain a selective advantage for translation under these conditions, including the mRNA encoding transcription factor ATF4. The ATF4 protein is a member of the bZIP family of transcription factors, which regulates the promoters of several genes implicated in the UPR. The importance of PERK-initiated signals for protection against ER stress has been documented by studies of *perk*$^{-/-}$ cells and of knock-in cells that express non-phosphorylatable eIF2α(S51A), both of which display hypersensitivity to ER stress (11, 12).

Ire1 similarly oligomerizes in ER membranes when released by Grp78. The Ire1α protein is a type I transmembrane protein, which contains both a Ser/Thr kinase domain and an endoribonuclease domain; the latter processes an intron from X box protein-1 (XBP-1)

Nonstandard abbreviations used: AD, Alzheimer disease; AβP, amyloid β-peptide; DED-L, death effector domain–like; eIF2α, eukaryotic initiation factor 2α; Htt, Huntingtin; IP3, inositol triphosphate; IP3R, IP3 receptor; NOS, nitric oxide synthase; PD, Parkinson disease; PERK, PKR-like ER kinase; polyQ, polyglutamine; PS-1, presenilin-1; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; UPR, unfolded protein response; XBP-1, X box protein-1.

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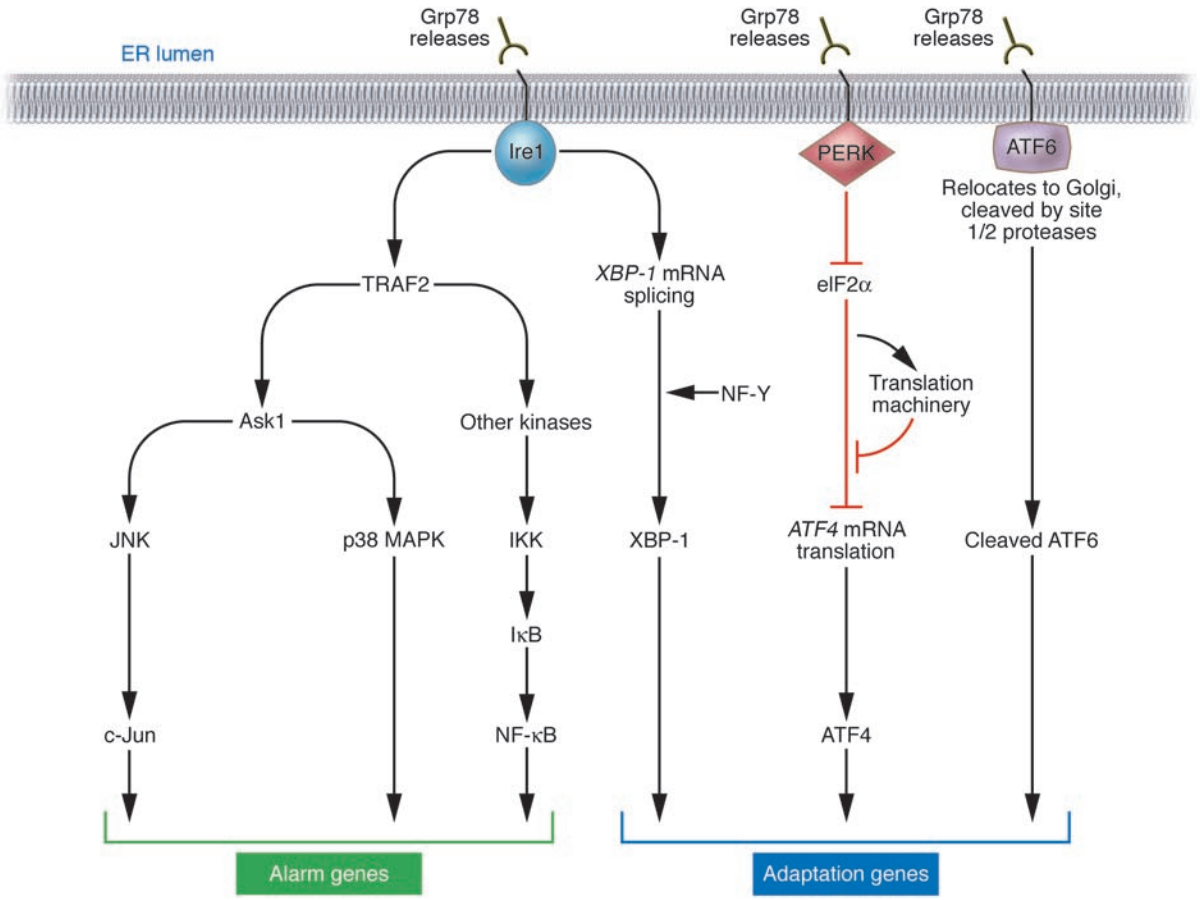

Figure 1
Signal transduction events associated with ER stress. Chaperone Grp78 binds the N-termini of Ire1, PERK, and ATF6, preventing their activation. Unfolded proteins in the ER cause Grp78 to release Ire1, PERK, and ATF6. Upon Grp78 release, Ire1 and PERK oligomerize in ER membranes. Oligomerized Ire1 binds TRAF2, signaling downstream kinases that activate NF-κB and c-Jun (AP-1), causing expression of genes associated with host defense (alarm). The intrinsic ribonuclease activity of Ire1 also results in production of XBP-1, a transcription factor that induces expression of genes involved in restoring protein folding or degrading unfolded proteins. Oligomerization of PERK activates its intrinsic kinase activity, resulting in phosphorylation of eIF2α and suppression of mRNA translation. Under these conditions, only selected mRNAs, including ATF4, are translated. ATF4 induces expression of genes involved in restoring ER homeostasis. Release of Grp78 from ATF6 allows this protein to translocate to the Golgi apparatus for proteolytic processing to release active ATF6, which controls expression of UPR genes.

