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REVIEW

Endoplasmic reticulum quality control and congenital pathology

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Summary
Quality control of the endoplasmic reticulum plays a critical role in protein folding, modification and modification of a secretory pathway. As endoplasmic reticulum chaperones, calreticulin and calnexin have similar substrate specificity and share several common features. Yet, surprisingly, mice bearing a disruption in the calreticulin gene die from a lesion in cardiac development and develop significant metabolic problems whereas calnexin-deficient mice are born alive with, yet not understood, neurological problems. Studies with calreticulin and calnexin gene knockout mice and calreticulin- and calnexin-deficient cell lines indicate that calnexin is unable to compensate for the loss of calreticulin and conversely, calreticulin cannot compensate for the loss of calnexin. Calreticulin or calnexin deficiency or reduction in the level of ERp57 protein (ERp57 heterozygote mice) leads to development of metabolic disorders as documented by sever changes serum lipids and carbohydrates composition in these animals. These observations indicate that calreticulin, calnexin and ERp57, in addition of being involved in maturation of glycoproteins in the endoplasmic reticulum, perform other distinct functions including affecting energy metabolism.

Keywords: endoplasmic reticulum – calreticulin – calnexin – chaperones – lipid metabolism

INTRODUCTION

Many human diseases are caused by mutations altering the folding pathway and final conformation of a protein. Many conformational diseases are caused by mutations in secretory proteins and leading to metabolic dysfunctions i.e. diabetes, to development and neurological diseases such as Alzheimer's. The endoplasmic reticulum (ER) is a processing place for the maturation, folding, transport and storage of proteins, and is also the most prominent intracellular Ca^{2+} store (Baumann and Walz 2001). The ER contains many proteins which carry out these diverse functions (Baumann and Walz 2001, Corbett and Michalak 2000, Meldolesi and Pozzan 1998, Trombetta and Parodi 2003). Calreticulin is an ER luminal Ca^{2+} binding chaperone involved in regulation of intracellular Ca^{2+} homeostasis and ER Ca^{2+} capacity (Arnaudeau et al. 2002, Nakamura et al. 2001). This is important because changes in the ER Ca^{2+} storage capacity affect its chaperone function and influence the quality control of the secretory pathway (Corbett et al. 2000). Calnexin is a type I integral

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membrane chaperone of the ER (Wada et al. 1991). The protein has a high degree of amino acid sequence and structural similarity (identity) to calreticulin (Bergeron et al. 1994, Michalak et al. 1999, Michalak et al. 2002). Calreticulin and calnexin, together with ERp57 (a PDI-like protein with thoredoxin domains which is also resident in the ER) constitute the calreticulin/calnexin cycle that is responsible for the folding and quality control of newly-synthesized glycoproteins (Trombetta and Parodi 2003). As ER chaperones, calreticulin and calnexin have similar substrate specificity and share several common features (Trombetta and Parodi 2003). Surprisingly, mice bearing a disruption in the calreticulin gene die from a lesion in cardiac development (Mesaeli et al. 1999), develop significant metabolic problems (Guo et al. 2002) whereas calnexin-deficient mice

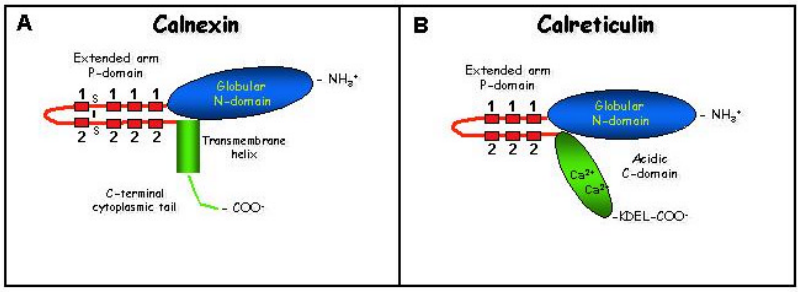

Fig. 1. **A model of calnexin (A) and calreticulin (B) domains.** Calreticulin contains a globular N-domain (blue) and central, proline-rich P-domain (red) which forms a characteristic loop. C-terminal C-domain (green) contains a large number of negatively charged amino acids and it is involved in high capacity Ca^{2+} storage. Similar domain arrangement is found in calnexin with exception that calnexin is an integral membrane protein with a C-terminal trasmembrane helix and cytoplasmic tail.

are born alive with, yet not understood, neurological problem (Denzel et al. 2002). Studies with calreticulin and calnexin gene knockout mice and calreticulin- and calnexin-deficient cell lines indicate that calnexin is unable to compensate for the loss of calreticulin (Knee et al. 2003, Mesaeli et al. 1999, Molinari et al. 2004, Nakamura et al. 2001) and conversely, calreticulin cannot compensate for the loss of calnexin (Denzel et al. 2002, Molinari et al. 2004, Zuppini et al. 2002). These observations indicate that calreticulin and calnexin are distinct proteins with very unique functions.

