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Review

Endoplasmic reticulum quality control and apoptosis✪

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The ER is one of the most important folding compartments within the cell, as well as an intracellular Ca^{2+} storage organelle and it contains a number of Ca^{2+} regulated molecular chaperones responsible for the proper folding of glycosylated as well as non-glycosylated proteins. The luminal environment of the ER contains Ca^{2+} which is involved in regulating chaperones such as calnexin and calreticulin, as well as apoptotic proteins caspase-12 and Bap31, which may play an important role in determining cellular sensitivity to ER stress and apoptosis. The ER quality control system consists of several molecular chaperones, including calnexin, that assist in properly folding proteins and transporting them through the ER as well as sensing misfolded proteins, attempting to refold them and if this is not possible, targeting them for degradation. Accumulation of misfolded protein in the ER leads to activation of genes responsible for the expression of ER chaperones. The UPR mechanism involves transcriptional activation of chaperones by the membrane-localized transcription factor ATF6, in conjunction with the ER membrane kinase IRE1, as well as translational repression of protein synthesis by another ER membrane kinase PERK. When accumulation of misfolded protein becomes toxic, apoptosis is triggered, potentially with IRE1 involved in signaling *via* caspase-12. Both the extrinsic and intrinsic apoptotic pathways appear to culminate in the activation of caspases and this results in the recruitment of mitochondria in an essential amplifying manner. Bap31 may direct pro-apoptotic crosstalk between the ER and the mitochondria *via* Ca^{2+} in conjunction with caspase-12 and calnexin. Accordingly, ER stress and the resultant Ca^{2+} release must be very carefully regulated because of their effects in virtually all areas of cell function.

Keywords: endoplasmic reticulum, molecular chaperones, intracellular organelle

ENDOPLASMIC RETICULUM

The endoplasmic reticulum (ER) is an essential intracellular organelle and in conjunction with Ca^{2+}, plays a vital role in many cellular processes including synthesis, folding, and post-translational modification of membrane associated, secreted, and integral membrane proteins and lipids (Baumann & Walz, 2001; Sitia & Braakman, 2003), contraction-relaxation, cell motility, cytoplasmic and mitochondrial metabolism, gene expression, cell cycle progression and apoptosis (Berridge, 2002). The lumen of the ER contains a large concentration of resident proteins involved in virtually all aspects

✪Paper dedicated to the memory of Professor Witold Drabikowski and Professor Gabriela Sarzała-Drabikowska.

Abbreviations: Apaf1, apoptotic protease activating factor-1; ATF, activating transcription factor 6; Bap31, Bcl-2-associated protein-31; BiP/GRP78, binding protein/glucose regulated protein 78; CAD, caspase activated DNase; CAI/cabin1, calcineurin inhibitor; CFTR, cystic fibrosis transmembrane conductance regulator; CKII, casein kinase II; DED, death effector domain; DD, death domain; DISC, death initiator signaling complex; Drp1, dynamin related protein-1; EDEM, ER degradation enhancing 1,2-mannosidase-like protein; eIF2, eukaryotic translation initiation factor 2; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERSE, ER stress element; FADD, Fas-associated death domain; FasR, Fas receptor; GRP94, glucose regulated protein 94; GT, UDP-glucose:glycoprotein glucosyltransferase; iCAD, inhibitor of CAD; IP_3R, inositol 1,4,5-trisphosphate receptor; IRE1, inositol requiring 1; JNK, cJun NH_2-terminal kinase pathway; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancer factor 2; NCX, sodium-Ca^{2+} exchanger; NF-AT, nuclear factor of activated T-cells; OST, oligosaccharyl transferase; PERK, ds-RNA-activated protein kinase-like ER kinase; PDK, proline-directed kinase; PKC, protein kinase C; PKCδ, protein kinase C δ; PMCA, plasma membrane Ca^{2+} ATPase; PS, phosphatidylserine; PTP, permeability transition pore; QC, quality control; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic reticulum calcium transporting ATPase; S1P (or S2P), site 1 (or 2) protease; TNFR, TNF receptor; TRADD, TNF receptor-associated death domain; TRAF2, TNF receptor-associated factor-2; UPR, unfolded protein response; VDAC, volatge-dependent anion channel; XBP1, X-box-binding protein-1.

of ER function (Corbett & Michalak, 2000; Molinari & Helenius, 2000; Baumann & Walz, 2001; Jakob *et al.*, 2001b). Many of these proteins bind and store Ca^{2+} as well as function as molecular chaperones. They are involved in the folding and quality control of newly synthesized proteins and prevent the formation of incorrectly folded intermediates (Molinari & Helenius, 2000). With the protein concentration in the ER being in the 100 mg/ml range, the ER demands constantly monitored protein processing to determine that the folding is being performed correctly (Kleizen & Braakman, 2004). If the ER becomes overwhelmed, either by a problem with protein folding or by overproduction of proteins (which compromises their correct processing), the cell then triggers a specific ER stress response. This adaptive response may in turn signal a change in cell status, such as growth, differentiation, or even promotion of apoptosis. Many severe protein folding diseases are the product of mutations resulting in impaired function of this quality control and ER stress response machinery including vascular diseases such as myocardial infarction (Brooks, 1997; 1999; Jakob *et al.*, 2001b; Sherman & Goldberg, 2001) and atherosclerosis (Graf *et al.*, 2004), neurodegenerative diseases, such as Alzheimer's (Soti & Csermely, 2002; Rutkowski & Kaufman, 2004) and Charcot-Marie Tooth Disease (Shames *et al.*, 2003), systemic disease, such as cystic fibrosis (Chevet *et al.*, 1999a), prion diseases such as bovine spongiform encephalopathy and the human variant, Creutzfeldt-Jakob disease (Jeffery *et al.*, 2000), as well as cancer (Li *et al.*, 2001; Dissemond *et al.*, 2004). A number of these chaperones are regulated by Ca^{2+} and as a significant portion of ER Ca^{2+} is free, this affects many Ca^{2+}-dependent processes in the lumen of the ER (Corbett & Michalak, 2000) including modulation of the quality and efficiency of protein folding (Corbett & Michalak, 2000). Disruption of ER homeostasis therefore results not only in cellular disease but has detrimental effects at organelle and systemic levels as well.

