Review

Calcium binding chaperones of the endoplasmic reticulum

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Abstract. The endoplasmic reticulum is a major Ca^{2+} store of the cell that impacts many cellular processes within the cell. The endoplasmic reticulum has roles in lipid and sterol synthesis, protein folding, post-translational modification and secretion and these functions are affected by intraluminal endoplasmic reticulum Ca^{2+}. In the endoplasmic reticulum there are several Ca^{2+} buffering chaperones including calreticulin, Grp94, BiP and protein disulfide isomerase. Calreticulin is one of the major Ca^{2+} binding/buffering chaperones in the endoplasmic reticulum. It has a critical role in Ca^{2+} signalling in the endoplasmic reticulum lumen and this has significant impacts on many Ca^{2+}-dependent pathways including control of transcription during embryonic development. In addition to Ca^{2+} buffering, calreticulin plays important role in the correct folding and quality control of newly synthesized glycoproteins.

Key words: Calcium binding — Chaperones — Endoplasmic reticulum

Introduction

The endoplasmic reticulum (ER) of eukaryotic cells is an extensive, continuous network of membrane tubules and is a separate metabolic compartment that houses many functions critical to the survival of a cell (Baumann and Walz 2001; Schroder 2008). The ER lumen provides a unique environment with a high concentration of Ca^{2+} binding proteins which directly influences the functioning of the ER such as its roles in Ca^{2+} storage and release, membrane and secretory protein synthesis and folding including post-translational modifications such as N-linked glycosylation and the formation of disulfide bonds, lipid and sterol synthesis and metabolism and signal transduction (Michalak et al. 2002; Schroder 2008). Many ER Ca^{2+} binding proteins have dual functions and are also molecular chaperones involved in protein folding and quality control (Ashby and Tepikin 2001). The functions of these chaperones and formation of folding complexes is dependent on Ca^{2+} concentrations (Ashby and Tepikin 2001). ER luminal Ca^{2+} impacts all downstream functions of the ER including apoptosis, stress response, organogenesis and transcriptional activity (Michalak et al. 2002). The protein folding machinery of the ER is highly sensitive to ER luminal Ca^{2+} fluctuations and the ER has evolved a sophisticated system of quality control as well as an unfolded protein response (UPR) pathway in order to deal with mis-folded proteins. Impaired function of the ER leads to many severe diseases (Michalak et al. 2002). This review will focus on ER luminal Ca^{2+} and the ER luminal Ca^{2+} buffering chaperones, their role in ER-dependent Ca^{2+} homeostasis and how they impact quality control in the secretory pathway.

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ER, a calcium storage organelle

Cytoplasmic Ca^{2+} is a versatile signalling molecule affecting many cellular functions including exocytosis, contraction, metabolism, transcription, fertilization and proliferation (Berridge et al. 2003). The ER is the major intracellular Ca^{2+} store in the cell. The total ER Ca^{2+} concentration is estimated to be 2 mmol/l while the free ER Ca^{2+} concentration varies from 50 to 500 μmol/l (Groenendyk 2006; Meldolesi and Pozzan 1998). This is magnitudes higher than the free cytoplasmic Ca^{2+} level which is approximately 100 nmol/l (Michalak et al. 2009). The ER Ca^{2+} stores play an essential role in Ca^{2+} signalling. The buffering of ER luminal Ca^{2+} is critical to the many diverse ER functions. Overload and depletion of ER Ca^{2+} stores have detrimental effects on the entire cell. Ca^{2+} release from ER stores is controlled by the inositol 1,4,5-triphosphate ($InsP_3$) receptor and the ryanodine receptor (RyR) (Taylor and Laude 2002). ER

Ca^{2+} store refilling is controlled by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Lipskaia et al. 2009) while the Na^{+}/Ca^{2+} exchanger and the plasma membrane Ca^{2+} ATPase actively remove Ca^{2+} from the cells (Rhodes and Sanderson 2009). Taken together, both the ER Ca^{2+} buffering proteins and the ER pumps and exchangers exert powerful effects via ER Ca^{2+} fluctuations on the varied functions of the ER (Frischauf et al. 2008). Store-operated calcium influx occurs when there is a depletion of ER Ca^{2+} stores which activates channels in the plasma membrane to refill the internal stores (Putney 1986). High throughput RNAi screens led to the identification of stromal interaction molecule 1 (STIM1) that functions as an ER Ca^{2+}-sensor that accumulates in punctuate close to the plasma membrane upon store-depletion (Frischauf et al. 2008). It clusters at the plasma membrane with the Ca^{2+} channel, Orai1, to activate Ca^{2+} influx (Frischauf et al. 2008).