CALRETICULIN AND CALNEXIN, ER LECTIN-LIKE CHAPERONES

Calreticulin is a Ca^{2+}-binding chaperone present in a number of extremely diverse species (Michalak et al. 1999). The protein binds/buffers Ca^{2+} in the ER lumen and participates in the folding of newly synthesized proteins and glycoproteins and it is an important constitute of a calreticulin/ calnexin cycle (Ellgaard et al. 1999, Jakob et al. 2001, Michalak et al. 1999, Saito et al. 1999). Figure 1 shows a model of calreticulin and calnexin. Calreticulin is made out of distinct structural and functional domains. The N-domain of calreticulin is globular with a disulfide bridge. This region binds heavy metals and may interact with a variety of molecules including proteins and nucleic acid. The P-domain comprises the proline-rich region, forms an extended arm structure and interacts with other ER luminal chaperones. The C-domain is highly acidic, binds Ca^{2+} with high capacity and it is involved in Ca^{2+} storage in the lumen of the ER.

Several functions have been described for calreticulin, including regulation of Ca^{2+} homeostasis and Ca^{2+}-dependent pathways (Arnaudeau et al. 2002, Camacho and Lechleiter 1995, Guo et al. 2002, Nakamura et al. 2001), lectin-like chaperone activity (Bergeron et al. 1994, Molinari and Helenius 2000, Nakamura et al. 2001), modulation of gene expression(Burns and Michalak 1993, Dedhar 1994, Michalak et al. 1999), nuclear transport (Holaska et al. 2001, Mesaeli and Phillipson 2004), and cell adhesion (Fadel et al. 1999, Fadel et al. 2001, Opas et al. 1996). It is generally believed that the protein has two principal functions: (**i**) molecular chaperone, and (**ii**) a modulator of Ca^{2+} homeostasis. Calreticulin functions as a molecular chaperone for

many proteins and glycoproteins (Nauseef et al. 1995, Saito et al. 1999). The protein binds $Glc_1Man_9GlcNAc_2$ oligosaccharide and recognizes the terminal glucose and four internal mannose in newly synthesized glycoproteins (Kapoor et al. 2003). Calreticulin binds Ca^{2+} and plays a role in regulation of Ca^{2+} homeostasis. Over-expression of calreticulin leads to increased amounts of Ca^{2+} in intracellular stores (Arnaudeau et al. 2002, Michalak et al. 1999, Nakamura et al. 2001), whereas calreticulin-deficient cells have reduced ER Ca^{2+} storage capacity (Nakamura et al. 2001). Store-operated Ca^{2+} influx across the plasma membrane is reduced in cells that express high level of calreticulin (Arnaudeau et al. 2002). Importantly, agonist-mediated Ca^{2+} release from the ER is inhibited in *crt*$^{-/-}$ cells (Mesaeli et al. 1999, Nakamura et al. 2001). Problems with Ca^{2+}-dependent signaling pathways must be responsible for embryonic (Mesaeli et al. 1999) and postnatal lethality seen in calreticulin-deficient mice (Guo et al. 2002).

Calnexin, is a type I integral membrane protein of the ER (Michalak et al. 2002, Wada et al. 1991). Calnexin can also be divided into distinct structural and functional domains (Fig. 1): N-terminal globular domain, extended arm P-domain, transmembrane domain and a short cytoplasmic tail (Wada et al. 1991). The N+P-domain of calnexin represents a "protein folding module" and it is attached to a membrane *via* a transmembrane domain. A major distinction between calreticulin and calnexin is that calnexin is an integral membrane protein (Fig. 1) whereas calreticulin is a luminal protein. Thus, calnexin interacts transiently with its protein-folding intermediates at the stationary phase of the ER membrane, whereas calreticulin interacts with its substrates in the mobile luminal environment. Different topological environments of calnexin and calreticulin must be critical in determining their distinct substrate specificities. calnexin has also been implicated to function as a modulator of ER Ca^{2+}-ATPase (SERCA) in *Xenopus* oocytes (Roderick et al. 2000).