CALCIUM HOMEOSTASIS AND THE ER

The ER plays a key role in maintaining Ca^{2+} homeostasis within the cell (Berridge, 2002) which directly influences protein folding and subsequently, ER stress and apoptosis. The ER also has an important role as being the largest physiological source of Ca^{2+} within the cell. This Ca^{2+} resource is vital for numerous signaling pathways including: fertilization, differentiation, proliferation, and transcription factor activation thereby influencing gene transcription and expression, protein and steroid synthesis, modification and folding of proteins, secretion, and apoptosis (Berridge *et al.*, 2000). Within the ER are three ubiquitously expressed Ca^{2+} transporters, the IP_3R (inositol 1,4,5-trisphosphate receptor) which releases Ca^{2+} from the ER, the RyR (ryanodine receptor) also involved in releasing Ca^{2+} from the ER, and the SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) transporter which acquires Ca^{2+} from the cytoplasm, transferring it into the ER. Subsequently, Ca^{2+} within the ER as well as being released from the ER can activate transcriptional and translational cascades that regulate the chaperones responsible for protein folding within the ER, but they may also regulate proteins responsible for ER stress, the UPR (unfolded protein response), and ERAD (ER-associated degradation), as well as regulating the proteins involved in the apoptotic pathway (Berridge, 2002; Breckenridge *et al.*, 2003a).

ENDOPLASMIC RETICULUM AND QUALITY CONTROL OF THE SECRETORY PATHWAY

The ER is one of the most important intracellular Ca^{2+} storage organelles containing a number of Ca^{2+} binding chaperones responsible for Ca^{2+} buffering as well as for the proper folding of glycosylated as well as non-glycosylated proteins (Michalak *et al.*, 2002). Ca^{2+} is known to directly regulate protein folding and this is done by controlling the chaperones responsible for quality control in the ER (Corbett *et al.*, 1999). About 30% of all cellular proteins are synthesized into the ER, where they interact with molecular chaperones and are transported as cargo

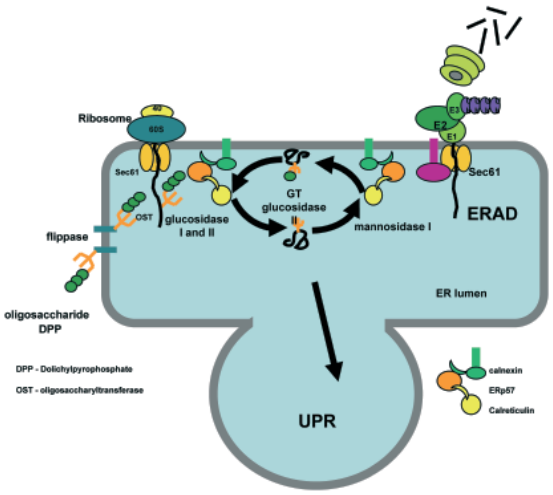

Figure 1. Nascent glycoprotein folding and ER-associated degradation.

Glycoproteins undergo extensive modification within the ER. This involves the QC cycle, in which calnexin, calreticulin, and GT participate. A protein will undergo repeated attempts at folding by interacting with calnexin, calreticulin and GT. If a protein is unable to fold properly, it is targeted for ERAD by cleavage of a single mannose and is recognized by the EDEM receptor, retro-translocated and degraded by the 26S proteosome.

through the ER to intracellular destinations as well as to the plasma membrane and the extracellular environment (Ghaemmaghami *et al.*, 2003). Molecular chaperones slow the folding rate of nascent proteins thereby preventing aggregation, as well as recruiting other folding chaperones (Fig. 1). This results in an increased yield of correctly folded proteins and their multi-protein complexes, and also an increase in the rate of correctly folding intermediates.

As proteins traverse the ER a number of them are also targeted for N-linked glycosylation at specific sites (Fig. 1). This hydrophilic oligosaccharide is involved in maintaining protein stability, solubility, charge and isoelectric point, membrane orientation and turnover rate, protection from proteolysis and denaturation, and mediates interaction with numerous pathogens. As well, N-linked glycans function as tags used for correct protein folding, efficient quality control, recognition for ERAD, lysosomal sorting, and transport (Helenius & Aebi, 2001; 2004). Initially, the oligosaccharide is generated on the cytoplasmic surface of the ER and is 'flipped' to the luminal side. This flipping is catalyzed by a bi-directional flippase that is not energy dependent (Hirschberg & Snider, 1987). The nascent oligosaccharide is then added to the protein by the translocon-associated enzyme, OST (oligosaccharyl transferase), and linked to the amino acid N (asparagine) in the amino-acid sequence NXS/T (asparagine-X-serine/threonine) (Fig. 1) *via* an *N*-glycosidic bond. This oligosaccharide is composed of $Glc_3Man_9GlcNAc_2$, in a flexible branched structure with the three branches labeled A, B, and C, where Glc is glucose, Man is mannose and GlcNAc is N-acetylglucosamine. Two enzymes, glucosidase I and II, subsequently cleave the glucose residues of the oligosaccharide. Glucosidase I specifically removes the terminal glucose residue almost immediately from the A chain while glucosidase II is responsible for cleaving the next two residues in succession, in a time dependent manner, both on the same chain as the first. Before glucosidase II cleaves the third glucose residue, the N-glycosylated protein with one terminal glucose residue on the A chain is recognized by the QC cycle, which manages the proper processing of most if not all N-linked glycoproteins that shuttle through the ER (Fig. 1) (Helenius & Aebi, 2004).

QC consists of a number of molecular chaperones, including calnexin and calreticulin, that assist in properly folding proteins and shuttling them through the ER as well as sensing misfolded proteins, attempting to refold them and if this is not possible, targeting them for degradation (Ellgaard & Helenius, 2003; Hirsch *et al.*, 2004) (Fig. 1). Once the protein has been properly folded, the third glucose is removed by glucosidase II, causing the protein to be released from the QC cycle and the protein is transported out of the ER. In some cases, this third glucose is prematurely removed and this misfolded or unfolded protein is recognized by another member of the QC, the enzyme GT (UDP-glucose glycoprotein glucosyltransferase), which replaces the removed glucose with a new glucose residue (Parodi, 2000). This allows the QC chaperones to rebind the nascent protein with the N-linked glycan containing a single glucose on the A chain and attempt to facilitate the folding of the protein again. This cycle may happen numerous times until the protein is folded correctly, or if the QC is unable to fold the protein, these misfolded and unfolded proteins accumulate and result in a variety of signaling pathways being turned on to control ensuing ER stress.

