Calcium, a signaling molecule in the endoplasmic reticulum?

Elaine F. Corbett and Marek Michalak

For many years now, it has been known that Ca^{2+} is an important signaling molecule in the cytosol of the cell, but emerging evidence suggests that Ca^{2+} might also play a signaling role in the endoplasmic reticulum. For example, agonist-induced fluctuations in free Ca^{2+} concentration in the endoplasmic reticulum can affect many functions of the endoplasmic reticulum, including protein synthesis and modification, and interchaperone interactions.

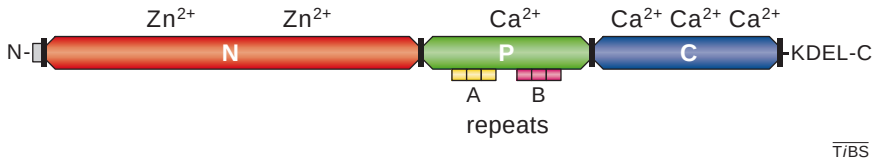
ALTERATIONS IN INTRACELLULAR Ca^{2+} homeostasis have profound effects on many cell functions, including secretion, contraction–relaxation, motility, metabolism, protein synthesis, modification and folding, gene expression, cell-cycle progression and apoptosis. Intracellular Ca^{2+} homeostasis is maintained primarily via the endoplasmic reticulum[1] (ER). In response to a variety of external stimuli, Ca^{2+} is released from the lumen of the ER via Ca^{2+} channels; these stimuli are mediated by the inositol-1,4,5-triphosphate [Ins(1,4,5)P_3] receptor and the ryanodine receptor[2,3]. Some Ca^{2+} also enters from the extracellular environment by crossing the plasma membrane[2,3]. The majority of Ca^{2+} released into the cytosol is subsequently transported back into the lumen of the ER via the sarcoplasmic–endoplasmic-reticulum Ca^{2+}-ATPase (SERCA)[2]. Some Ca^{2+} is removed from the cytosol by the plasma-membrane Ca^{2+}-ATPase and the Na^+–Ca^{2+} exchanger[2].

It is widely accepted that, when Ca^{2+} is released into the cytosol, it is an extremely important signaling molecule. There is an enormous range of known responses to increased cytosolic Ca^{2+} concentrations, some occurring within seconds, whereas others are far more prolonged[1–3]. The release and reuptake of Ca^{2+} results in a continuous fluctuation in the free concentration of Ca^{2+} in the lumen of the ER[1,4] (free $[Ca^{2+}]_{ER}$). When Ca^{2+} has been taken up from the cytoplasm into the ER, the Ca^{2+} stores are said to be full; when it has been released from the ER into the cytoplasm, the Ca^{2+} stores are said to be empty.

It is now known that changes in the free $[Ca^{2+}]_{ER}$ affect (and perhaps control) many functions of the ER, including the synthesis and secretion of proteins[5], the interactions of chaperones with one another and with their substrates[6–10], and the activation of Ca^{2+} influx via plasma-membrane channels[11]. These studies suggest that changes in the free $[Ca^{2+}]_{ER}$ play a signaling role in the lumen of the ER and that Ca^{2+}-binding chaperones could be involved in 'sensing' these changes.

The free concentration of Ca^{2+} fluctuates in the lumen of the ER in agonist-stimulated cells

The total concentration of Ca^{2+} in the lumen of the ER is estimated to be 1–3 mM (Ref. 1). A significant portion of this Ca^{2+} is free[1], but the free $[Ca^{2+}]_{ER}$ is extremely difficult to measure and is



N domain	P domain	C domain
Highly conserved amino acid sequence	High-affinity Ca^{2+}-binding site	High-capacity Ca^{2+}-binding sites
Contains signal sequence to target the protein to ER lumen	Lectin-like chaperone domain	Contains KDEL ER retrieval signal
Interacts with PDI and inhibits PDI activity	Interacts with PDI	Ca^{2+} 'sensor' for calreticulin–protein interactions
Interacts with ERp57	Interacts with perforin	Binds factor IX and X
Weak interactions with perforin	Amino acid sequence similarity to calnexin, calmegin and CALNUC	Antithrombotic activity
Binds Zn^{2+}	Putative glycosylation site (*Leishmania* protein)	Binds to cell surface
Potential phosphorylation site		Putative glycosylation site
Potential glycosylation site (bovine protein)		Prevents retenosis
Tumor- and peptide-specific immunity		
Binds the DNA-binding domain of steroid receptors and α subunit of integrin *in vitro*		
Binds *Rubella* RNA		