mRNA, rendering it competent for translation to produce the 41-kDa XBP-1 protein, a bZIP-family transcription factor. XBP-1 binds to promoters of several genes involved in retrograde transport of misfolded proteins from ER to cytosol and in ER-induced protein degradation (reviewed in ref. 8). XBP-1 heterodimerizes with protein NF-Y and binds at least 2 types of *cis*-acting elements in gene promoters, including the ER stress enhancer (ERSE) and unfolded protein response element (UPRE) (13). Ablation of Ire1α in mice produces an embryonic lethal phenotype. Fibroblasts from *Ire1α*$^{-/-}$ embryos are defective in activation of UPRE-driven reporter genes, thus showing a cause-and-effect linkage of Ire1α to this *cis*-acting element (14).

Release of Grp78 from the N-terminus of ATF6 triggers a different mechanism of protein activation, compared with PERK and Ire1. Instead of oligomerizing, release of Grp78 frees ATF6 to translocate to the Golgi apparatus, where resident proteases (site 1 and site 2 protease) cleave ATF6 at a juxtamembrane site, releasing this transcription factor into the cytosol and allowing it to migrate into the nucleus to regulate gene expression (15). ATF6 collaborates with Ire1, where ATF6 induces transcription to increase XBP-1 mRNA, and Ire1's endoribonuclease activity then processes that mRNA so that XBP-1 protein is produced.

Sounding the alarm in response to ER stress: NF-κB activation mechanisms

Given the massive glycoprotein production associated with many viral infections, it is not surprising that ER stress activates some of the same signal transduction pathways associated with innate immunity. In this regard, Ire1 shares in common with many members of the TNF receptor family the ability to bind adapter protein TRAF2. TRAF2 is an E3 ligase that binds Ubc13, resulting in non-canonical polyubiquitination of substrates involving lysine 63 rather than the canonical lysine 48 as a linking site (16). TRAF2 activates protein kinases implicated in immunity and inflammation, including Ask1, which activates JNK, and kinases linked to NF-κB activation. Also recruited to Ire1 is the c-Jun N-terminal inhibitory kinase (JIK), responsible for posttranslational modification of components of the Ire1α/TRAF2/Ask1 complex (17, 18).

Apoptosis induced by ER stress: so many mechanisms, so little clarity

The adaptive responses to misfolded proteins in the ER provide protection from cell death, inasmuch as gene transfer–mediated overexpression of Grp78 or protein-disulfide isomerase (PDI) reduces cell death induced by oxidative stress, Ca^{2+} disturbances, and hypoxia (19, 20). However, when protein misfolding is persistent or excessive, ER stress triggers cell death, typically apoptosis. Several mechanisms, described below, have been proposed for linking the distressed ER to cell death (Figure 2), including direct activation of proteases, kinases, transcription factors, and Bcl-2–family proteins and their modulators.

Proteases. Caspases are required for apoptosis, and certain members of this family of cysteine proteases associate with the ER (reviewed in ref. 21). In rodents, caspase-12 associates with activated Ire1, resulting in proteolytic processing of caspase-12. Mice lacking caspase-12 genes display partial resistance to pharmacological inducers of ER stress, such as tunicamycin (inhibitor of N-linked protein glycosylation) and thapsigargin (inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases [SERCAs], which pump Ca^{2+} into the ER) (22). Because proteolytic activity has been difficult to demonstrate for caspase-12 (23), it is unknown whether the proteolytic processing of caspase-12 that occurs during ER stress results in its activation. Also, the mechanisms responsible for proteolysis of caspase-12 may be indirect, involving calpains activated by Ca^{2+} released in the vicinity of the ER (24), instead of an induced proximity mechanism where oligomers of Ire1 provide a scaffold for clustering caspase-12 zymogens. Caspase-7 also may activate caspase-12 by translocating from cytosol to ER (25). However, the relevance of caspase-12 to ER-induced apoptosis has been questioned because of an absence of caspase-12 in most humans. In this regard, the ancestral human *CASPASE-12* gene is disrupted by a termination codon and thus is inactive (26). For persons with hereditary polymorphisms that leave the open reading frame intact (estimated at ~1% of African populations), caspase-12 operates as a *trans*-dominant inhibitor of proinflammatory caspases, lacking conserved residues required for catalytic activity (23).

Human caspase-4, one of the closest paralogs of rodent caspase-12, may associate with ER (27), raising the possibility that this protease can perform the functions normally ascribed to rodent caspase-12 in the context of ER stress. But caspase-4 belongs to the group of proinflammatory caspases responsible for proteolytic activation of cytokines, rather than the apoptotic caspases. Nevertheless, small interfering RNA–mediated knock down of caspase-4 in human neuroblastoma cells partially reduces cell death caused by the ER stress inducers thapsigargin and amyloid β-peptide (AβP), but not inducers of mitochondria-dependent cell death (e.g., UV irradiation, DNA-damaging drugs). However, caspase-4 knock down in HeLa cells had little effect on apoptosis induced by ER stress, implying that the relevance of this protease to ER stress is tissue-specific.