Two critical ER residents are calnexin (Bergeron *et al.*, 1994) and calreticulin (Michalak *et al.*, 1999), both lectin-like chaperones and members of the protein folding machinery found in the ER involved in the recognition of the glycoprotein containing the $Glc_1Man_9GlcNAc_2$ oligosaccharide (Michalak *et al.*, 2002; Ellgaard & Helenius, 2003;) (Fig. 2). Calnexin and calreticulin, in conjunction with ERp57 and GT are an essential part of the ER QC system that prevents the export of misfolded and incompletely folded glycoproteins as well as non-glycosylated proteins (Johnson *et al.*, 2001; Ellgaard & Helenius, 2003). Calnexin, a type I membrane protein, and its soluble homolog calreticulin, share approx. 30% sequence identity and 41% sequence similarity and as such, contain similar regions and appear to perform similar functions. Calnexin is a 90 kDa, integral membrane protein that contains a single transmembrane domain near the C terminus as well as a C-terminal charged ER retention signal (RKPRRE) (Jackson *et al.*, 1990; Rajagopalan *et al.*, 1994). The luminal domain of calnexin contains four repeat mo-

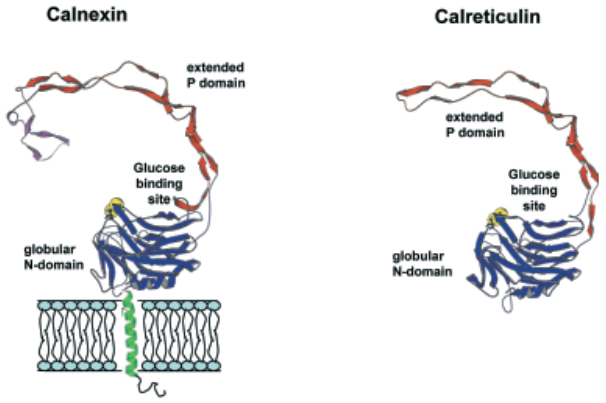

Figure 2. A model of 3D structure of calnexin and calreticulin.

Calnexin is composed of a globular N-terminal domain, involved in glycoprotein binding, an extended P-domain necessary for chaperone function, a single transmembrane region responsible for membrane localization and a cytoplasmic C-terminus containing an ER retention motif.

tifs of 17 amino acids (Ohsako *et al.*, 1994) and numerous consensus sites for phosphorylation by PKC (protein kinase C), CKII (casein kinase II), PDK (proline-directed kinase), and MAPK (mitogen-activated protein kinase) in the cytoplasmic domain (Tjoelker *et al.*, 1994; Wong *et al.*, 1998; Chevet *et al.*, 1999b; Roderick *et al.*, 2000). Calnexin has been specifically observed to be involved in numerous disease states such as the misfolding of the chloride channel termed CFTR, resulting in cystic fibrosis (Amaral, 2004), sitosterolemia, caused by reduced trafficking of a sterol receptor resulting in sterol accumulation and premature atherosclerosis (Graf *et al.*, 2004), and Charcot-Marie Tooth Disease, caused by misfolded pmp22 (Shames *et al.*, 2003). As well, significant upregulation of calnexin is observed in breast cancer (Li *et al.*, 2001), while metastatic melanoma lesions exhibited considerable down-regulation of calnexin (Dissemond *et al.*, 2004).

The structure of calnexin has recently been solved by X-ray crystallography (Schrag *et al.*, 2001) and consists of a globular, Ca^{2+}-binding domain that interacts with the glycosylated substrate, as well as an extended arm region that contains the conserved proline residues, the four tandem repeats, and interacts with ERp57 in a Zn^{2+}-dependent manner (Leach *et al.*, 2002) (Fig. 2). Using NMR, the ERp57 binding site was narrowed down to a small peptide derived from the proline-rich domain (Pollock *et al.*, 2004). Calnexin is very similar in sequence to calreticulin, a molecular chaperone that binds N-linked glycoproteins and promotes their correct folding (Saito *et al.*, 1999).

Calreticulin is found in the lumen of the ER and has been determined to bind Ca^{2+} (Johnson *et al.*, 2001) and resultantly, is involved in several processes that comprise cellular Ca^{2+} homeostasis such as Ca^{2+} uptake into the ER *via* SERCA (Camacho & Lechleiter, 1995), Ca^{2+} storage within the ER (Nakamura *et al.*, 2001) and Ca^{2+} release from the ER (Mesaeli *et al.*, 1999), as well as influencing store operated Ca^{2+} influx at the plasma membrane (Arnaudeau *et al.*, 2002). The NMR structure of the P-domain of calreticulin was reported (Ellgaard *et al.*, 2001) as a flexible, extended, finger-like region that interacts with ERp57 in a chaperone dependent manner (Frickel *et al.*, 2002) (Fig. 2). Calreticulin is also associated with several cancer disease states, with downregulation in metastatic melanoma (Dissemond *et al.*, 2004), and squamous cell carcinoma (Ogino *et al.*, 2003), while being significantly upregulated in colon cancer (Brunagel *et al.*, 2003), and is presented as an auto antigen in hepatitis, celiac and other autoimmune diseases (Sanchez *et al.*, 2000; 2003). It is also observed to be important in Alzheimer's disease (Nicolls *et al.*, 2003). It was recently documented that in calreticulin-deficient mouse fibroblast cells, folding was accelerated and there was significantly more partially folded proteins produced, while in calnexin-deficient cells, folding was severely impaired (Molinari *et al.*, 2004). This indicates that even though the level of sequence identity between calreticulin and calnexin leaves no doubt that they are structurally similar; it has yet to be conclusively determined whether their functions are also similar.

ER STRESS, UNFOLDED PROTEIN RESPONSE AND ER ASSOCIATED DEGRADATION

Considering that the ER is constantly monitoring protein processing *via* the QC machinery, what is the response if this process is compromised, either by over production of protein, by the accumulation of misfolded protein, or by a reduction in the Ca^{2+} concentration within the ER? In reaction to these conditions, the cell triggers a specific ER stress response (Rutkowski & Kaufman, 2004) that leads to the subsequent activation of genes responsible for the expression of ER chaperones (Gething & Sambrook, 1992). ER stress also activates ER-nuclear signaling, termed UPR which in turn inhibits protein synthesis (Prostko *et al.*, 1995; Brostrom *et al.*, 1996; Harding *et al.*, 1999) as well as facilitating protein degradation (Jeffery *et al.*, 2000).