Figure 1

The structure and function of calreticulin. Calreticulin is a 46-kDa Ca^{2+}-binding chaperone located in the lumen of the endoplasmic reticulum (ER). The protein contains an N-terminal signal sequence and a C-terminal KDEL ER-retrieval signal. It has at least three structural and functional domains: N, P and C. The N domain is the N-terminal half of the molecule and is the most conserved region of the protein. The P domain comprises a proline-rich sequence with three repeats of the amino acid sequence PxxIxDPDAxKPEDWDE (repeat A) followed by three repeats of the sequence GxWxPPxIxNPxYx (repeat B). The P domain of mammalian calreticulin binds Ca^{2+} with high affinity and low capacity [K_d = 1 μM; B_{max} = 1 (mole Ca^{2+}) (mole protein)$^{-1}$]. Repeats A and B are essential for this high-affinity Ca^{2+} binding and for the lectin-like activity of calreticulin. This region of the protein has a similar amino acid sequence to calnexin, calmegin and CALNUC, a Golgi Ca^{2+}-binding protein. The C-terminal quarter of the protein (the C domain) is highly acidic and negatively charged. This region of the protein binds Ca^{2+} with low affinity and high capacity [K_d = 2 nM; B_{max} = 25 (mole Ca^{2+}) (mole protein)$^{-1}$]. Proposed functions for these domains of calreticulin are listed in the bottom section of the figure. Abbreviation: PDI, protein disulfide isomerase.

E.F. Corbett and **M. Michalak** are in the MRC Group in Molecular Biology of Membranes and the Department of Biochemistry, University of Alberta, Edmonton, AL, Canada T6G 2H7.
Email: marek.michalak@ualberta.ca

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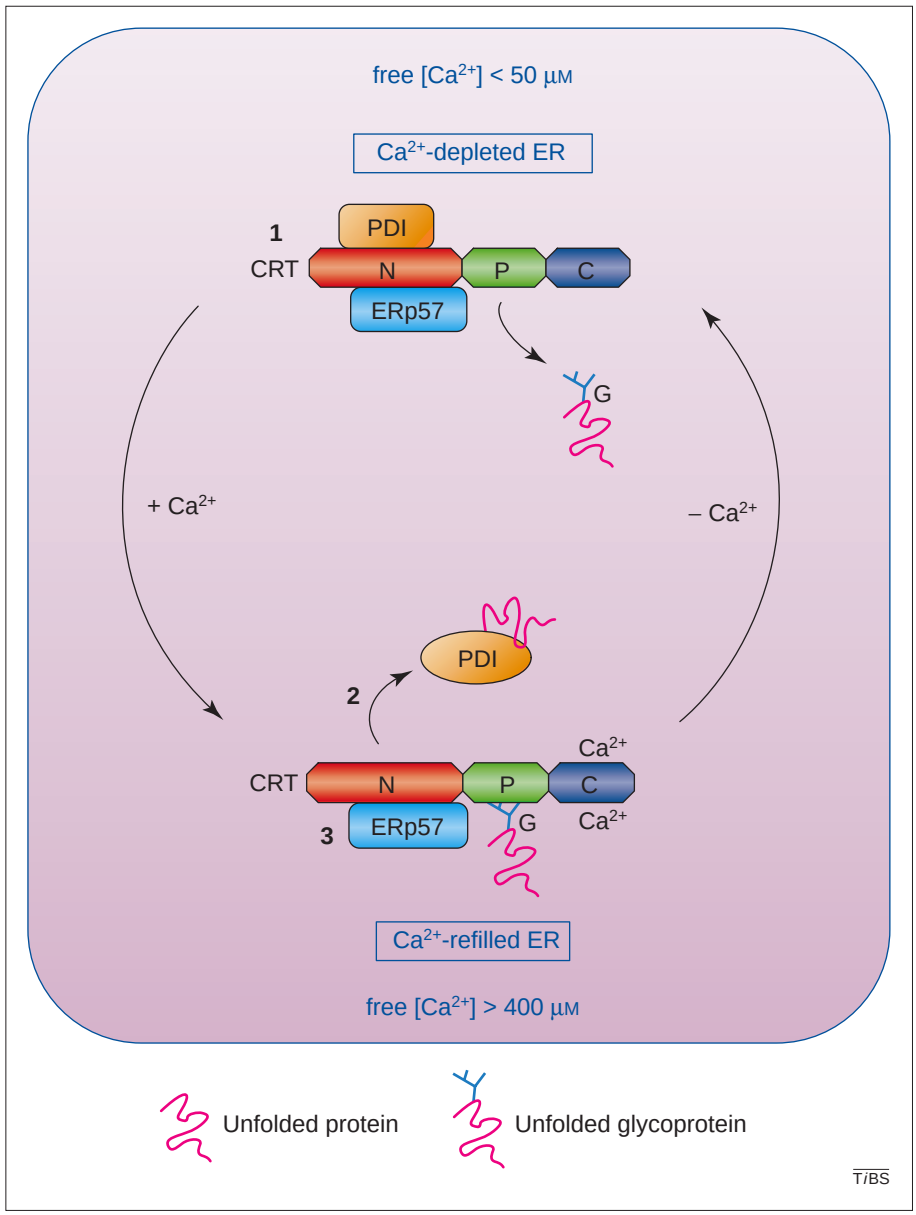