The ER resident protein Bap31 contains 3 predicted transmembrane domains, followed by a leucine zipper and a death effector domain–like (DED-L) region that associates with certain isoforms of procaspase-8 in the cytosol (28). Bap31 can display either pro- or antiapoptotic phenotypes, depending on whether its cytosolic tail is removed by cleavage by caspases. Overexpression of full-length Bap31 blocks apoptosis induced by anti-Fas antibody and cycloheximide, while expression of the truncated 20-kDa protein induces apoptosis (29, 30). A mutant of Bap31 in which the caspase-8 cleavage site was mutated suppressed Fas-induced apoptosis (29). Since proximal steps in Fas signaling were not blocked by mutant Bap31, this suggests that the ER participates as an intermediary in death receptor–induced apoptosis in some cells.

The DED-L domain of Bap31 also binds a homologous DED-L domain in BAR, another ER-associated apoptosis regulator (31). The 52-kDa BAR protein contains a RING domain that binds ubiquitin-conjugating enzymes, followed by an α-helical region that binds Bcl-2 and Bcl-x_L, the DED-L domain, and a C-terminal membrane-anchoring domain (32). Like the DED-L domain of Bap31, the DED-L domain of BAR binds caspase-8, sequestering it and thwarting apoptosis induction initiated by TNF/Fas-family death receptors (32, 33). BAR also binds the apoptosis regulators Hip and Hippi, which contain DED-L domains homologous to those found in BAR and Bap31. Hip associates with Huntingtin (Htt), the protein implicated in Huntington disease that causes degeneration of neurons containing Htt polyglutamine (polyQ) expansions (34). Hippi is a DED-L domain–containing Hip-interacting protein that binds procaspase-8. Htt with polyQ expansion has reduced affinity for Hip compared with the normal Htt protein, a circumstance under which it has been proposed that Hip is free to bind Hippi and trigger caspase-8 activation (35). Interactions of BAR, Bap31, Hip, and Hippi deserve further investigation on a number of fronts, including whether these proteins represent substrates for the E3 ligase activity of BAR, elucidation of their agonistic and antagonistic relations among each other, and evaluation of effects of these protein interactions on nonapoptotic functions of Htt and its interacting proteins.

The ability of BAR and Bap31 to bind procaspase-8 prompts speculation that perhaps these ER proteins could promote rather than inhibit caspase-8 activation, if induced to aggregate in ER membranes, thereby constituting a novel ER-associated "apoptosome." If so, then the parallel ability of BAR and Bap31 to bind Bcl-2 and Bcl-x_L through domains separate from the DED-L domain might supply a mechanism for preventing caspase activation, providing a long-sought analogy to the paradigm for caspase regulation seen in *Caenorhabditis elegans*, where the Bcl-2 ortholog Ced9 binds caspase activator Ced4, preventing activation of Ced3 protease (36).

Kinases. The kinase Ask1 has been implicated in apoptosis induction in the context of signaling by TNF-family receptors (reviewed in ref. 37). During ER stress, Ask1 is recruited to oligomerized Ire1 complexes containing TRAF2, activating this kinase and causing downstream activation of JNK and p38 MAPK. Consistent with a key role for Ask1 in apoptosis induced by ER stress, studies of *ask1*$^{-/-}$ neurons subjected to inducers of ER stress indicate a requirement for this kinase for JNK activation and cell death (38). The downstream death effectors of Ask1 are not clear. The kinase pathway initiated by Ask1 leads to JNK activation, and JNK-mediated phosphorylation activates the proapoptotic protein Bim (39–41), while inhibiting the antiapoptotic protein Bcl-2 (42).

Thus, Ire1 plays roles in all 3 of the ER responses to unfolded proteins (adaptation, alarm, and apoptosis), through its actions upon XBP-1 (adaptation), TRAF2 (alarm [NF-κB]), and apoptosis effectors caspase-12 and Ask1. How these 3 functions of Ire1 are integrated remains unclear.

The protein tyrosine kinase c-Abl can translocate from the ER surface to mitochondria in response to ER stress (43). Moreover, a functional role for c-Abl has been suggested by studies of *c-Abl*$^{-/-}$ fibroblasts, which display resistance to cell death induced

by Ca^{2+} ionophores, brefeldin A, and tunicamycin (43). How c-Abl promotes apoptosis is unknown at present.

Transcription factors. CHOP (GADD153) is a member of the C/EBP family of bZIP transcription factors, and its expression is induced to high levels by ER stress (reviewed in ref. 44). The *chop* gene promoter contains binding sites for all of the major inducers of the UPR, including ATF4, ATF6, and XBP-1, and these transcription factors play causative roles in inducing *chop* gene transcription. Cause-and-effect roles in *chop* gene induction have been demonstrated for signaling molecules involved in ER stress by genetic manipulation of mice, showing that *perk*$^{-/-}$ and *atf4*$^{-/-}$ cells and eIF2α(S51A) knock-in cells fail to induce *chop* during ER stress (11, 12, 45). Cross-talk between the PERK/eIF2α pathway and the Ire1/TRAF2/Ask1 pathway may also enhance CHOP activity at a posttranscriptional level, given that Ask1 activates both JNK and p38 MAPKs, and phosphorylation of the CHOP protein on serine 78 and serine 81 by p38 MAPKs increases its transcriptional and apoptotic activity (46). In addition to the aforementioned regulators, upstream activators of *chop* also include ATF2, which is induced by hypoxia and which is required for *chop* induction during amino acid starvation (47).