Three resident transmembrane proteins, in combination with the ER molecular chaperone BiP/GRP78, are primarily responsible for the response to ER stress. The UPR mechanism involves transcriptional activation of chaperones and members of the ERAD by the transcription factor ATF6, in conjunction with the ER membrane kinase and endoribonuclease IRE1, as well as translational repression of protein synthesis by the ER kinase PERK (Rutkowski & Kaufman, 2004) (Fig. 3).

ATF6 is a transcription factor that is retained in the ER with BiP/GRP78. When there is an accumulation of unfolded protein, BiP/GRP78 is sequestered away, resulting in ATF6 transit to the Golgi where it undergoes cleavage and activation by S1P and S2P proteases. This yields a soluble transcription factor that translocates to the nucleus and is involved in the upregulation of ER resident chaperones, such as BiP/GRP78, proteins involved in ERAD, such as the EDEM receptor, and XBP1 mRNA (Mori *et al.*, 1996). As well, BiP/GRP78 is also sequestered away from IRE1 and PERK, resulting in dimerization and conformational change in these kinases that is transmitted across the membrane, leading to activation of their kinase activity (Bertolotti *et al.*, 2000) (Fig. 3). PERK activation leads to the phosphorylation of the translation initiation factor eIF2, resulting in inhibition of protein synthesis by sequestration of GTP-$tRNA_{Met}$ (Kudo *et al.*, 2002). When IRE1 senses the accumulation of unfolded proteins in the ER, it dimerizes and autophosphorylates to activate the ki-

nase domain. This signals downstream transcriptional upregulation of BiP/GRP78 and other chaperones to compensate for the amount of unfolded protein. As well, IRE1 also has endoribonuclease activity used to cleave 28S rRNA and inhibit protein synthesis as well as splice and activate XBP1 mRNA, recently upregulated by ATF6. The nascent XBP1 splice variant binds to the specific promoter, ERSE to upregulate chaperone expression (Yoshida *et al.*, 2001; Calfon *et al.*, 2002; Lee *et al.*, 2002; Yamamoto *et al.*, 2004) (Fig. 3). IRE1 was also recently observed to recruit TRAF2 and stimulate the stress activated JNK (c-Jun amino-terminal kinase pathway) (Urano *et al.*, 2000). These three ER resident transmembrane proteins combine to upregulate ER chaperones, capable of handling the elevated amount of unfolded proteins, as well as proteins involved in ERAD, which in turn degrade the misfolded proteins and in addition, down regulate protein synthesis, reducing the influx of nascent protein into the ER.

ERAD, one of the mechanisms that a cell regulates to control the elevated amount of misfolded protein, is triggered when these misfolded proteins are targeted by the ER α1,2-mannosidase I which cleaves a single mannose in a time-dependent manner from the B chain of the oligosaccharide (Yoshida *et al.*, 2003). This involves recognition of this mannose trimmed, misfolded protein by EDEM which appears to accept the protein from calnexin (Molinari *et al.*, 2003; Oda *et al.*, 2003; Yoshida, 2003) and transport it to the Sec61 translocation pore (Hosokawa *et al.*, 2001; Jakob *et al.*, 2001a). The misfolded protein is then unfolded and retro-translocated into the cytoplasm where it is deglucosylated by N-glycanase, poly-ubiquitinated and subsequently targeted to the 26S proteosome for degradation (Tsai *et al.*, 2002; Hirsch *et al.*, 2004).

If at some point the cell is overwhelmed with unfolded protein and is unable to recover, the feedback mechanism facilitates a signal to the apoptotic pathway from the ER in a Ca^{2+}-dependent manner potentially *via* the pro-apoptotic protein, caspase-12, possibly by means of an interaction with IRE1 and TRAF2 (Yoneda *et al.*, 2001), or by an interaction with Bap31 and calnexin (unpublished; (Zuppini *et al.*, 2002) and the cell is targeted for apoptosis (Nakagawa *et al.*, 2000) (Fig. 3). It appears that the Ca^{2+} released from the ER may activate transcriptional and translational cascades that regulate the chaperones responsible for protein folding within the ER, but may also regulate transcriptional cascades involved in apoptosis as well as directly regulating the proteins involved in the apoptotic pathway (Berridge, 2002; Breckenridge *et al.*, 2003a; Rao *et al.*, 2004).

APOPTOSIS

Apoptosis, a process of conserved and programmed cellular suicide, is composed of a complex cascade of closely synchronized factors that interact in a tightly controlled and genetically regulated manner and are present within the cell, ready for activation by death inducing signals. These apoptotic cascades can be stimulated by a variety of extrinsic and intrinsic signals as well as pathological cellular insults that culminate in the activation of cysteine proteases, termed caspases, that communicate with diverse pathways in the cell to transmit the apoptotic signal (Strasser *et al.*, 2000; Ferrari *et al.*, 2002; Green & Evan, 2002; Breckenridge *et al.*, 2003a; Hajnoczky *et al.*, 2003; Siegel *et al.*, 2003; Danial & Korsmeyer, 2004) (Fig. 4). Apoptosis is utilized by an organism to remove unwanted or damaged cells and this plays an important role in development, tissue homeostasis and is used as a defense against foreign pathogens and defective cells (Meier *et al.*, 2000; Siegel *et al.*, 2003; Danial & Korsmeyer, 2004; Norbury & Zhivotovsky, 2004). In addition, apoptosis plays a fundamental role in the pathogenesis of human diseases. This can be through multiple pathways, including gene suppression, activation

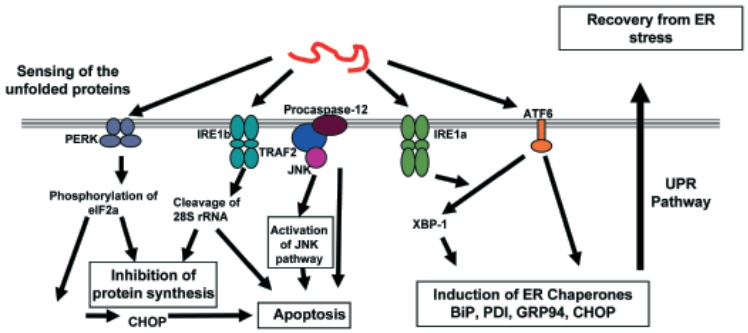

Figure 3. Accumulation of unfolded proteins in the ER induces stress.