Calreticulin, a major calcium buffering chaperone of the ER

Calreticulin is a 46-kDa ER resident Ca^{2+} binding and buffering protein and molecular chaperone (Michalak et al. 2009). Calreticulin contains ER targeting signal sequence and it terminates with ER retrieval signal, Lys-Asp-Glu-Leu-COOH (KDEL) (Fliegel et al. 1989). The protein has been implicated to play a role in many diverse cellular processes (Michalak et al. 2009). Many of these functions are due to calreticulin's role as an ER Ca^{2+} buffering protein (Michalak et al. 2009). The protein can be divided into three major structural and functional domains (Fliegel et al. 1989; Nakamura et al. 2001b; Ostwald and MacLennan 1974). The N-domain (residues 1-180) of calreticulin contains both polypeptide- and carbohydrate binding sites and, together with the P-domain, it is critical to the chaperone function of the protein (Kapoor et al. 2004; Leach et al. 2002). Within the N-domain, there are specific amino acid residues that contribute to oligosaccharide binding and conformational stability of the protein (Kapoor et al. 2004; Leach and Williams 2003; Martin et al. 2006; Thomson and Williams 2005). The P-domain (residues 181-290) immediately follows the N-domain and forms a flexible arm domain (Ellgaard et al. 2001a; Ellgaard et al. 2001b). This central proline-rich core is characterized by three copies of two repeat amino acid sequences (denoted type 1 and 2) and are arranged in a "111222" pattern (Ellgaard et al. 2001a). These repeats may play a role in oligosaccharide binding contributing to the lectin-like function of calreticulin. They may also be involved in forming complexes between calreticulin and ERp57, an oxidoreductase folding enzyme of the ER (Vassilakos et al. 1998). Indeed, NMR studies revealed that ERp57 docks on the tip of the P-domain of calreticulin (Frickel et al. 2002). This may involve specific amino acid residues including Glu^{239}, Asp^{241}, Glu^{243}, Trp^{244} (Frickel et al. 2002; Leach et al. 2002; Martin et al. 2006). Interestingly, *in vitro* studies indicate that the P-domain binds Ca^{2+} with a high affinity (K_d = 1 μmol/l) and a low capacity (1 mol of Ca^{2+} per mol of protein) (Baksh and Michalak 1991). The third domain of calreticulin is the low affinity (K_d = 2 mol/l), high capacity (25 mol of Ca^{2+} per mol of protein) Ca^{2+} binding C-domain (residues 291-400) (Baksh and Michalak 1991; Nakamura et al. 2001b). This domain is responsible for binding over 50% of Ca^{2+} in the ER (Nakamura et al. 2001b). The Ca^{2+} binding capacity of the C-domain is derived from large clusters of acidic amino acid residues consisting of aspartic and glutamic acid interrupted with basic residues of lysine and arginine (Breier and Michalak 1994). Interruption of the basic residues, interestingly, results in a reduction in the Ca^{2+} binding capacity of calreticulin (Breier and Michalak 1994).

Mutational analysis revealed that specific amino acid residues are required for the chaperone function of calreticulin (Guo et al. 2003; Martin et al. 2006). Trp^{302} in the carbohydrate binding N-domain as well as Trp^{244} at the tip of the P-domain are both critical to the chaperone function of calreticulin (Martin et al. 2006). The N-domain amino acid His^{153} was found to not only be essential for chaperone function but also important in the structure of calreticulin (Guo et al. 2003). Surprisingly, when the disulfide bridge in the N-domain was disrupted (Cys^{88} and Cys^{120}) there was only partial disruption of the chaperone function of calreticulin (Martin et al. 2006). Overall, conformational changes of calreticulin resulting from mutations in specific amino acids residues of the protein or due to fluctuations in Ca^{2+} concentrations of the ER lumen impact the functionality of the chaperone function of the protein (Corbett et al. 2000; Guo et al. 2003; Martin et al. 2006).

Calreticulin, loss-of-function and gain-of-function

The direct link of calreticulin to Ca^{2+} signalling is highlighted by data that shows that changes in calreticulin expression level directly correlates to changes in Ca^{2+} signalling (Nakamura et al. 2001b). It is not surprising, therefore, that the regulation of expression of calreticulin impacts on ER Ca^{2+} release and storage capacity and ultimately results in abnormal embryonic development (Li et al. 2002; Mesaeli et al. 1999; Nakamura et al. 2001a). Key to understanding the chaperoning functions and Ca^{2+} binding properties of calreticulin comes from studies involving animal and cellular models of calreticulin-deficient systems and over-expressing calreticulin (Michalak et al. 2009). These studies further demonstrate the role of calreticulin as a buffering protein and a regulator of Ca^{2+} homeostasis might be far

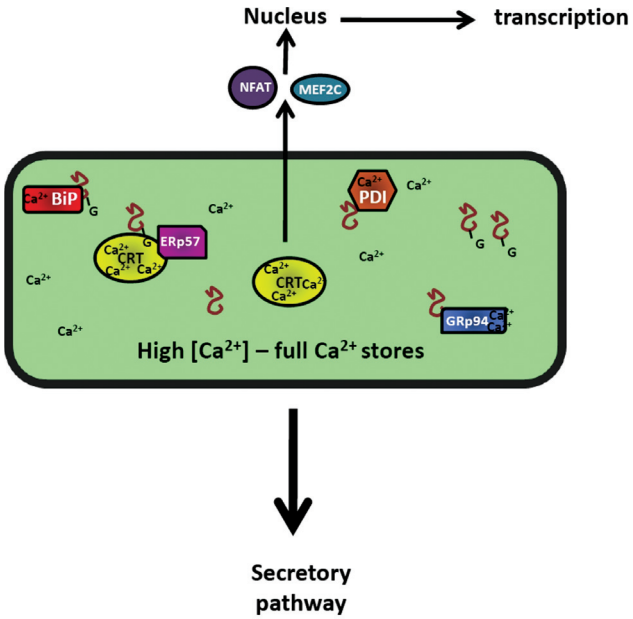

Figure 1. Calcium binding chaperones in the endoplasmic reticulum. Under conditions of high [Ca^{2+}] or full Ca^{2+} stores, the chaperones, calreticulin (CRT), glucose-regulated protein 78 (Grp78/BiP), glucose-regulated protein 94 (Grp94), protein disulfide isomerase (PDI) and the oxidoreductase ERp57 are fully active to bind mis-folded proteins. Both the secretory pathway and Ca^{2+} dependent transcriptional processes are also fully active.