Overexpression of CHOP protein induces apoptosis, through a Bcl-2–inhibitable mechanism (48, 49). Moreover, *chop*$^{-/-}$ mice are resistant to kidney damage induced by tunicamycin and to brain injury resulting from cerebral artery occlusion, demonstrating a role for CHOP in cell destruction when ER stress is involved (48, 50). How CHOP induces apoptosis is unclear. CHOP forms heterodimers with other C/EBP-family transcription factors via bZIP-domain interactions, which suppresses their binding to C/EBP sites in DNA, while promoting binding to alternative DNA sequences for target gene activation (51). Consequently, CHOP inhibits expression of genes responsive to C/EBP-family transcription factors, while enhancing expression of other genes containing the consensus motif 5′-(A/G)(A/G)(A/G)TGCAAT(A/C)CCC-3′. One relevant target may be *bcl-2*, whose expression is suppressed by CHOP, at least in some cellular contexts (49). CHOP may also have nontranscriptional actions, still poorly defined (44). While capable of inducing apoptosis and contributing to cell death in several scenarios involving ER stress, CHOP is not essential for cell death induced by ER stress, as demonstrated by the observation that *perk*$^{-/-}$ and eIF2α(S51A) knock-in cells are hypersensitive to ER stress–induced apoptosis but fail to induce *chop* gene expression (12, 45).

Scotin is another ER-targeted apoptosis inducer (52). The gene encoding Scotin is a direct target of p53, suggesting a way to link DNA damage to ER-mediated cell death mechanisms.

Bcl-2–family proteins and their modulators. Association of certain Bcl-2/Bax–family proteins with ER membranes dates back to the initial discovery of Bcl-2 (53). Though better known for their actions upon mitochondria, Bcl-2/Bax–family proteins also integrate into ER membranes, where they modulate ER Ca^{2+} homeostasis and control cell death induced by ER stress agents, including tunicamycin, brefeldin A (an inhibitor of ER-Golgi transport), thapsigargin, and oxidants (reviewed in ref. 54). Experiments in which the normal C-terminal transmembrane domain of Bcl-2 was swapped with membrane-targeting domains from ER resident proteins suggested that Bcl-2 targeted exclusively to the ER (as opposed to both ER and mitochondria) is more restricted in its antiapoptotic actions, suppressing cell death induced by ER stress agents and by c-Myc. Recent findings that apoptosis induced by c-Myc may be attributable to its induction of Bim suggest that ER-targeted Bcl-2 may sequester this BH3-only protein, preventing it from interacting with other members of the Bcl-2/Bax family (55).

Spike is a BH3-only protein anchored in the ER (56). The BH3-like domain of Spike is required for apoptosis induction, but dimerization partners among Bcl-2/Bax–family proteins have yet to be found. Several other Bcl-2/Bax–family proteins reside at least in part in association with or integrated into ER membranes, with some, such as the antiapoptotic protein Mcl-1 and proapoptotic Bik, found predominantly in the ER (57, 58). Given the preferences of certain BH3 domains for interactions with particular members of the Bcl-2/Bax family (59), it seems likely that a network of interactions among a subset of this family of apoptosis regulators takes place on ER membranes, the functional consequences of which are not yet fully understood. Recently, expression of at least 1 of the Bcl-2/Bax–family genes was linked to ER stress. The BH3-only protein Puma is induced by tunicamycin and thapsigargin in a p53-independent manner, with *Puma*$^{-/-}$ cells showing resistance to apoptosis induced by ER stress (60).

The BI-1 protein contains 6 transmembrane domains, resides in the ER (61), interacts functionally or physically with Bcl-2–family members, and is induced by hypoxia (62). This protein blocks cell death induced by oxidative stress in yeast, plants, and animals (63). Mice lacking BI-1 display increased sensitivity to tunicamycin-induced kidney damage and to stroke injury, implying a role for BI-1 in protection from insults known to trigger ER stress. In cultured cells, overexpression of BI-1 selectively reduces, while BI-1 deficiency selectively increases, sensitivity to cell death induced by agents that trigger ER stress, while having far less effect on apoptosis induced by agents that trigger cell death pathways linked to mitochondria (intrinsic pathway) or TNF/Fas-family death receptors (extrinsic pathway) (64). BI-1 associates with the antiapoptotic proteins Bcl-2 and Bcl-x_L, but not proapoptotic Bax and Bak (61). Nevertheless, BI-1 inhibits cell death induced by Bax overexpression, in animal cells, plants, and yeast.

The ER protein Bap31 lacks homology with Bcl-2/Bax–family proteins and contains no recognizable BH3 dimerization domain, but it binds Bcl-2 and Bcl-x_L and regulates apoptosis. BAR also binds Bcl-2 and Bcl-x_L, and the responsible domain is required for BAR-mediated suppression of cell death (32). Interestingly, BAR is capable of suppressing Bax-induced death of yeast, implying caspase-independent functions for this protein, given that yeast lack bona fide caspases. This suggests that, mechanistically, BAR may share something in common with Bcl-2 and BI-1, which also suppress Bax-induced killing of yeast.