Figure 2

Ca^{2+}-dependent interactions between calreticulin, protein disulfide isomerase (PDI), ERp57 and unfolded glycoproteins. (1) At the concentrations of Ca^{2+} found during agonist-dependent Ca^{2+} depletion of the ER (<50 μM), PDI and ERp57 interact with calreticulin. (2) At the concentrations of Ca^{2+} found when ER Ca^{2+} stores are full (>400 μM), PDI and calreticulin do not associate and PDI carries out its chaperone functions. (3) Increased free $[Ca^{2+}]_{ER}$ promote the binding of monoglucosylated glycoproteins to the P domain of calreticulin (and the central domain of calnexin). Under these conditions, calreticulin and ERp57 are associated, forming fully functional chaperoning complexes. Abbreviations: CRT, calreticulin; G, glucose.

still a point of considerable controversy. In the past, depending on the method used, values as low as 1 μM and as high as 3 mM have been reported[1].

More recently, Tsien *et al.* have developed fusion-protein constructs of green-fluorescent protein and blue-fluorescent protein that they have named 'cameleons'. These can be targeted to the lumen of the ER to measure the free $[Ca^{2+}]_{ER}$[4]. This group found that, when Ca^{2+} stores were full, the free $[Ca^{2+}]_{ER}$ ranged from 60 μM to 400 μM (Ref. 4). Upon agonist-dependent depletion of the stores, free $[Ca^{2+}]_{ER}$ ranged from 1 μM to 50 μM (Ref. 4). Using cameleons targeted to the ER of HEK293 and mouse embryonic fibroblasts, we have measured the free $[Ca^{2+}]_{ER}$ to be approximately 240–288 μM in full stores and approximately 18–30 μM after agonist-induced Ca^{2+} depletion (N. Demaurex and M. Michalak, unpublished). The relatively large range of values reported in these studies might reflect differences between cell types and the heterogeneity of the environment in the lumen of the ER. However, these cameleon studies clearly support the hypothesis that, during agonist activation, the free $[Ca^{2+}]_{ER}$ fluctuates from values as high as 400 μM to as low as 1 μM (Ref. 4).