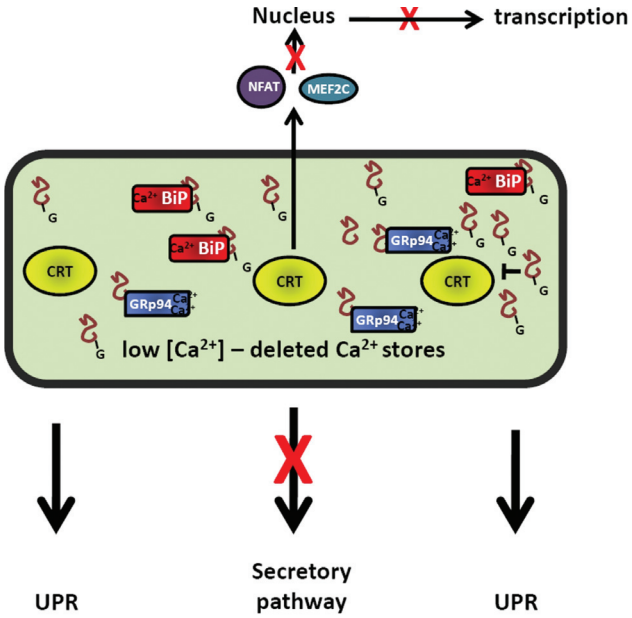

Figure 2. Calcium binding chaperones in the endoplasmic reticulum. When there is low [Ca^{2+}] or empty Ca^{2+} stores, there is an increase in accumulation of mis-folded proteins, activation of the unfolded protein response (UPR) and subsequent increase in expression of BiP, Grp94 and calreticulin (CRT). There is also significant inhibition of the secretory pathway and Ca^{2+} dependent transcriptional processes. NFAT, nuclear factor of activated T-cells; MEF, myocyte-specific enhancer factor; G, glucose.

more critical than its role as molecular chaperone (Figs. 1 and 2) (Michalak et al. 2009).

Loss-of-Function

Studies with calreticulin-deficient mice and embryonic stem cells show a critical role for calreticulin in maintenance of ER Ca^{2+} (Li et al. 2002; Mesaeli et al. 1999; Nakamura et al. 2001a). Calreticulin-deficient mice are embryonic lethal at 14.5 post-coitum due to impaired cardiac development (Mesaeli et al. 1999). Specifically, there is a marked decrease in ventricular wall thickness and deep intertrabecular recesses in the ventricular walls, however, development of all other tissues is normal (Mesaeli et al. 1999; Rauch et al. 2000). Interestingly, fibroblast cells derived from calreticulin-deficient embryos show significantly reduced ER Ca^{2+} capacity although free ER Ca^{2+}, as measured by the ER-targeted "cameleon" reporter, remains unchanged (Nakamura et al. 2001b). Also, in *crt*$^{-/-}$ derived fibroblasts, there is inhibition of bradykinin-induced Ca^{2+} release. This is likely due to the impairment of bradykinin binding to its cell surface receptor suggesting that calreticulin plays a role in the correct folding of the bradykinin receptor (Mesaeli et al. 1999; Nakamura et al. 2001b). This inability for bradykinin Ca^{2+} release results in aberrant nuclear translocation of a number of factors such as NF-AT (nuclear translocation of nuclear factor of activated T-cells) and MEF2C (myocyte-enhancer factor 2C) (Guo et al. 2001; Lynch et al. 2005; Mesaeli et al. 1999). These transcription factors are all essential during vertebrate cardiac morphogenesis and hypertrophy (Chien and Olson 2002; Lynch et al. 2005; Qiu and Michalak 2009; Srivastava and Olson 2000). These studies suggest that the lethality observed in the calreticulin-deficient mice is due to the Ca^{2+} buffering role of calreticulin but not its chaperone function (Michalak et al. 2009; Nakamura et al. 2001b). Interestingly, the embryonic lethality of the calreticulin-deficient mice is rescued by over-expression of the serine/threonine phosphatase, calcineurin (Guo et al. 2002). These mice have rescued cardiac development however they show impeded growth, hypoglycaemia, increased levels of serum triacylglycerols and cholesterol indicating an important role of calreticulin in postnatal energy metabolism (Guo et al. 2002). Most importantly, over-expression of activated calcineurin rescues the nuclear localization of NF-AT and MEF2C that is aberrant in the absence of calreticulin (Guo et al. 2002; Lynch et al. 2005). The rescue of these calreticulin-deficient mice with activated calcineurin underscores the importance of the calreticulin and calcineurin relationship in the Ca^{2+}

signalling cascade for normal cardiac development (Guo et al. 2002).

Studies using calreticulin-deficient embryonic stem cells further support the role of the ER, calreticulin and Ca^{2+} in cardiogenesis (Li et al. 2002). *crt*$^{-/-}$ ES-derived cardiomyocytes have a severe disruption of myofibrillogenesis due to insufficient expression and Ca^{2+} -dependent phosphorylation of ventricular myosin light chain 2 (MLC2v) (Li et al. 2002). Myofibrillogenesis is rescued in *crt*$^{-/-}$ ES-derived cardiomyocytes when they are supplied with a Ca^{2+} ionophore highlighting the importance of calreticulin and ER Ca^{2+} signalling in cardiac development (Li et al. 2002).

Gain-of-Function

Increased expression of calreticulin results in significant increase in Ca^{2+} capacity of the ER (Arnaudeau et al. 2002). Transgenic mice over-expressing calreticulin in the heart display bradycardia, complete heart block and sudden death, cardiac edema and abnormal sarcomere structure of the heart, dilated ventricular chamber and atria, thinner ventricular walls and disarrayed cardiomyocytes (Nakamura et al. 2001a; Hattori et al. 2007). Over-expressing mice also had reduced HCN1 (hyperpolerization-activated cyclic nucleotide-gated channel1) activation, which regulates cardiac pacemaker activity (Hattori et al. 2007). A decrease in the protein level of connexin40 (Cx40) and connexin43 (Cx43), components of gap junction, and MEF2C were also observed (Hattori et al. 2007). Together, decreased levels of HCN1, Cx40 and MEF2C results in impaired structure and function of the heart in calreticulin over-expressing mice (Hattori et al. 2007). Interestingly, calreticulin autoantibody has been detected in patients suffering from congenital heart block and transgenic mice over-expressing calreticulin in the heart have a similar phenotype to children suffering from congenital heart block (Nakamura et al. 2001a; Hattori et al. 2007; Orth et al. 1996; Moak et al. 2001). This suggests a role for calreticulin in the pathogenesis of adult and paediatric congenital heart block (Orth et al. 1996; Moak et al. 2001).