Other apoptosis regulators. Given that mitochondria release apoptogenic proteins into the cytosol, the ER might use similar mechanisms for linking ER stress to cell death. In insect cells, at least one example has been uncovered of a protein, called Jafrac2, that is normally sequestered in the ER but is released into the cytosol during apoptosis induced by certain stimuli (65). Like most proteins imported into the ER, the N-terminal leader peptide of Jafrac2 is removed by proteolysis. This proteolytic processing exposes an IAP-binding motif in Jafrac2, poising it to attack antiapoptotic IAP-family proteins upon accessing the cytosol, thereby freeing caspases. It remains to be determined whether examples of apoptogenic protein release from the ER of mammalian cells will be discovered.

Ca^{2+} and apoptosis induced by ER stress

Release of Ca^{2+} from the ER plays critical roles in cellular signaling mediated by the second messengers inositol triphosphate

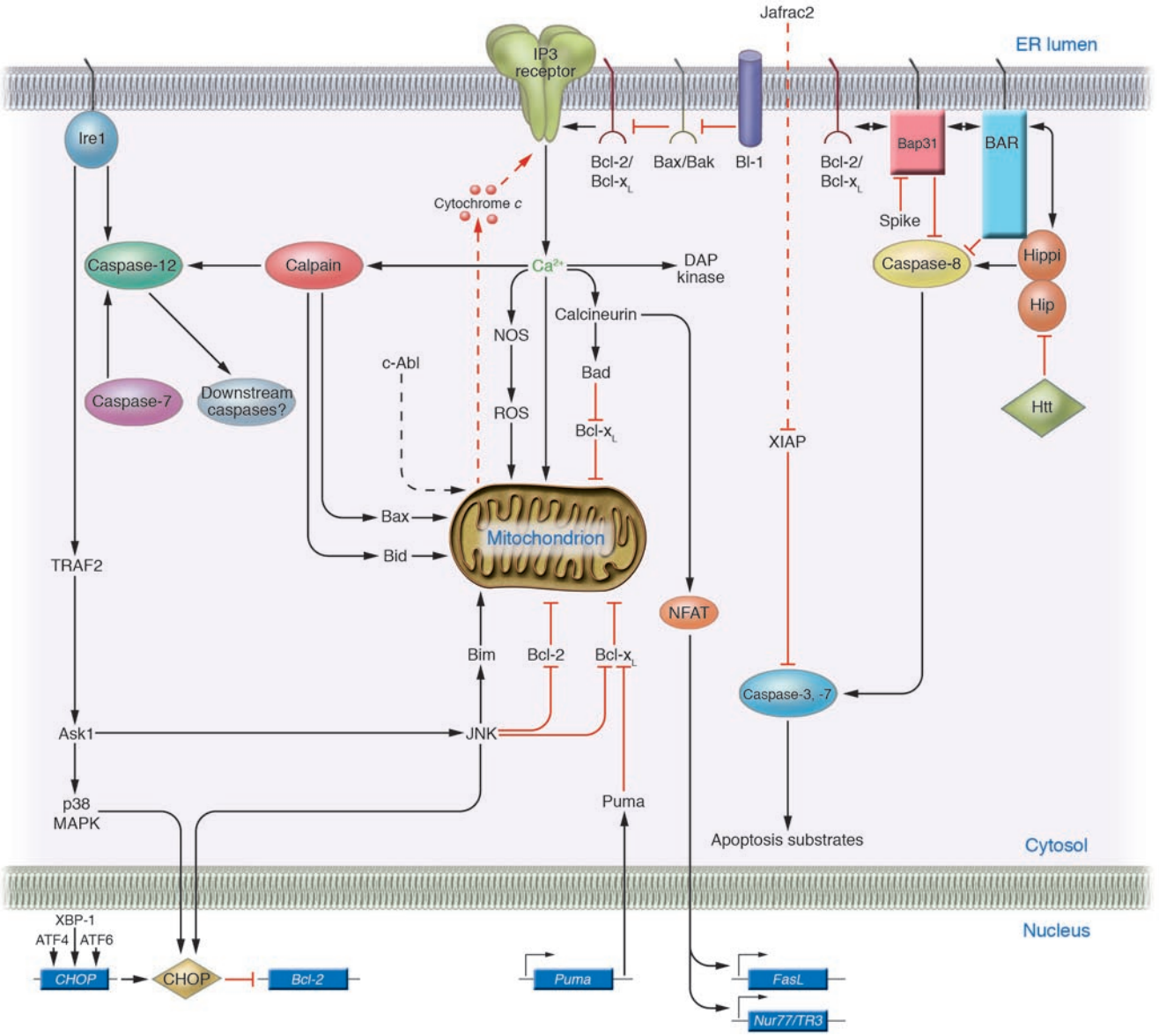

Figure 2
Cell death mechanisms induced by ER stress. Several of the proposed pathways linking ER stress to cell death are depicted. Dashed lines indicate protein translocation events (c-Abl, Jafrac2). The mitochondrial permeability transition pore complex, which is Ca^{2+}-sensitive, is not shown in the diagram. See the text for additional details.