The lectin-like chaperone activity of calreticulin depends on Ca^{2+} binding

Calreticulin is a Ca^{2+}-binding chaperone[12] that is located in the lumen of the ER (Fig. 1). It interacts in a Ca^{2+}-dependent manner with other ER chaperones and modulates their function[8,9]. Calreticulin also undergoes dynamic, Ca^{2+}-dependent interactions with newly synthesized proteins and with ER proteins involved in Ca^{2+} transport[13,14]. Importantly, calreticulin affects Ca^{2+} transport across the ER membrane[13–15]. This means that it can modulate the free $[Ca^{2+}]_{ER}$ and, therefore, Ca^{2+}-dependent protein–protein interactions in the ER.

Calreticulin is similar to calnexin[16,17]; both bind $Glc_1Man_9GlcNAc_2$ oligosaccharides in a Ca^{2+}-dependent manner, recognizing the terminal glucose and four internal mannose moieties[10,18]. Recent evidence indicates that calreticulin and calnexin also interact with the polypeptide portion of newly synthesized proteins and so they can be considered to be true molecular chaperones[19,20]. However, the role of $[Ca^{2+}]_{ER}$ in these interaction is not yet understood.

In the lumen of the ER, the carbohydrate attached to newly synthesized proteins is $Glc_3Man_9GlcNAc_2$. Two of the three glucose moieties are then removed by glucosidase I and II, allowing the glycoproteins to bind to calreticulin and calnexin. These two proteins work by a similar mechanism to assist in the folding of newly synthesized glycoproteins[16,17]. Once a glycoprotein is folded correctly, it escapes from the folding cycle, but if a glycoprotein is not correctly folded, terminal glucose(s) are reattached by the enzyme UDP–glucose glycoprotein glucosyltransferase. Unfolded glycoproteins can thus undergo cycles of binding to and release from calnexin and calreticulin.

The lectin-binding site in calreticulin and calnexin is located in the proline-rich P domain of calreticulin (Fig. 1) and the proline-rich central domain of calnexin[10]. Importantly, in both calreticulin and calnexin, Ca^{2+} is essential for their lectin-like behavior[10]. *In vitro* studies indicate that both proteins bind carbohydrate at the high Ca^{2+} concentration observed when Ca^{2+} stores are full and that this binding is significantly reduced at the low free $[Ca^{2+}]_{ER}$ observed when

Ca^{2+} stores are empty (<50 μM)[10] (Fig. 2). In both proteins, the carbohydrate-binding proline-rich domain also binds Ca^{2+} with high affinity (K_d = 1 μM)[12]. As the free $[Ca^{2+}]_{ER}$ might not decrease below this level, it is unlikely that Ca^{2+} binding to the high-affinity Ca^{2+} binding, proline-rich domain of calreticulin and calnexin regulate these protein–carbohydrate interactions (Fig. 1). It is not clear at present which Ca^{2+}-binding site is essential for the lectin-like activity of calreticulin and calnexin, but it is conceivable that Ca^{2+} binding to carbohydrate might also play an important role.

There are many other components of the calreticulin–calnexin cycle, including glucosidase II and UDP–glucose transferase, which might also be sensitive to changes in the free $[Ca^{2+}]_{ER}$. However, because there is a direct relationship between high-affinity Ca^{2+} binding and the lectin-like chaperone function of calreticulin and calnexin, it is likely that the folding of newly synthesized glycoproteins is highly sensitive to changes in the free $[Ca^{2+}]_{ER}$.

The activities of PDI and ERp57 are affected by Ca^{2+} binding to calreticulin

The ER chaperones calreticulin and protein disulfide isomerase (PDI) interact with one another in a Ca^{2+}-dependent fashion. Interestingly, the Ca^{2+} dependency varies over the range of free $[Ca^{2+}]_{ER}$ measured during the emptying and refilling of ER Ca^{2+} stores[8,9] (Fig. 2). Most importantly, Ca^{2+} binding to the physiologically relevant high-capacity, low-affinity Ca^{2+}-binding site in calreticulin (Fig. 1) is responsible for control of these interactions. For example, calreticulin binds reversibly to PDI *in vitro* at the low free $[Ca^{2+}]_{ER}$ observed when Ca^{2+} stores are empty (<50 μM), and this protein–protein interaction results in a reduction of PDI chaperone activity[8,21] (Fig. 2a). By contrast, at the high free $[Ca^{2+}]_{ER}$ observed when Ca^{2+} stores are refilled, PDI does not interact with calreticulin and has a high chaperone activity[8,9,22] (Fig. 2b). PDI activity might also be affected directly by changes in the ER lumenal $[Ca^{2+}]_{ER}$[23].