A set of ER resident transmembrane proteins together with the ER molecular chaperone BiP/GRP78, plays critical role in ER stress. The UPR mechanism involves transcriptional activation of chaperones and members of the ERAD by the transcription factor ATF6, in conjunction with the ER membrane kinase and endoribonuclease IRE1, and translational repression of protein synthesis by the ER kinase PERK.

or mutation. Augmented apoptosis leads to, among others, Alzheimer's and Parkinson's disease (Mattson, 2000), while an inhibition in apoptosis leads to diverse cancers and autoimmune disorders (Green & Evan, 2002).

One of the key factors involved in this complex apoptotic cascade is a family of proteins termed caspases (Fig. 4). Caspases are cysteine-dependent aspartate-specific proteases that contain a highly conserved pentapeptide active site, QACRG and are involved in both extrinsic and intrinsic apoptotic pathways. The importance of this group of proteins has been primarily observed using mouse models. Mice that are deficient in caspase-3 and caspase-9 resulted in embryonic lethality as a result of insufficient brain development (Kuida *et al.*, 1996; 1998). Caspase-8 deficient mice were only viable until day 12, dying from impaired heart muscle development and congested accumulation of erythrocytes (Varfolomeev *et al.*, 1998). In fact, caspase-2, -3, -6, -7, -8, -9, -10 and -12 all have been recognized to play an important role in the apoptotic signaling pathway (Earnshaw *et al.*, 1999). Considering the important role caspases have on cell survival, the cell needs to have extremely tight control over their activity. To do this, caspases are initially produced as inactive zymogens or pro-enzymes that must be cleaved to be activated. There are two groups of caspases, the initiator caspases and the effector caspases. Initiator caspases such as caspase-2, -8, -9 and -10 contain a long prodomain and act to cleave activator caspases while the effector caspases only have a short prodomain and act directly on cellular components to cause cell death (Nicholson & Thornberry, 1997; Thornberry & Lazebnik, 1998; Earnshaw *et al.*, 1999; Slee *et al.*, 1999; Saraste & Pulkki, 2000; Wang, 2000; Denault & Salvesen, 2002; Creagh *et al.*, 2003).

Initiator and effector caspases are central figures in both the extrinsic and intrinsic apoptotic signaling pathways. In the extrinsic apoptotic pathway, an initiator caspase such as caspase-8, is recruited to the cytoplasmic domain of the Death Receptors, which include the TNF Receptor (TNFR) and the Fas Receptor (FasR) (Ashkenazi, 2002). Once a ligand has bound the receptor, the receptors form trimers and auto-activate (Naismith & Sprang, 1998), recruiting an adaptor protein such as TRADD or FADD *via* their DD. The adaptor protein also contains a DED that recruits the initiator caspase-8 leading to formation of a DISC (Sartorius *et al.*, 2001), resulting in proximity-induced dimerization (Boatright *et al.*, 2003; Donepudi *et al.*, 2003) and activation of caspase-8 that signals the caspase cascade (Earnshaw *et al.*, 1999; Kam & Ferch, 2000; Sartorius *et al.*, 2001; Denault & Salvesen, 2002) (Fig. 4). Effector caspases such as caspase-3, -6, and -7 are cleaved and activated by an initiator caspase and directly act on cellular components such as the cytoskeleton (Liu *et al.*, 1996; Kluck *et al.*, 1997; Yang *et al.*, 1997) or ion transporters (Remillard & Yuan, 2004) that subsequently achieve cellular death (Slee *et al.*, 1999). The intrinsic apoptotic pathway involves the mitochondria and utilizes another initiator caspase, caspase-9. A death stimulus signals through the Bcl-2 fam-

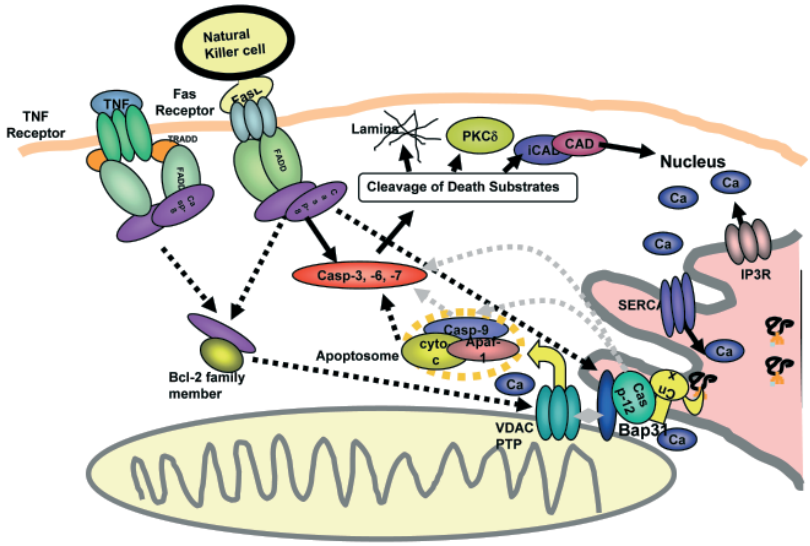

Figure 4. ER membrane and apoptosis.

Apoptosis can be signaled through two different but interconnected pathways, the receptor mediated pathway (extrinsic) and the ER stress pathway (intrinsic). The extrinsic pathway utilizes caspase-8 dependent cleavage of caspase-3 as well as facilitating the involvement of the mitochondria *via* caspase-8 dependent cleavage of Bap31 and recruitment of Bcl-2 family members which target the mitochondria, resulting in the release of cytochrome *c* and subsequent formation of a caspase-9 complex, leading to the apoptotic cascade. The intrinsic pathway mobilizes caspase-12, resulting in direct activation of caspase-9 without a need for the mitochondria, but may choose to recruit the mitochondria, potentially *via* caspase-12 dependent cleavage of Bap31, facilitating an amplified caspase cascade.