Calcium binding chaperones and folding enzymes of the ER

In addition to calreticulin there are a number of other Ca^{2+} buffering chaperones and folding enzymes that affect ER-dependent Ca^{2+} homeostasis. These include calnexin, BiP/Grp78, glucose-regulated protein 94 (Grp94), protein disulfide isomerase (PDI)/Calcistorin, and ERp72. BiP/Grp78 binds Ca^{2+} at relatively low capacity (1-2 mol of Ca^{2+} per mol of protein) but is responsible for as much as 25% of the Ca^{2+} binding capacity of the ER (Lievremont et al. 1997). Grp94 is one of the most abundant Ca^{2+} buffering proteins of the ER (Argon and Simen 1999). It is a low-affinity, high-capacity Ca^{2+} binding protein with 15 moderate-affinity sites (K_d =~ 2 μM) with low capacity (1 mol Ca^{2+} per mol of protein) and 11 low-affinity sites (K_d ~ 600 μM) with high capacity (10 mol of Ca^{2+} per mol of protein) (Argon and Simen 1999). Grp94 is highly expressed in the early stages of embryonic hearts and suggests that it may have a role in the process of myocardial cell differentiation and heart development (Barnes and Smoak 1997). It has been found that the selective increase in Grp94 in response to Ca^{2+} levels protects cardiomyocytes in ischemia (Vitadello et al. 2003).

There are several ER oxidoreducatases in the ER lumen that also have roles buffering ER Ca^{2+}. PDI is a 58-kDa protein that binds Ca^{2+} with a high capacity (19 mol Ca^{2+} per mol of protein) and weak affinity (K_d =2-5mM) (Lebeche et al. 1994). ERcalcistorin/PDI is an ER luminal calsequestrin-like protein that binds Ca^{2+} with a high capacity (23 mol of Ca^{2+} per mol of protein) and low affinity (K_d = ~1mM) (Lucero and Kaminer 1999). ERp72, a 72-kDa member of the PDI family, is known to bind with a high capacity (12 mol of Ca^{2+} per mol of protein) and low affinity (Lucero et al. 1998).

ER Ca^{2+} and quality control in the secretory pathway

The ER is a multifunctional organelle and aside from its role in Ca^{2+} storage it is also well-known for its role in the synthesis, folding and post-translational modification of all secreted and integral membrane proteins (Groenendyk 2006). The critical importance of the ER as a site for protein storage machinery is underscored by the numerous diseases that result from impaired protein folding or post-translational machinery (Groenendyk 2006). Many of the ER Ca^{2+} buffering proteins also have dual functions as folding chaperones for newly synthesized proteins, so it is not surprising that their structure, complex formation with other foldases as well as substrates is dependent on fluctuations within the ER lumen (Corbett et al. 1999; Corbett and Michalak 2000). In addition, exit of properly folded proteins from the ER and trafficking to the Golgi apparatus is also an ER Ca^{2+} dependent process (Lodish and Kong 1990).

Calreticulin, calnexin and other ER Ca^{2+} binding chaperons and folding enzymes are important component of protein folding and quality control. A lectin-like chaperone function of calreticulin and calnexin is especially important in this process. Both proteins assist in folding of glycosylated proteins via their interaction with mono-glucosylated protein (Oliver et al. 1999). The protein is released from the folding machinery when the glucose residue is removed by glucosidase II (Hebert and Molinari 2007). Monoglucosylated carbohydrate binding to calreticulin or calnexin is dependent on the presence of Ca^{2+} (Williams 2006). In the

absence of Ca^{2+}, these interactions are broken resulting in accumulation of mis-folded proteins, activation of UPR and frequently cell death (Michalak et al. 2009). The interaction of the nascent polypeptide to calnexin and calreticulin is a Ca^{2+} -dependent process (Corbett et al. 2000). Under conditions of low ER luminal Ca^{2+} (<100 μmol/l), calreticulin is rapidly degraded by trypsin while under high luminal Ca^{2+} concentrations (500 μmol/l to 1 mmol/l) calreticulin formed an N-domain protease resistant core (Corbett et al. 2000). This suggests that fluctuations in Ca^{2+} within the ER lumen can affect the conformation of calreticulin and this will inevitably impact on the function of this protein (Corbett et al. 2000). Correctly folded proteins are transported to their biological destinations. However, if the protein is still mis-folded, it is recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT1) which specifically re-glucosylates allowing the protein to re-enter the calreticulin/calnexin/ERp57 cycle for another round of protein folding (Hebert and Molinari 2007). If the protein is terminally mis-folded it is removed from the ER by ER associated degradation (ERAD) which involves translocation of the mis-folded proteins to the cytoplasm where they are degraded by ubiquitine-dependent pathway (Vembar and Brodsky 2008). Native proteins, however, are transported from the ER through the Golgi to their correct location within the cell (Malhotra and Kaufman, 2007).