Ca^{2+} binding to the high-Ca^{2+}-capacity C domain of calreticulin also plays a role in recently described interactions between calreticulin and ERp57 (Refs 9,22). ERp57 belongs to the PDI family of proteins and is involved in chaperoning and disulfide-bond formation. Calreticulin interacts with ERp57 *in vitro* and *in vivo*[9,22] (Fig. 2c), and the disulfide-isomerase activity of ERp57 is increased in

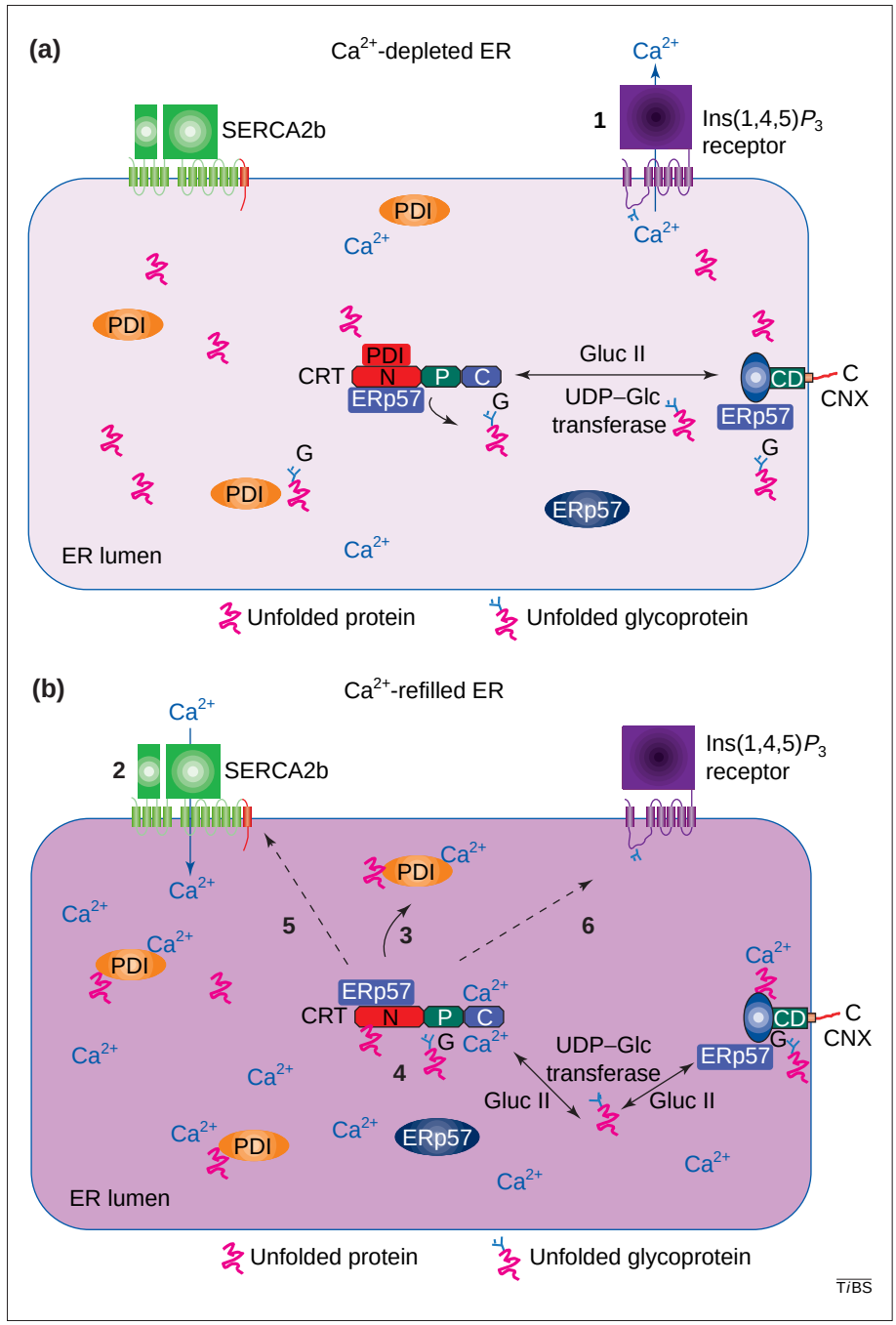

Figure 3

Ca^{2+}-dependent dynamics in the lumen of the ER. This diagram shows a model of events occurring in response to agonist-activated Ca^{2+} fluctuations in the lumen of the endoplasmic reticulum (ER). These events affect the interactions between and the function of chaperones. **(a)** During agonist activation, Ca^{2+} is released from the ER via the Ins(1,4,5)P_3 receptor Ca^{2+} channel (1), resulting in a decreased free $[Ca^{2+}]_{ER}$ (<50 μM). Under these conditions, there are only limited interactions between proteins in the lumen of the ER and unfolded polypeptides. **(b)** When Ca^{2+} is taken up by SERCA2b (2), ER stores are refilled ($[Ca^{2+}]_{ER}$ >400 μM), leading to full activation of chaperone function. Interactions between calreticulin and ER chaperones (e.g. PDI) are regulated by Ca^{2+} binding to calreticulin (3). Interactions between calreticulin and monoglucosylated unfolded glycoproteins are regulated by Ca^{2+} binding to calreticulin (4). Calreticulin might also interact, in a Ca^{2+}-dependent manner, with the unique C-terminal tail of SERCA2b, to modulate its ability to refill ER Ca^{2+} stores (5). Calreticulin might also interact with the Ins(1,4,5)P_3 receptor (6). Interactions between calreticulin (or calnexin) and misfolded proteins are also depicted. The role of Ca^{2+} in these interactions is not presently understood. Broken arrows indicate that there is presently no direct evidence for the indicated interactions [between calreticulin and SERCA2b, and between calreticulin and the Ins(1,4,5)P_3 receptor]; solid arrows indicate demonstrated pathways. The differing intensity of the background represents differences in Ca^{2+} concentration in depleted and refilled Ca^{2+} stores. Abbreviations: CD, central domain of calnexin; CNX, calnexin; CRT, calreticulin; G, glucose residue; Gluc II, glucosidase II; PDI, protein disulfide isomerase; UDP–Glc transferase, UDP–glucose glycoprotein glucosyltransferase.