ily of proteins and targets the mitochondria where several members of the family, such as Bax, interact with the VDAC (Marzo *et al.*, 1998) or with the PTP (Brini *et al.*, 2000), causing a small amount of cytochrome *c* to be released from the mitochondria (Fig. 4). Cytochrome *c* then binds to and promotes Ca^{2+} release through the IP_3R in the ER membrane. The released Ca^{2+} triggers the extrusion of a large amount of cytochrome *c* from all the mitochondria in the cell (Boehning *et al.*, 2003). Caspase-9 is then activated downstream of the mitochondria by binding cytochrome *c* and Apaf-1 to form an apoptosome (Salvesen & Renatus, 2002), resulting in proximity-induced dimerization and activation (Katoh *et al.*, 2004). This complex then signals the effector caspases such as caspase-3, -6 and -7 to cleave and destroy cellular structures such as lamin (Earnshaw *et al.*, 1999; Ferraro *et al.*, 2003), and cellular proteins such as DNA repair enzymes, gelsolin and PKCδ (Nicholson & Thornberry, 1997; Thornberry & Lazebnik, 1998). The effector caspases also activate other destructive proteins such as CAD by cleaving its inhibitor, iCAD, leaving CAD able to target to the nucleus and degrade DNA as well as removing the inhibitory domain of p21-activated protein kinase 2 leading to plasma membrane blebbing (Sakahira *et al.*, 1998). One of these effector caspases, caspase-3, when activated, mediates the cleavage of the IP_3R leading to an signal independent Ca^{2+} release, resulting in an increase in cytoplasmic Ca^{2+} concentration, promoting apoptosis (Nakayama *et al.*, 2004). In conjuction, caspase-3 dependent cleavage of PMCA is also partially responsible for the increase in cytoplasmic Ca^{2+} concentration (Chami *et al.*, 2003). This cleavage renders PMCA unable to extrude Ca^{2+}, leading to Ca^{2+} overload. In conjunction with the inactivation of Ca^{2+} pumps, caspase-3 also cleaves the B subunit of NaK-ATPase (Mann *et al.*, 2001; Dussmann *et al.*, 2003). This cleavage leads to inactivation, loss of ion regulation and resultant volume shrinkage.

Prolonged ER stress can be responsible for the activation of apoptosis through both a mitochondrial dependent and independent pathway. The mitochondrial independent pathway is thought to occur through initiator caspase-12. Caspase-12 is ubiquitously expressed and localized to the ER membrane and like other caspases, is synthesized as an inactive proenzyme consisting of a regulatory prodomain and two catalytic subunits. Activation of caspase-12 appears to be triggered only by various stimuli that activate ER stress (Nakagawa *et al.*, 2000). Caspase-12 deficient mice are partially resistant to apoptosis induced by ER stress but not by other apoptotic stimuli (Nakagawa *et al.*, 2000) indicating that there is a distinction between the mitochondrial dependent pathway and the independent pathway. Caspase-12 is localized at the cytoplasmic face of the ER and is cleaved and activated by the Ca^{2+}-dependent protease m-calpain. Caspase-12, once activated, directly cleaves caspase-9 activating it without the need for cytochrome *c* or Apaf-1, which in turn cleaves the effector caspase-3 (Morishima *et al.*, 2002; Rao *et al.*, 2002). Caspase-12 also interacts with TRAF2 and is activated by proximity-induced dimerization (Shi, 2004), followed by the signaling of the JNK pathway (Yoneda *et al.*, 2001). Caspase-12 may potentially interact with another pro-apoptotic protein, Bap31, a 28 kDa integral ER membrane protein containing a cytoplasmic domain that preferentially associates with caspase-8, Bcl-XL, and Bcl-2 (Ng *et al.*, 1997). This interaction may be responsible for the feedback mechanism involving the release of cytochrome *c* following the mitochondrial independent pathway activation by ER stress. Active caspase-8 can cleave Bap31, with the p20 N-terminal transmembrane portion retained in the ER membrane and involved in induction of apoptosis *via* an interaction with Drp1 located at the mitochondria (Breckenridge *et al.*, 2003b). Overexpression of the transmembrane fragment causes an early release of Ca^{2+} from the ER, with an associated uptake of Ca^{2+} into the mitochondria, resulting in mitochondria strongly sensitized to caspase-8 induced cytochrome *c* release (Ng *et al.*, 1997). Bap31 is also observed to interact with calnexin, an ER integral membrane protein. In calnexin-deficient cells, there was a significant decrease in Bap31 cleavage and a resultant resistance to ER stress-induced apoptosis but there was normal caspase activity and cytochrome *c* release. Also observed was a direct interaction between calnexin and Bap31, indicating a role for calnexin and Bap31 communication (Zuppini *et al.*, 2002). In conjunction, calnexin has been observed to interact with both the SERCA transporter (Munch *et al.*, 2002), preventing the uptake of Ca^{2+} from the cytoplasm into the ER lumen and the IP_3R (Joseph *et al.*, 1999), regulating Ca^{2+} release *via* its cytoplasmic tail. Bap31, caspase-12 and calnexin may be part of the ER stress-induced apoptotic pathway and therefore may also be dependent on Ca^{2+}. ER stress has been determined to signal apoptosis through a mitochondrial dependent pathway by the activation of BAD, perhaps through the dephosphorylation of a serine by Ca^{2+}-dependent phosphatase, calcineurin, which is activated by an increase in cytoplasmic Ca^{2+} concentration (Carlberg *et al.*, 1996; Wang *et al.*, 1999). The ER-mitochondrial communication is possibly supported by physical links between the two organelles.

Both the extrinsic and intrinsic pathways appear to culminate in the activation of caspase-9, but the intrinsic pathway can choose to facilitate the need for mitochondrial involvement, presumably to augment apoptosis. Ca^{2+}-dependent, ER stress-activated caspase-12 may facilitate this intrinsic mitochondrial independent apoptotic cascade by inter-

acting and cleaving the ER localized, pro-apoptotic protein, Bap31 to signal the mitochondrial dependent apoptotic cascade in an effort to escalate the apoptotic cascade. It appears that the Bap31, p20 fragment can direct pro-apoptotic crosstalk between the ER and the mitochondria *via* Ca^{2+} (Nguyen *et al.*, 2000), perhaps in conjunction with caspase-12 and calnexin.

Caspases are also indirectly regulated by a group of proteins, the Bcl-2 family (Strasser *et al.*, 2000). They have both pro-apoptotic and pro-survival functions, are membrane localized and soluble proteins, and are found in numerous locations within the cell, including the cytoplasm (Schinzel *et al.*, 2004), the mitochondria (Schinzel *et al.*, 2004), and the ER (Zong *et al.*, 2003; Annis *et al.*, 2004). Bcl-2 family members are able to form homo and heterodimers resulting in conformational change and are either inhibited or activated (Borner, 2003; Burlacu, 2003) which suggests a form of internal regulation over apoptosis (Adams & Cory, 2002). At the membrane, they integrate and perform their pro-apoptotic function such as targeting to the mitochondria (Wang, 2001) where they interact with the PTP (Gross *et al.*, 1999) resulting in the release of cytochrome *c*, signaling the apoptotic cascade (Hsu *et al.*, 1997; Wolter *et al.*, 1997; Goping *et al.*, 1998; Griffiths *et al.*, 1999; Schinzel *et al.*, 2004). It is reasonable to assume that the fundamental role of Bcl-2 family members is to guard the integrity of the mitochondria, the ER and the nucleus and to control the release of ions and proteins from these organelles (Wang, 2001; Cory *et al.*, 2003).