(IP3) and cytosolic ADP-ribose and other regulators via effects on IP3 receptors (IP3Rs) and ryanodine receptors (66, 67). Opposing these gated Ca^{2+} channels are the SERCA-family proteins, Ca^{2+} ATPases that pump Ca^{2+} into the ER, which are regulated by phosphorylation and interactions with other proteins (e.g., phospholamban and sarcolipin). Various stimuli that cause the ER to dump Ca^{2+} precipitate cell death, including hypoxia, oxidants, stimulators of IP3 production, and pharmacological antagonists of SERCA. The downstream effectors of Ca^{2+}-induced cell death are potentially myriad and could minimally include (a) induction of mitochondrial permeability transition, induced upon entry of excessive amounts of Ca^{2+} into the matrix of mitochondria (68, 69); (b) local activation near the ER of calpains, a family of Ca^{2+}-dependent cysteine proteases implicated in pathological cell death (70, 71), whose substrates include Bax and Bid (which are activated) (72–74), Bcl-2 and Bcl-x_L (which are inhibited), and several caspases (reviewed in ref. 4); (c) alterations in Ca^{2+}-dependent phospholipid scramblases, which alter membrane biology to promote apoptosis or necrosis, including transferring phosphatidylserine to the outer leaflet of the plasma membrane (a signal for clearance of cells by phagocytosis) and transferring cardiolipin from the inner to outer membrane of mitochondria (a signal for targeted insertion of proapoptotic Bcl-2–family proteins Bid and Bax into membranes) (75–77); (d) Ca^{2+}/calmodulin–mediated activation of the protein phosphatase calcineurin, which dephosphorylates the proapoptotic protein Bad, allowing it to dimerize with and antagonize Bcl-x_L (78), and which dephosphorylates NFAT-family transcription factors, allowing entry into the nucleus and trans-

activation of proapoptotic genes encoding FasL and Nur77/TR3 (79); (e) stimulation of Ca^{2+}-sensitive isoforms of nitric oxide synthase (NOS), exacerbating oxidative stress (reviewed in ref. 5); (f) activation of death-associated protein kinase (DAP kinase) and its relative DRP-1, which contain calmodulin-binding domains (reviewed in ref. 80); (g) activation of Ca^{2+}-sensitive mitochondrial fission protein DRP-1 (81), which has been implicated in Bax-induced release of cytochrome *c* from mitochondria; and possibly (h) alterations in the Ca^{2+}-binding protein TCTP (fortilin), a putative modulator of antiapoptotic Bcl-2/Bax–family proteins such as Mcl-1 (82). In addition, ectopic expression of the proapoptotic mammalian protein Bak in yeast induces cell death through a calnexin-dependent pathway, correlating with Bak binding to this Ca^{2+}-dependent ER chaperone (83).

A role for Bcl-2 in modulating intracellular Ca^{2+} was first demonstrated over a decade ago (84), but only recently have clues about the mechanisms involved begun to emerge. Based on data from a variety of techniques, it appears that overexpression of antiapoptotic proteins Bcl-2 and Bcl-x_L lowers the basal amounts of Ca^{2+} in the ER, because of increased leakage of Ca^{2+} under resting conditions. The consequence of this is that upon exposure to stimuli that precipitously dump Ca^{2+} from internal stores, less Ca^{2+} enters the cytosol, resulting in lower peak concentrations of cytosolic Ca^{2+} and less overall cytosolic Ca^{2+} accumulation (54, 85–88). Downstream, less Ca^{2+} enters mitochondria, which possibly explains the inhibition of mitochondrial depolarization and the suppression of cytochrome *c* release. Like overexpression of Bcl-2 or Bcl-x_L, ablation of the expression of proapoptotic Bax and Bak also reduces basal Ca^{2+} in the ER, implying a role for these proapoptotic proteins in setting cellular ER Ca^{2+} concentrations (89). Interestingly, Bcl-2 remains competent in its ability to reduce ER Ca^{2+}, even in cells lacking Bax and Bak, implying that Bcl-2 operates downstream of Bax/Bak with respect to ER Ca^{2+} regulation, unlike the situation with mitochondria-dependent cell death, where genetic evidence indicates that Bcl-x_L and Bcl-2 function upstream of Bax/Bak (90).

Attempts to establish whether these changes in ER Ca^{2+} handling mediated by Bcl-2/Bax–family proteins are causally linked to cell death regulation have failed to provide firm answers, but supporting evidence has been obtained from a variety of experimental approaches, including genetic manipulations of Ca^{2+}-regulating proteins in the ER (88, 89, 91, 92).

Because several Bcl-2/Bax–family proteins share structural similarity with the pore-forming domains of bacterial toxins, they may function as ion channels, thus explaining the ability of Bcl-2/Bax–family proteins to modulate ER Ca^{2+} (reviewed in ref. 93). However, mutations designed to impair the putative pore-forming regions of Bcl-2 do not affect its ability to regulate ER Ca^{2+} (94); this suggests alternative mechanisms. In this regard, Bcl-2 was reported to bind IP3Rs several years ago (95), and recently Bcl-2 has been implicated in regulating IP3R activity (96). IP3R knock down inhibits the ability of Bcl-2 to promote leakage of Ca^{2+} from the ER, suggesting that Bcl-2 relies on IP3Rs to reduce luminal ER Ca^{2+}. The mechanism by which Bcl-2 modulates IP3Rs has yet to be defined, particularly the issue of whether this is a direct effect of these proteins on IP3Rs or an indirect effect on unidentified IP3R-interacting proteins present in ER membranes. Interestingly, cytochrome *c*, an apoptosis-inducing protein released from mitochondria, binds IP3Rs and induces Ca^{2+} release from ER, thereby triggering ER stress (97) and providing another potential link between IP3Rs and cell death regulation. Also, reduction in or ablation of expression of certain IP3Rs (e.g., IP3R1 and IP3R3) decreases sensitivity of some types of cells (e.g., lymphocytes, neurons) to apoptosis (98–100), suggesting further links between Ca^{2+} dysregulation by IP3Rs and apoptosis induction.