the presence of either calreticulin or calnexin[21]. Calreticulin is thought to bind ERp57 in a chaperone complex, thereby assisting disulfide-bond formation in newly synthesized glycoproteins[21,22,24].

Although the initial interaction between calreticulin and ERp57 is independent of $[Ca^{2+}]$, ERp57 is affected indirectly in two ways. First, at high free $[Ca^{2+}]_{ER}$, Ca^{2+} binding to calreticulin induces conformational changes in ERp57, presumably allowing closer interaction of the protein with folding intermediates[9]. Second, at low free $[Ca^{2+}]_{ER}$, calreticulin binds monoglucosylated glycoproteins only weakly[10]. This suggests that these chaperone complexes are probably not functional in Ca^{2+}-depleted ER, whereas they are likely to be functional when Ca^{2+} stores are full and glycoproteins are bound to calreticulin or calnexin, or both.

Figure 3 shows how changes in the free $[Ca^{2+}]_{ER}$ in agonist-stimulated cells might regulate interactions between certain chaperones via Ca^{2+} binding to calreticulin. In addition to these effects, it is likely that the function of other chaperones in the lumen of the ER is sensitive to fluctuations in free $[Ca^{2+}]_{ER}$. For example, at low free $[Ca^{2+}]_{ER}$, the activity of immunoglobulin-binding protein (BiP) is inhibited[25,26]. At low free $[Ca^{2+}]_{ER}$, protein synthesis, glycoprotein processing and transport are blocked[25,26]. It appears that these effects might be mediated via Ca^{2+}-dependent changes in protein–protein interactions in the lumen of the ER. Overall, changes in the free $[Ca^{2+}]_{ER}$ might result in cycles of Ca^{2+}-dependent association and dissociation, and this is important because it might facilitate the passage of certain newly synthesized proteins from one chaperone to another during the folding process.