A great deal of interest has recently been directed towards the involvement of Ca^{2+} in apoptosis and it has been established that Ca^{2+} plays a vital executioner role in the regulation of apoptosis (Duchen, 2000; Ermak & Davies, 2002; Ferrari *et al.*, 2002; Pinton *et al.*, 2002; Breckenridge *et al.*, 2003a; Hajnoczky *et al.*, 2003; Mattson & Chan, 2003; Oakes *et al.*, 2003; Rizzuto *et al.*, 2003; 2004; Distelhorst & Shore, 2004). This has been observed undr many apoptotic stimulation conditions, including genetic stress in human lung carcinoma A549 cells (Amuthan *et al.*, 2002), drug treatment of MDCK cells with betulinic acid (Chou *et al.*, 2000) and treatment of mouse lymphoma cells with the SERCA inhibitor, thapsigargin (Distelhorst & McCormick, 1996). Within the cell are numerous transporters responsible for controlling the Ca^{2+} concentration in the cytoplasm. The ER membrane contains three Ca^{2+} transporters, the IP_3R and the RyR which are involved in Ca^{2+} release from the ER, and the SERCA transporter, which utilizes ATP energy to renew the Ca^{2+} in the ER. If the IP_3R is activated, there is an immediate release of Ca^{2+} and the cytoplasmic Ca^{2+} concentration increases. The importance of the ER Ca^{2+} levels can be observed in cells that are deficient in IP_3R, consequently having an increased resistance to apoptosis (Jayaraman & Marks, 1997). As well, stable expression of the RyR in CHO cells, subsequently treated with caffeine stimulates depletion of Ca^{2+} stores from the ER, resulting in increased apoptosis (Pan *et al.*, 2000). Additionally, over-expression of SERCA, leading to an increase in ER Ca^{2+} levels, is associated with an increase in the sensitivity to ceramide-induced apoptosis indicating augmented ER Ca^{2+} levels result in increased apoptosis upon an apoptotic trigger (Brini *et al.*, 2000). In contrast, if the SERCA pump is inhibited, for example by thapsigargin, the reduced ER Ca^{2+} levels as well as the increased cytoplasmic Ca^{2+} levels, result in ER stress, Ca^{2+} toxicity and cellular death. It appears that the amount of releasable Ca^{2+} from the ER rather than the free Ca^{2+} level, or any Ca^{2+} available from other sources (Diaz-Horta *et al.*, 2002) and its effect on the mitochondria may dictate the fate of a cell (Rizzuto *et al.*, 2004). Accordingly, ER stress and the resultant Ca^{2+} release must be very carefully regulated because of their effects in virtually all areas of cell function.

The plasma membrane also plays a crucial role in the regulation of intracellular Ca^{2+} levels. Outside the cell, the Ca^{2+} concentration is in the 1–2 mM concentration range and the plasma membrane has an important job in keeping the Ca^{2+} from entering the cell. This is achieved by several transporters, including the PMCA which utilizes the energy of ATP to transport Ca^{2+} out of the cell, and the NCX (Sodium-Ca^{2+} Exchanger), which utilizes the sodium gradient to transport Ca^{2+} out of the cell. Over-expression of the PMCA results in reduced sensitivity to ceramide induced apoptosis *via* the rapid transport of Ca^{2+} out of the cell (Brini *et al.*, 2000). If the Ca^{2+} concentration in the cytoplasm is increased, there is a resultant increase in nuclear Ca^{2+} as well. The nuclear pore is large enough to accommodate the Ca^{2+} ion and the nuclear environment would equilibrate with the cytoplasm. In the nuclei, Ca^{2+} is shown to modulate gene transcription (Gong *et al.*, 1995) and nucleases (Ermak & Davies, 2002) that are involved in cellular apoptosis as well as directly affecting the nuclear pore complex itself, either by controlling the phosphorylation state or by binding members of the pore complex (Kehlenbach & Gerace, 2000; Zaidi *et al.*, 2004). As well, Ca^{2+} release from the ER and uptake into the mitochondria is critical in triggering apoptotic signals. Mitochondrial Ca^{2+} accumulation affects the respiratory chain (McClintock *et al.*, 2002) and the ATP synthase itself (Shchepina *et al.*, 2002) as well as being responsible for the release of cytochrome *c* (Darios *et al.*, 2003). This Ca^{2+} signal from the ER, generated by IP_3R mediated Ca^{2+} release, is transmitted effectively to the mitochondria, and occurs between closely associated if not contacting domains of the ER and the mitochondria. It has been determined that targeting of a pro-apoptotic Bcl-2

family member, Bid, to the mitochondrial membrane facilitates this uptake of Ca^{2+} from the ER into the mitochondria, effectively recruiting the mitochondria in the apoptotic cascade. In contrast, Bid failed to assist the delivery of Ca^{2+} signals from the cytoplasm to the mitochondria (Csordas *et al.*, 2002).

The release of Ca^{2+} from the ER is also involved in the "eat me" PS (phosphatidylserine) signal projected by a cell undergoing apoptosis (Draper *et al.*, 2004). PS is a membrane lipid found on the inside leaflet of the plasma membrane, and is constantly flipping to the outside leaflet but rarely stays there. If a cell is undergoing apoptotis, PS stays on the outside leaflet where it can be recognized by receptors on a T-cell that bind PS and trigger phagocytosis of the apoptotic cell (Fadok *et al.*, 2000; Savill & Fadok, 2000). This recognition has recently been found to include an adaptor protein, annexin I. It is rapidly upregulated upon Fas stimulation is Ca^{2+} and caspase-dependent and is recruited by exposed PS (Arur *et al.*, 2003). The flipping of PS is reliant on three enzymes, a Ca^{2+}-dependent scramblase that randomly deposits lipids on the outer and inner leaflets, an ATP-dependent aminophospholipid translocase that transfers lipids from the outer to the inner leaflet and a third as of yet unidentified enzyme responsible for the transfer of lipid to the outer membrane (Balasubramanian & Schroit, 2003). This flipping appears to be Ca^{2+}-dependent in a variety of cell types (Dachary-Prigent *et al.*, 1995), and occurs before the activation of caspases (Draper *et al.*, 2004).