ER Ca^{2+} and the unfolded protein response

Disruption of any of the protein folding machinery results in an increase in mis-folded proteins in the ER lumen and this is termed ER stress (Schroder and Kaufman 2005a). In order to deal with this increased load of proteins in the ER, the cell has evolved a sophisticated system called the unfolded protein response (UPR) (Kozutsumi et al. 1988; Schroder and Kaufman 2005a). UPR is modulated by three ER transmembrane proteins called activating transcription factor-6 (ATF6), inositol-requiring kinase 1 (IRE) and double-stranded RNA-activated protein kinase-like ER kinase (PERK) (Schroder and Kaufman 2005b). These proteins work to alleviate stress on the ER by decreasing protein load through three simple adaptive mechanisms. First, there is an up-regulation of chaperone proteins and foldases as well as an increase in the size of the ER. Secondly, there is an inhibition of translation of newly synthesized proteins in the ER and, thirdly, there is an increase in ERAD machinery to rapidly clear mis-folded proteins from the ER lumen (Schroder and Kaufman 2005b). Activation of the three sensors is maintained by the regulatory protein, glucose regulated protein-78 (Grp78) or BiP (Hendershot 2004). Therefore, BiP has been referred to the as the master regulator of the ER because this role in the ER that prevents aggregation of newly synthesized proteins and associates with UPR sensors to prevent their activation (Hendershot 2004; Groenendyk and Michalak 2005; Hebert and Molinari 2007). Interestingly, drugs that interfere with intracellular Ca^{2+} stores are known to activate UPR (Tombal et al. 2002). For example, thapsigargin, a Ca^{2+}-ATPase inhibitor, decreases the intracellular Ca^{2+} concentration resulting in activation of all branches of the UPR pathway (Lytton et al. 1991; Li et al. 2000). Additionally, the ionophore, ionomycin, which increases intracellular Ca^{2+} levels, is also known to induce the UPR pathway and its treatment of cells is marked with an increase in expression of BiP/Grp78 (Miyake et al. 2000).

PERK, an ER transmembrane kinase, is the first sensor activated in mammalian UPR and its function is to transiently attenuate mRNA translation decreasing the load of newly synthesized proteins into the already stressed ER (Malhotra and Kaufman 2007; Lin et al. 2009). When there is an accumulation of mis-folded proteins in the ER lumen, BiP is sequestered by mis-folded proteins, released from PERK which results in the dimerization of PERK and eventual *trans*-autophosphorylation (Malhotra and Kaufman 2007). This autophosphorylation event causes the activation of its alpha subunit of eukaryotic initiation factor 2 (eIF2α) phosphorylation activities where PERK goes on to phosphorylate eIF2α at Ser^{51} (Malhotra and Kaufman 2007; Lin et al. 2009). Phosphorylated eIF2α inhibits translation of mRNA and consequently reduces protein synthesis (Malhotra and Kaufman 2007). Phosphorylated eIF2α also plays a selective role in the transcription by inducing the translation of activating transcription factor 4 (ATF4) mRNA which results in the transcription of genes involved in mechanisms such as apoptosis and anti-oxidative stress response (Ameri and Harris 2008).

In mammalian cells, IRE1 is a bi-functional protein with not only a cytosolic carboxy-terminal kinase domain but also an endoribonuclease domain (Back et al. 2005). Under conditions of no stress, IRE1, like PERK, is maintained as an inactive homodimer by the protein chaperone Grp78/BiP. However, when mis-folded proteins accumulate in the ER lumen, IRE1 is released from the Grp78/BiP protein and it homodimerizes and *trans*-autophosphorylates to activate its endoribonuclease (RNase) activity (Malhotra and Kaufman 2007). The RNase domain of IRE1 targets the mRNA of the basic leucine zipper domain (bZIP) containing transcription factor, X-box binding factor-1 (Xbp1) (Malhotra and Kaufman 2007). There is splicing of a 26-nucleotide intron from the mRNA of Xbp1 causing a translational frameshift to an active and stable transcription factor (Back et al. 2005; Malhotra and Kaufman 2007). The splicing of the mRNA results in the activation of a potent transcription factor whose protein, *XBP1*, translocates to the nucleus where it activates the transcription of UPR element (UPRE) containing genes to alleviate ER stress (Malhotra and Kaufman 2007). There is activation in the transcription of genes involved in ER associ-

ated degradation (ERAD) in order to alleviate the build up of mis-folded proteins in the ER lumen (Back et al. 2005).

Upon ER stress and UPR, ATF6 is released from Grp78/BiP, cleaved and activated in response to ER stress and their bZip domains allow them to bind ERSEs as homodimers or as a heterodimer and this modulates the ER stress response (Kondo et al. 2005; Murakami et al. 2006; Thuerauf et al. 2004). Under normal conditions, the luminal domain of ATF6, like PERK and IRE1, is bound by Grp78/BiP however, it is also bound by calreticulin (through its three glycosylation sites) and upon ER stress, the luminal domain of ATF6 is released from Grp78/BiP and from calreticulin (due to underglycosylation of the three luminal glycosylation sites), revealing non-consensus Golgi localization sites (GLSs) (Shen et al. 2002; Hong et al. 2004; Shen et al. 2005). Since carbohydrate binding to calreticulin is Ca^{2+}-dependent it is conceivable that calreticulin-ATF6 interactions may be regulated by ER luminal Ca^{2+}. Once free of calreticulin and Grp78/BiP, ATF6 then translocates to the Golgi apparatus where it is subject to proteolytic processing (Shen et al. 2002; Hong et al. 2004) to release N-ATF6 portion of the ATF6. N-ATF6 translocates to the nucleus to activate promoters containing ERSE's (Thuerauf et al. 2007). The involvement of players such as Grp78/BiP and calreticulin in UPR suggest that Ca^{2+} may have role in the mediation of this stress response. It is of interest that these sensors are maintained in their "off" states by binding Grp78/BiP (PERK, IRE, ATF6) and calreticulin (ATF6) (Hendershot 2004; Hong et al. 2004).