Calreticulin affects cellular processes outside the ER via an ER signaling network

Calreticulin has been implicated in diverse cellular functions, including gene expression and cell adhesion[12] (Fig. 1). Originally, it was hypothesized that this is due to the interaction that was found *in vitro* between calreticulin and the DNA-binding domain of steroid receptors or between calreticulin and the cytoplasmic tail of α integrin[12]. However, because calreticulin is in the lumen of the ER, there has always been some controversy about its *in vivo* involvement in cellular processes occurring outside the ER. In many years of investigation, calreticulin has never been detected in the cytoplasm, and cytoplasmically targeted calreticulin has no effect on the function of steroid receptors or cell adhesion *in vivo*[27–29].

Fortunately, recent reports are helping to resolve this conundrum. They indicate that calreticulin influences gene expression and cell adhesion indirectly, from within the lumen of the ER[28,29]. This is via either modulation of adhesion-related molecules or changes in integrin-dependent Ca^{2+} signaling, or both[27–29]. It seems likely that calreticulin participates in a signaling network within the lumen of the ER[30]. An obvious and exciting possibility is that this signaling network is regulated by changes in the free $[Ca^{2+}]_{ER}$.

Calreticulin plays a role in regulating $[Ca^{2+}]_{ER}$

If, in agonist-stimulated cells, fluctuating Ca^{2+} concentrations are involved in controlling ER functions, the ability of ER proteins to regulate the free $[Ca^{2+}]_{ER}$ is potentially important. Recent evidence indicates that calreticulin affects the Ca^{2+}-storage capacity of the ER by modulating the function of the Ins(1,4,5)P_3-receptor Ca^{2+}-release channel and the SERCA2b Ca^{2+} ATPase[13–15]. Camacho *et al.* have shown that calreticulin affects the generation of the complex cytosolic Ca^{2+} signals (Ca^{2+} waves) that occur in response to Ins(1,4,5)P_3 microinjection in *Xenopus* oocytes[13]. Also, coexpression of calreticulin with SERCA2b results in a sustained elevation of Ca^{2+} release, without concomitant oscillations[13,14].

Other studies show that Ins(1,4,5)P_3-dependent Ca^{2+} release from the lumen of the ER is impaired in calreticulin-deficient fibroblasts isolated from calreticulin-deficient mouse embryos[15]. By contrast, in cultured calreticulin-deficient embryonic stem cells, Ca^{2+} handling by the ER is not changed[27,28]. However, there is a significant reduction in the integrin-mediated influx of extracellular Ca^{2+} in these cells[27]. The reasons for this discrepancy are not clear at present but might be related to the harsh selection procedure used on the calreticulin-deficient cells. Nevertheless, the literature clearly indicates that calreticulin plays a role in the processes that deplete and refill the ER Ca^{2+} store.

Conclusion

It is well established that Ca^{2+} is one of the most important signaling molecules in the cytosol[2]. It now appears that Ca^{2+} levels are also of central importance in dictating the function of proteins that reside in the ER. Thus, Ca^{2+} might also have been chosen as a signaling molecule in the ER. Recent evidence indicates that calreticulin 'senses' changes in the free $[Ca^{2+}]_{ER}$ via its high-capacity Ca^{2+}-binding site and, in response to these changes, alters its interactions with other chaperones. In addition, Ca^{2+} binding to its high-affinity Ca^{2+}-binding site affects its interaction with unfolded glycoproteins. Calreticulin, therefore, could be a key player in Ca^{2+}-signaling processes within the ER.