Within the ER, Ca^{2+} concentrations are buffered by the ER chaperone, calreticulin. To observe the effect of the ER luminal environment on apoptosis, HeLa cells over-expressing calreticulin were generated and were observed to have an increased sensitivity to thapsigargin and staurosporine induced apoptosis (Nakamura *et al.*, 2000). As well, over-expression of calreticulin in human embryonic kidney cells (HEK-293 cells) using the pTeT-On inducible system resulted in augmented ER Ca^{2+} levels, increased amount of Ca^{2+} available for release, amplified cytochrome *c* release as well as greater susceptibility of the cells to apoptosis (Arnaudeau *et al.*, 2002). In contrast, calreticulin-deficient cells had a decreased amount of cytochrome *c* release, decreased caspase-3 activity, decreased amount of Ca^{2+} available for release, as well as a drastically reduced sensitivity to apoptosis (Nakamura *et al.*, 2000). It appears that calreticulin, *via* its Ca^{2+} binding capabilities, increases the amount of Ca^{2+} available for release from the ER lumen. In addition, over-expression of the pro-survival protein Bcl-2 in LNCaP prostate cancer epithelial cells results in a reduction in calreticulin as well as SERCA, consequently less Ca^{2+} was present in the ER and available for release (Vanden Abeele *et al.*, 2002), all resulting in a significant resistance to apoptosis. This mobilization of ER Ca^{2+} stores initiates the activation of the apoptotic pathway and sensitizes mitochondria to directed pro-apoptotic stimuli (Breckenridge *et al.*, 2003b). This mobilization can also be triggered by over-expression of the SERCA pump in liver-derived cells and is responsible for augmented Ca^{2+} uptake into the ER, with amplified ER Ca^{2+} concentration available for mobilization, resulting in increased sensitivity to apoptosis (Ma *et al.*, 1999). If Ca^{2+} release is inhibited by loss of the IP_3R, as seen in Jurkat T lymphocytes deficient in IP_3R, the cells are unable to release Ca^{2+} (Jayaraman & Marks, 1997), and are resistant to apoptosis. This resistance to apoptosis may be related to defects in calcineurin, the Ca^{2+}-dependent cytoplasmic phosphatase, as well as to inactivation of caspases due to lack of intracellular Ca^{2+} release after stimulation (Tantral *et al.*, 2004). Both extrinsic and intrinsic signals that trigger apoptosis involve a disruption in Ca^{2+} homeostasis.

Ca^{2+} is also known to regulate another family of proteins involved in the control of apoptosis, termed calpains. Calpains are cytoplasmic, Ca^{2+}-dependent cysteine proteases similar to caspases that are ubiquitously expressed in all animal cells. Unlike caspases, which function only during apoptosis, calpains are active in normal cellular activities such as cell cycle (Choi *et al.*, 1997; Pariat *et al.*, 1997) and cellular remodeling (Potter *et al.*, 1998). Calpains are activated secondary to caspases (Wood & Newcomb, 1999) by the sustained increase in cytoplasmic Ca^{2+} levels (Nath *et al.*, 1996) and are usually found to deactivate caspases. The calpain family is composed of at least six members, which can be divided into two groups: ubiquitous and tissue specific. Two of these family members, μ and m-calpain, are ubiquitously expressed and can be distinguished by their *in vitro* requirement for different levels of Ca^{2+} required for activation (μ-calpain and m-calpain require micro- and millimolar concentrations of Ca^{2+}, respectively). Calpain-mediated proteolysis, unlike caspases, does not require a specific amino-acid residue for cleavage. Calpain is observed to influence signal transduction processes by cleaving cytoskeletal proteins, membrane proteins, and enzymes (Saido *et al.*, 1994). Calpains are also found to be involved in cell death and cleave several substrates of the apoptotic pathway. Ca^{2+}-dependent activation of calpains has been implicated in activating caspases with different apoptotic stimuli (Wang, 2000) including ER stress (Nakagawa & Yuan, 2000), B-cell receptor (Ruiz-Vela *et al.*, 1999) and radiation-induced apoptosis (Waterhouse *et al.*, 1998). For example, upon apoptotic stimulation, Ca^{2+}-dependent calpain cleaves the prodomain, activating ER-localized caspase-12 which is involved in the ER stress pathway (Nakagawa & Yuan, 2000; Kusakawa *et al.*, 2000; Lee *et al.*, 2000; Mandic *et al.*, 2002). Several other pro-apoptotic proteins, such as

Bax (Wood *et al.*, 1998; Choi *et al.*, 2001), and Bid (Mandic *et al.*, 2002), are also cleaved by calpain to increase their activity, and in addition, calpain may collaborate with caspases in the execution of apoptosis (Wood & Newcomb, 1999). The calpain protease activity is dependent on Ca^{2+} and this activation occurs downstream of the cytoplasmic rise in Ca^{2+} (Hajnoczky *et al.*, 2003). Another substrate of calpain is the endogenous calcineurin inhibitor, CAIN/cabin1 that binds via its C-terminus and inhibits calcineurin (Sun *et al.*, 1998; Lai *et al.*, 1998; 2000). Cleavage of CAIN within this C-terminus generates a cleavage product with a molecular mass of 32 kDa, allowing dissociation from calcineurin, and resultant activation of calcineurin, leading to de-phosphorylation and activation of transcription factors such as MEF2 and NF-AT (Kim *et al.*, 2002). Calpain has also been shown to cleave the ER chaperone GRP94 which in turn may also affect Ca^{2+} signaling during apoptosis (Reddy *et al.*, 1999). Calpain activity can be directly regulated by its endogenous inhibitor, calpastatin. Calpastatin is cleaved by caspase-3 and this cleavage is essential for the regulation of calpain activity during cell death (Porn-Ares *et al.*, 1998; Zhu *et al.*, 1998).

A disturbance in Ca^{2+} homeostasis appears to be accountable for the development of the apoptotic pathways. Future development will hopefully help elaborate on the essential role of the ER during apoptosis. ER membrane may be the future target for therapeutic innervation for numerous diseases.

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