Conclusions

The ER is a continuous, dynamic and multifunctional organelle and is the major Ca^{2+} store of the cell. It plays a vital role in many cellular processes of the cell including protein folding and secretion, post-translational modification, lipid and sterol biosynthesis and Ca^{2+} buffering and homeostasis. Calreticulin, an ER resident protein, is the major Ca^{2+} binding protein of the ER. Modulation of this protein is tightly controlled to impact Ca^{2+} stores and signalling. Ca^{2+} fluctuations as a result of calreticulin modulation impact the cells at the molecular level, as seen with impacts on protein folding machinery, as well as at the whole tissue level, specifically cardiac development. Normal cardiac development is ultimately controlled by calreticulin and its mis-regulation (loss-of-function or gain-of-function) leads to lethal cardiac pathologies. It is exceedingly important to continue to study the role calreticulin plays in the ER as it is a valuable player in normal development.

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References

Ameri K., Harris A. L. (2008): Activating transcription factor 4. Int. J. Biochem. Cell Biol. **40**, 14–21

Argon Y., Simen B. B. (1999): GRP94, an ER chaperone with protein and peptide binding properties. Semin. Cell Dev. Biol. **10**, 495–505

Arnaudeau S., Frieden M., Nakamura K., Castelbou C., Michalak M., Demaurex N. (2002): Calreticulin differentially modulates calcium uptake and release in the endoplasmic reticulum and mitochondria. J. Biol. Chem. **277**, 46696–46705

Ashby M. C., Tepikin A. V. (2001): ER calcium and the functions of intracellular organelles. Semin. Cell Dev. Biol. **12**, 11–17

Back S. H., Schroder M., Lee K., Zhang K., Kaufman R. J. (2005): ER stress signaling by regulated splicing: IRE1/HAC1/XBP1. Methods **35**, 395–416

Baksh S., Michalak M. (1991): Expression of calreticulin in *Escherichia coli* and identification of its Ca^{2+} binding domains. J. Biol. Chem. **266**, 21458–21465

Barnes J. A., Smoak I. W. (1997): Immunolocalization and heart levels of GRP94 in the mouse during post-implantation development. Anat. Embryol. **196**, 335–341

Baumann O., Walz B. (2001): Endoplasmic reticulum of animal cells and its organization into structural and functional domains. Int. Rev. Cytol. **205**, 149–214

Berridge M. J., Bootman M. D., Roderick H. L. (2003): Calcium signalling: dynamics, homeostasis and remodelling. Nat. Rev. Mol. Cell Biol. **4**, 517–529

Breier A., Michalak M. (1994): 2,4,6-Trinitrobenzenesulfonic acid modification of the carboxyl-terminal region (C-domain) of calreticulin. Mol. Cell. Biochem. **130**, 19–28

Chien K. R., Olson E. N. (2002): Converging pathways and principles in heart development and disease: CV@CSH. Cell **110**, 153–162

Corbett E. F., Michalak K. M., Oikawa K., Johnson S., Campbell I. D., Eggleton P., Kay C., Michalak M. (2000): The conformation of calreticulin is influenced by the endoplasmic reticulum lumenal environment. J. Biol. Chem. **275**, 27177–27185

Corbett, E. F., Michalak, M. (2000): Calcium, a signaling molecule in the endoplasmic reticulum? Trends Biochem. Sci. **25**, 307–311

Corbett, E. F., Oikawa, K., Francois, P., Tessier, D. C., Kay, C., Bergeron, J. J. M., Thomas D. Y., Krause K. H., Michalak M. (1999): Ca^{2+} regulation of interactions between endoplasmic reticulum chaperones. J. Biol. Chem. **274**, 6203–6211

Ellgaard L., Riek R., Braun D., Herrmann T., Helenius A., Wuthrich K. (2001a): Three-dimensional structure topology of the calreticulin P-domain based on NMR assignment. FEBS Lett. **488**, 69–73

Ellgaard L., Riek R., Herrmann T., Guntert P., Braun D., Helenius A., Wuthrich K. (2001b): NMR structure of the calreticulin P-domain. Proc. Natl. Acad. Sci. U.S.A. **98**, 3133–3138

Fliegel L., Burns K., MacLennan D. H., Reithmeier R. A. F., Michalak M. (1989): Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. **264**, 21522–21528

Frickel E. M., Riek R., Jelesarov I., Helenius A., Wuthrich K., Ellgaard L. (2002a): TROSY-NMR reveals interaction between ERp57 and the tip of the calreticulin P-domain. Proc. Natl. Acad. Sci. U.S.A. **99**, 1954–1959

Frischauf I., Schindl R., Derler I., Bergsmann J., Fahrner M., Romanin C. (2008): The STIM/Orai coupling machinery. Channels (Austin) **2**, 261–268

Groenendyk J., Opas M., Michalak M. (2006): Protein folding and calcium homeostasis in the endoplasmic reticulum. Calcium Binding Proteins **1**, 77–85

Groenendyk J., Michalak M. (2005): Endoplasmic reticulum quality control and apoptosis. Acta Biochim. Pol. **52**, 381–395

Guo L., Groenendyk J., Papp S., Dabrowska M., Knoblach B., Kay C., Parker J. M. R., Opas M., Michalak M. (2003): Identification of an N-domain histidine essential for chaperone function in calreticulin. J. Biol. Chem. **278**, 50645–50653

Guo L., Lynch J., Nakamura K., Fliegel L., Kasahara H., Izumo S., Komuro I., Agellon L. B., Michalak M. (2001): COUP-TF1 antagonizes Nkx2.5-mediated activation of the calreticulin gene during cardiac development. J. Biol. Chem., **276**, 2797–2801

Guo L., Nakamura K., Lynch J., Opas M., Olson E. N., Agellon L. B., Michalak M. (2002): Cardiac-specific expression of calcineurin reverses embryonic lethality in calreticulin-deficient mouse. J. Biol. Chem. **277**, 50776–50779