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The Adaptor hypothesis revisited

Michael Ibba, Hubert D. Becker, Constantinos Stathopoulos, Debra L. Tumbula and Dieter Söll

As originally postulated in Crick's Adaptor hypothesis, the faithful synthesis of proteins from messenger RNA is dependent on the presence of perfectly acylated tRNAs. The hypothesis also suggested that each aminoacyl-tRNA would be made by a unique enzyme. Recent data have now forced a revision of this latter point, with an increasingly diverse array of enzymes and pathways being implicated in aminoacyl-tRNA synthesis. These unexpected findings have far-reaching implications for our understanding of protein synthesis and its origins.

PROTEIN SYNTHESIS SPECIFIED by a mRNA template is one of the central facets of gene expression. During protein synthesis, the sequential series of triplet codons found in mRNA is used to direct the ribosomal synthesis of a polypeptide of corresponding amino acid sequence. Amino acids are provided for protein synthesis in the form of aminoacyl-tRNAs. The identity of an amino acid inserted at a particular position in a nascent polypeptide is principally determined by two factors: the interaction of the aminoacyl-tRNA anticodon with an appropriate codon in mRNA and the correct pairing of amino acid and tRNA anticodon in the aminoacyl-tRNA. Consequently, the faithful synthesis of proteins is dependent on the presence in the cell of a complete set of correctly aminoacylated tRNAs (reviewed in Ref. 1).

Before the individual components of the protein synthesis machinery were identified experimentally, its basic constituents were predicted by Crick in the Adaptor hypothesis[2] (Box 1). A central premise of the Adaptor hypothesis is that each aminoacyl-tRNA is synthesized by a unique amino-acid-specific enzyme, and therefore the cell is expected to contain 20 such proteins. This prediction was borne out by the discovery of the 20 aminoacyl-tRNA synthetases (AARSs) in the decades that followed the publication of the Adaptor hypothesis[3] and has subsequently been elaborated on by a vast body of genetic, biochemical and structural data (Box 2; reviewed in Refs 4,5). Although the first exception to the '20 AARS' rule was reported as early as 1968 in bacteria[6] and subsequently in archaea[7] and eukaryotic organelles[8,9], this was assumed to be no more than an evolutionary anomaly. However, recent discoveries, mainly arising from functional genomics studies in bacterial and archaeal systems, have shown that, contrary to all expectations, numerous organisms do not use a full complement of 20 canonical AARS enzymes to synthesize aminoacyl-tRNAs for protein synthesis. In such cases, a reduced number of AARSs [the minimal known complement is 16 (Refs 10,11)] work in tandem with a variety of novel enzymes and pathways to provide the full complement of aminoacyl-tRNAs required for protein synthesis. Here, we will review recent advances in our understanding of the various pathways and enzymes currently known to be involved in aminoacyl-tRNA synthesis. The implications of these findings for interpretation of the Adaptor hypothesis and for our understanding of the origins of protein synthesis are also discussed.

tRNA-dependent amino acid transformations provide Asn-tRNA and Gln-tRNA

The most widespread exception to Crick's Adaptor hypothesis is found in the tRNA-dependent amino acid transformation pathways (Fig. 1). This two-step indirect route to glutaminyl-tRNA^{Gln} (Gln-tRNA^{Gln}) and asparaginyl-tRNA^{Asn} (Asn-tRNA^{Asn}) generally exists when glutaminyl-tRNA synthetase (GlnRS) or asparaginyl-tRNA synthetase (AsnRS) are absent. In forming Gln-tRNA^{Gln}, tRNA^{Gln} is first misaminoacylated with glutamate by a non-discriminating (i.e. owing to relaxed tRNA specificity) glutamyl-tRNA synthetase, which, in addition to generating Glu-tRNA^{Glu}, will also form Glu-tRNA^{Gln}.

M. Ibba is at the Center for Biomolecular Recognition, Dept of Medical Biochemistry and Genetics, Laboratory B, The Panum Institute, Blegdamsvej 3c, DK-2200, Copenhagen N, Denmark; **H.D. Becker, C. Stathopoulos** and **D.L. Tumbula** are at the Dept of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520-8114, USA; and **D. Söll** is at the Depts of Molecular Biophysics and Biochemistry, Chemistry, and Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520-8114, USA.
Email: soll@trna.chem.yale.edu

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