Curiously, the antiapoptotic protein BI-1 also regulates ER Ca^{2+} homeostasis in a manner analogous to that of Bcl-2 and Bcl-x_L. Overexpression of BI-1 reduces basal ER Ca^{2+} concentrations, while ablation of the genes encoding BI-1 increases amounts of thapsigargin-releasable Ca^{2+} from internal stores (64). Since BI-1 associates with Bcl-2 and Bcl-x_L in ER membranes (61), it will be interesting to determine whether BI-1 also interacts with and regulates IP3Rs.

The truncated Bap31, resembling the caspase-cleavage product, induces Ca^{2+} efflux from the ER and induces apoptosis (30), providing further correlative connections between modulation of ER Ca^{2+} dynamics and cell death regulation. Interestingly, Bap31 was reported to bind an ER-associated putative ion channel called A4, but the relevance of this protein interaction to regulation of ER Ca^{2+} remains unclear (101).

ER stress and diseases

ER stress is associated with a range of diseases, including ischemia/reperfusion injury, neurodegeneration, and diabetes (reviewed in ref. 44), making ER stress a probable instigator of pathological cell death and dysfunction.

ER stress and neurodegeneration. AβP is a proteolytic product of amyloid β-precursor protein that is causally associated with Alzheimer disease (AD). Mice lacking caspase-12 are partially resistant to apoptosis induced by exposure to AβP (22), raising the possibility of a functional link between ER stress and AβP-induced toxicity. Mutant versions of the AβP-interacting protein presenilin-1 (PS-1), previously associated with AD, interfere with the UPR (102) and may render neurons more susceptible to cell death induced by ER stress (103). The brains of mice harboring AD mutants of PS-1 also have increased CHOP protein (104). Interestingly, PS-1 induces cleavage of Ire1α, releasing the cytosolic domain to translocate to the nucleus, suggesting further interactions between molecules involved in AD and ER stress responses (105).

Hereditary mutations in the ER-associated E3 ubiquitin ligase Parkin have also been associated with ER stress–induced cell death and are found in patients with familial Parkinson disease (PD) (106, 107). Overexpression of wild-type Parkin suppresses cell death induced by several ER stress–inducing agents, and by α-synuclein, the principal component of pathological Lewy bodies seen in PD (107). Parkin expression is induced by ER stress, suggesting a role for it in adaptation to ER stress, presumably functioning in the ER-associated protein degradation pathway to clear misfolded proteins.

Neurodegenerative diseases associated with inclusion body formation and protein aggregation have also been linked to ER stress, including amyotrophic lateral sclerosis, PD, Huntington disease, and others (reviewed in refs. 44, 108). Htt variants with polyQ expansions, for example, induce classical signal transduction events associated with the UPR and cause proteolytic processing of caspase-12 (109), as well as cause global reductions in proteasome activity (110). Thus, by exhausting the cytosolic protein degradation machinery, inclusion body diseases probably cause a back-up of misfolded proteins in the ER, triggering ER stress.

ER stress and ischemia/reperfusion injury. Reduced blood flow resulting from arterial occlusion or cardiac arrest results in tissue

hypoxia and hypoglycemia, which cause protein misfolding and ER stress. Reperfusion of the affected tissues then triggers oxidative stress, with production of NO, and other reactive oxygen species that result in protein misfolding. NO and other reactive molecules also may modify oxidizable residues (cysteines) in ER-associated Ca^{2+} channels, including ryanodine receptors and SERCAs, causing ER Ca^{2+} depletion, yet another cause of protein misfolding.

Brain ischemia/reperfusion injury activates the PERK/eIF2α pathway and induces *chop* expression in rodents (111, 112). Moreover, *chop*$^{-/-}$ mice suffer less tissue loss after stroke, implying a causal role for this mediator of ER stress in neuronal cell death (113). NO, a known mediator of brain injury during stroke, induces *chop* expression in cultured neurons. Furthermore, a NOS inhibitor is protective in a rodent model of brain ischemia (114), and mice lacking the gene encoding iNOS display decreased sensitivity to brain ischemia (115), suggesting a causal role for this ER stress inducer in stroke damage.

A role for the antiapoptotic protein BI-1 in protection from cerebral ischemia has been demonstrated by studies of *bi-1*$^{-/-}$ mice, which suffer larger infarcts following cerebral artery occlusion (64). Given that hypoxia has been implicated in *bi-1* gene induction (62), these findings imply a role for endogenous *bi-1* in survival of cells traumatized by ER stress.

ER stress and heart disease. The role of ER stress in heart disease has not been extensively studied, but Ask1 kinase activity increases in mice following myocardial infarction or aortic constriction, and *ask1*$^{-/-}$ mice showed reduced cardiomyocyte apoptosis, in addition to better preservation of left ventricular function, compared with wild-type animals (116).