Hattori K., Nakamura K., Hisatomi Y., Matsumoto S., Suzuki M., Harvey R. P., Kurihara H., Hattori S., Yamamoto T., Michalak M., Endo F. (2007): Arrhythmia induced by spatiotemporal overexpression of calreticulin in the heart. Mol. Genet. Metab. **91**, 285–293

Hebert D. N., Molinari M. (2007): In and out of the ER: protein folding, quality control, degradation, and related human diseases. Physiol. Rev. **87**, 1377–1408

Hendershot L. M. (2004): The ER function BiP is a master regulator of ER function. Mt. Sinai J. Med. **71**, 289–297

Hong M., Luo S. Z., Baumeister P., Huang J. M., Gogia R. K., Li M. Q., Lee A. S. (2004): Underglycosylation of ATF6 as a novel sensing mechanism for activation of the unfolded protein response. J. Biol. Chem. **279**, 11354–11363

Kapoor M., Ellgaard L., Gopalakrishnapai J., Schirra C., Gemma E., Oscarson S., Helenius A., Surolia A. (2004): Mutational analysis provides molecular insight into the carbohydrate-binding region of calreticulin: pivotal roles of tyrosine-109 and aspartate-135 in carbohydrate recognition. Biochemistry **43**, 97–106

Kondo S., Murakami T., Tatsumi K., Ogata M., Kanemoto S., Otori K., Iseki K., Wanaka A., Imaizumi K. (2005): OASIS, a CREB/ATF-family member, modulates UPR signalling in astrocytes. Nat. Cell Biol. **7**, 186–194

Kozutsumi Y., Segal M., Normington,K., Gething M. J., Sambrook J. (1988): The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. Nature **332**, 462–464

Leach M. R., Cohen-Doyle M. F., Thomas D. Y., Williams D. B. (2002): Localization of the lectin, ERp57 binding, and polypeptide binding sites of calnexin and calreticulin. J. Biol. Chem. **277**, 29686–29697

Leach M. R., Williams D. B. (2003): Lectin-deficient calnexin is capable of binding class I histocompatibility molecules in vivo and preventing their degradation. J. Biol. Chem. **279**, 9072–9079

Lebeche D., Lucero H. A., Kaminer B. (1994): Calcium binding properties of rabbit liver protein disulfide isomerase. Biochem. Biophys. Res. Commun. **202**, 556–561

Li J. A., Puceat M., Perez-Terzic C., Mery A., Nakamura K., Michalak M., Krause K. H., Jaconi M. E. (2002): Calreticulin reveals a critical Ca^{2+} checkpoint in cardiac myofibrillogenesis. J. Cell Biol. **158**, 103–113

Li M. Q., Baumeister P., Roy B., Phan T., Foti D., Luo S. Z., Lee A. S. (2000): ATF6 as a transcription activator of the endoplasmic reticulum stress element: thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. Mol. Cell. Biol. **20**, 5096–5106

Lievremont J. P., Rizzuto R., Hendershot L., Meldolesi J. (1997): BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of Ca^{2+}. J. Biol. Chem. **272**, 30873–33089

Lin J. H., Li H., Zhang Y., Ron D., Walter P. (2009): Divergent effects of PERK and IRE1 signaling on cell viability. PLoS ONE **4**, e4170

Lipskaia L., Hulot J. S., Lompre A. M. (2009): Role of sarco/endoplasmic reticulum calcium content and calcium ATPase activity in the control of cell growth and proliferation. Pflugers Arch. **457**, 673–685

Lodish H. F., Kong N. (1990): Perturbation of cellular calcium blocks exit of secretory proteins from the rough endoplasmic reticulum. J. Biol. Chem. **265**, 10893–10899

Lucero H. A., Kaminer B. (1999): The role of calcium on the activity of ERcalcistorin/protein-disulfide isomerase and the significance of the C-terminal and its calcium binding. A comparison with mammalian protein-disulfide isomerase. J. Biol. Chem. **274**, 3243–3251

Lucero H. A., Lebeche D., Kaminer B. (1998): ERcalcistorin/protein-disulfide isomerase acts as a calcium storage protein in the endoplasmic reticulum of a living cell. Comparison with calreticulin and calsequestrin. J. Biol. Chem. **273**, 9857–9863

Lynch J., Guo L., Gelebart P., Chilibeck K., Xu J., Molkentin J. D., Agellon L. B., Michalak M. (2005): Calreticulin signals upstream of calcineurin and MEF2C in a critical Ca^{2+}-dependent signaling cascade. J. Cell Biol. **170**, 37–47

Lytton J., Westlin M., Hanley M. R. (1991): Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase family of calcium pumps. J. Biol. Chem. **266**, 17067–17071

Malhotra J. D., Kaufman R. J. (2007): The endoplasmic reticulum and the unfolded protein response. Semin. Cell Dev. Biol. **18**, 716–731

Martin V., Groenendyk J., Steiner S. S., Guo L., Dabrowska M., Parker J. M. R., Muller-Esterl W., Opas M., Michalak M. (2006): Identification by mutational analysis of amino acid residues essential in the chaperone function of calreticulin. J. Biol. Chem. **281**, 2338–2346

Meldolesi J., Pozzan T. (1998): The endoplasmic reticulum Ca^{2+} store: a view from the lumen. Trends Biochem. Sci. **23**, 10–14

Mesaeli N., Nakamura K., Zvaritch E., Dickie P., Dziak E., Krause K.-H., Opas M., MacLennan D. H., Michalak M. (1999): Calreticulin is essential for cardiac development. J. Cell Biol. **144**, 857–868

Michalak M., Parker J. M. R., Opas M. (2002): Ca^{2+} signaling and calcium binding chaperones of the endoplasmic reticulum. Cell Calcium **32**, 269–278

Michalak M., Groenendyk J., Szabo E., Gold L. I., Opas M. (2009): Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. Biochem. J. **417**, 651–666

Miyake H., Hara I., Arakawa S., Kamidono S. (2000): Stress protein GRP78 prevents apoptosis induced by calcium ionophore, ionomycin, but not by glycosylation inhibitor, tunicamycin, in human prostate cancer cells. J. Cell. Biochem. **77**, 396–408

Moak J. P., Barron K. S., Hougen T. J., Wiles H. B., Balaji S., Sreeram N., Cohen M. H., Nordenberg A., Van Hare G. F., Friedman R. A, Perez M., Cecchin F., Schneider D. S., Nehgme R. A., Buyon J. P. (2001): Congenital heart block: development of late-onset cardiomyopathy, a previously underappreciated sequela. J. Am. Coll. Cardiol. **37**, 238–242

Murakami T., Kondo S., Ogata M., Kanemoto S., Saito A., Wanaka A., Imaizumi K. (2006): Cleavage of the membrane-bound transcription factor OASIS in response to endoplasmic reticulum stress. J. Neurochem. **96**, 1090–1100

Nakamura K., Robertson M., Liu G., Dickie P., Guo J. Q., Duff H. J., Opas M., Kavanagh K., Michalak M. (2001a): Complete heart block and sudden death in mouse over-expressing calreticulin. J. Clin. Invest. **107**, 1245–1253

Nakamura K., Zuppini A., Arnaudeau S., Lynch J., Ahsan I., Krause R., Papp S., De Smedt H., Parys J. B., Muller-Esterl W., Lew D. P., Krause K.-H., Demaurex N., Opas M., Michalak M. (2001b): Functional specialization of calreticulin domains. J. Cell Biol. **154**, 961–972

Oliver J. D., Roderick H. L., Llewellyn D. H., High S. (1999): ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin. Mol. Biol. Cell. **10**, 2573–2582

Orth T., Dorner T., Meyer Zum Buschenfelde K.-H., Mayet W.-J. (1996): Complete congenital heart block is associated with increased autoantibody titers against calreticulin. Eur. J. Clin. Invest. **26**, 205–215

Ostwald T. J., MacLennan D. H. (1974): Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. J. Biol. Chem. **249**, 974–979

Putney J. W. Jr. (1986): A model for receptor-regulated calcium entry. Cell Calcium **7**, 1–12

Qiu Y., Michalak M. (2009): Transcriptional control of the calreticulin gene in health and disease. Int. J. Biochem. Cell Biol. **41**, 531–538

Rauch F., Prud'homme J., Arabian A., Dedhar S., St-Arnaud R. (2000): Heart, brain, and body wall defects in mice lacking calreticulin. Exp. Cell Res. **256**, 105–111

Rhodes J. D., Sanderson J. (2009): The mechanisms of calcium homeostasis and signalling in the lens. Exp. Eye Res. **88**, 226–234

Schroder M. (2008): Endoplasmic reticulum stress responses. Cell. Mol. Life Sci. **65**, 862–894

Schroder M., Kaufman R. J. (2005a): ER stress and the unfolded protein response. Mutat. Res. **569**, 29–63

Schroder M., Kaufman R. J. (2005b): The mammalian unfolded protein response. Annu. Rev. Biochem. **74**, 739–789

Shen J., Chen X., Hendershot L., Prywes R. (2002): ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. Dev. Cell **3**, 99–111

Shen J., Snapp E. L., Lippincott-Schwartz J., Prywes R. (2005): Stable binding of ATF6 to BiP in the endoplasmic reticulum stress response. Mol. Cell. Biol. **25**, 921–932

Srivastava D., Olson E. N. (2000): A genetic blueprint for cardiac development. Nature **407**, 221–226

Taylor C. W., Laude A. J. (2002): IP3 receptors and their regulation by calmodulin and cytosolic Ca^{2+}. Cell Calcium **32**, 321–334

Thomson S. P., Williams D. B. (2005): Delineation of the lectin site of the molecular chaperone calreticulin. Cell Stress Chaperones **10**, 242–251

Thuerauf D. J., Marcinko M., Belmont P. J., Glembotski C. C. (2007): Effects of the isoform-specific characteristics of ATF6 alpha and ATF6 beta on endoplasmic reticulum stress response gene expression and cell viability. J. Biol. Chem. **282**, 22865–22878

Thuerauf D. J., Morrison L., Glembotski C. C. (2004): Opposing roles for ATF6alpha and ATF6beta in endoplasmic reticulum stress response gene induction. J. Biol. Chem. **279**, 21078–21084

Tombal B., Denmeade S. R., Gillis J. M., Isaacs J. T. (2002): A supramicromolar elevation of intracellular free calcium $[Ca^{2+}]_i$ is consistently required to induce the execution phase of apoptosis. Cell Death Differ. **9**, 561–573

Vassilakos A., Michalak M., Lehrman M. A., Williams D. B. (1998): Oligosaccharide binding characteristics of the molecular chaperones calnexin and calreticulin. Biochemistry **37**, 3480–3490

Vembar S. S., Brodsky J. L. (2008): One step at a time: endoplasmic reticulum-associated degradation. Nat. Rev. Mol. Cell Biol. **9**, 944–957

Vitadello M., Penzo D., Petronilli V., Michieli G., Gomirato S., Menabo R., Di Lisa F., Gorza L. (2003): Overexpression of the stress protein Grp94 reduces cardiomyocyte necrosis due to calcium overload and simulated ischemia. FASEB J. **17**, 923–925

Williams D. B. (2006): Beyond lectins: the calnexin/calreticulin chaperone system of the endoplasmic reticulum. J. Cell Sci. **119,** 615–623

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