ER stress in diabetes. Pancreatic β cells have a well-developed ER, reflecting their role in secreting large amounts of insulin and various glycoproteins. This function of β cells may explain why mice lacking PERK are susceptible to diabetes, showing apoptosis of their β cells and progressive hyperglycemia with aging (117). Moreover, *PERK* gene mutations in association with infant-onset diabetes occur in humans with the autosomal recessive disorder Wolcott-Rallison syndrome (118). At autopsy, these patients show massive β cell loss, resembling the pathology of *perk*$^{-/-}$ mice. Similarly, eIF2α(S51A) knock-in mice suffer from β cell depletion, which begins in utero, suggesting a more rapid course than that in *perk*$^{-/-}$ mice (12). The failure of *perk*$^{-/-}$ to phenocopy eIF2α(S51A) raises the possibility that other kinases besides PERK inhibit eIF2α during ER stress. Pancreatic β cell apoptosis induced by NO, a mediator of inflammation relevant to autoimmune diabetes, is CHOP-dependent, further implicating ER stress as an instigator of β cell death (50). Also, in rodent models of diabetes caused by a nonsecreted insulin mutant, homozygous deletion of *chop* delays disease onset (119), implying a role for this gene in β cell depletion in vivo. Recently, *xbp-1*$^{+/-}$ heterozygous mice have been shown to be more sensitive to diabetes caused by obesity and high-fat diet (120). The underlying mechanism is related to the requirement of XBP-1 for dampening of JNK activation caused by ER stress, which correlates with phosphorylation of IRS-1 and reduced tyrosine phosphorylation of IRS-1 in insulin-stimulated cells. Interestingly, signs of ER stress were found in liver and adipose tissue of obese mice and mice fed high-fat diets, indicating that the metabolic abnormalities associated with obesity and unhealthy diets cause ER stress in vivo.

Other cells that secrete proteins in large quantities may also be at risk for ER stress–induced apoptosis. For example, studies of *perk*$^{-/-}$ mice indicate a requirement for differentiation (or survival) of plasma cells, known for their production of immunoglobulins (121).

Therapeutic targets. Several mediators of ER-initiated cell death are candidates for drug discovery efforts, though some are better validated than others. Gene ablation studies in mice argue that agents inhibiting Ask1 and CHOP are attractive, because mice lacking these genes are phenotypically normal but exhibit reduced sensitivity to cell death induced by ER stressors, such as stroke and polyQ-expanded proteins associated with neurodegeneration (reviewed in refs. 38, 44). Presumably Ask1 is also responsible for the hyperactivity of JNK associated with insulin resistance in the context of ER stress caused by high-fat diet. Ask1 theoretically could be attacked by small molecules targeting the ATP-binding site of the kinase domain, analogous to other kinase inhibitors recently approved for other indications. The CHOP protein may be difficult to attack with small-molecule drugs. However, since p38 MAPK augments CHOP activity, small-molecule antagonists of this kinase currently in development for inflammatory diseases might find utility as cytoprotective agents in clinical scenarios involving ER stress. Also, c-Abl inhibitors such as imatinib (Gleevec) could be examined for cytoprotective activity in ischemic and degenerative diseases, given recent evidence that c-Abl may relay death signals from ER to mitochondria (43).

Compounds that augment the PERK/eIF2α pathway may also protect against cell death by ER stress. Indeed, a recent screen for inhibitors of neuronal death induced by tunicamycin identified compounds that suppress protein phosphatases responsible for dephosphorylation of eIF2α on serine 51, thus increasing accumulation of phosphorylated eIF2α and providing protection from apoptosis induced by several inducers of ER stress (122). Interestingly, the prototype compound characterized (Salubrinal) apparently is not an active-site inhibitor of the phosphatase but rather specifically disrupts complexes containing GADD35 and protein phosphatase-1 (PP1), thereby preventing GADD34-mediated targeting of PP1 onto substrate phospho-eIF2α.

It remains to be determined whether broad-spectrum inhibitors of caspase-family cell death proteases would preserve cell survival in the face of ER stress, given that culture experiments have shown that nonapoptotic cell death still occurs in the presence of compounds such as benzoyl-valinyl-alaninyl-aspartyl-fluoromethylketone (zVAD-fmk), at least when strong pharmacological inducers of ER stress are used (64). However, mice lacking various individual caspases, including caspase-1, caspase-2, and caspase-11 (a probable caspase-4 or -5 ortholog), are resistant to stroke injury (123), a condition in which ER stress participates in the cell death mechanism.

Provided that side effects from vascular instability are not an issue, inhibitors of NOS are also attractive, since mice that lack iNOS show decreased sensitivity to brain ischemia and reduced CHOP expression (115). Finally, inhibitors of proapoptotic Bcl-2/Bax–family proteins that operate upon ER membranes could be useful for ameliorating tissue loss due to stimulators of ER stress.

Conclusions

ER stress has been implicated in several diseases, and pathways linking ER stress to cell death have been reported. The principal challenge with any strategy for blocking cell death caused by ER stress lies with the multitude of parallel pathways potentially leading to downstream cell death mechanisms. Thus, blocking only 1 cell death pathway emanating from the ER may be inadequate to

preserve cell survival. Further studies of genes and gene products involved in ER-initiated cell death are needed to fully validate targets for drug discovery.

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