CALRETICULIN/CALNEXIN CYCLE

Calreticulin and calnexin, together with ERp57 constitute the calreticulin/calnexin cycle that is responsible for the folding and quality control of newly-synthesized glycoproteins (Fig. 2) (High et al. 2000, Zapun et al. 1999). There are other Ca^{2+}-binding chaperones that reside in the lumen of the ER, including Grp94, the PDI family of proteins (PDI, ERp72), and BiP, but these proteins appear to play an important role in the folding and posttranslational modification of non-glycosylated proteins (Gething 1999, Nicchitta 1998). Calreticu-

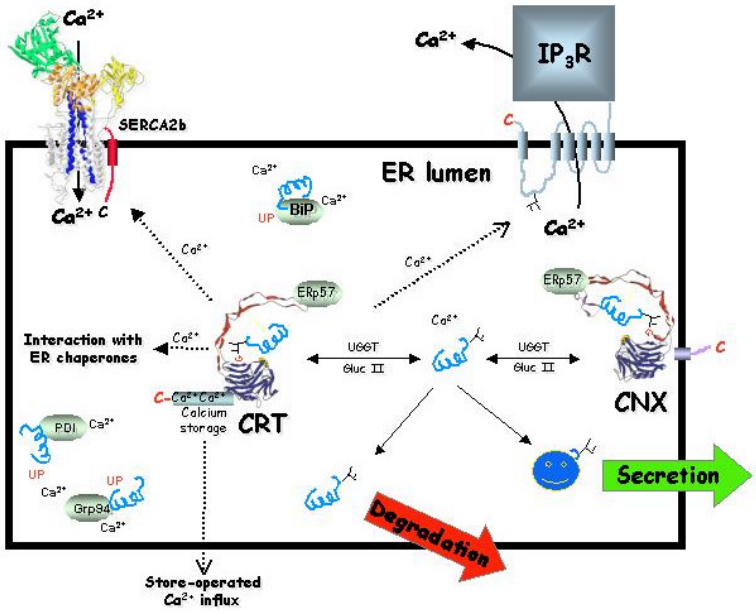

Fig. 2. **Calreticulin/calnexin cycle.** Calreticulin and calnexin bind monoglucosylated carbohydrate on newly-synthesized glycoproteins. Glucosidase II removes terminal glucose and UGGT recognizes misfolded glycoproteins and re-glucosylate them. This de-glucosylation-glucosylation cycle may be repeated several times before a newly synthesized glycoprotein is properly folded or sent for degradation. Calreticulin and calnexin also affect Ca^{2+} homeostasis. Calreticulin is a major Ca^{2+} storage protein and calnexin affects function of SERCA2b. Solid arrows indicate demonstrated pathways. Broken arrows indicate pathways not yet shown for mammalian cells. UP, unfolded glycoproteins, G, glucose residue.

lin and calnexin are perhaps the most important chaperones because they directly affect the folding and posttranslational modification of virtually all glycosylated, secreted, or integral membrane proteins that pass through the ER.

Fig. 2 shows a diagram of calreticulin/calnexin cycle with emphasis on the role of calreticulin and calnexin in protein folding and modulation of Ca^{2+} homeostasis. Calreticulin and calnexin bind monoglucosylated carbohydrate on newly-synthesized glycoproteins (Trombetta 2003). The carbohydrate binding is sensitive to changes in Ca^{2+} concentration, suggesting that ER luminal Ca^{2+} may affect the folding of glycoproteins. ERp57 associates with the substrates recognized by both calreticulin and calnexin and catalyzes rearrangements of disulfide-bonds within the substrate proteins (Zapun et al. 1998). ERp57 docks onto the extended, P-domain of calreticulin and calnexin (Russell et al. 2004, Silvennoinen et al. 2004). Changes within the ER lumen, such as fluctuation in the concentration of Ca^{2+}, Zn^{2+} or ATP, may affect the formation of these chaperone complexes and their ability to assist in protein folding (Corbett et al. 2000). For example, Zn^{2+}-dependent conformational changes in calreticulin are known to enhance its carbohydrate-independent chaperone function (Saito et al. 1999).

CALRETICULIN- AND CALNEXIN-DEFICIENT MICE

Calreticulin deficiency is embryonic lethal due to a lesion in cardiac development (Guo et al. 2002, Mesaeli et al. 1999). Cells isolated from *crt*$^{-/-}$ embryos have impaired agonist-induced Ca^{2+} release (Nakamura et al. 2001), inhibited nuclear import of the transcription factor NF-ATc1, Mef2c and p53, modified sensitivity to apoptosis, compromised function of calnexin, and activated unfolded proteins response (UPR) (Li et al. 2002, Mesaeli et al. 1999, Mesaeli and Phillipson 2004, Nakamura et al. 2000, Nakamura et al. 2001) indicating a major impact of calreticulin deficiency on ER and cellular functions. Remarkably, *crt*$^{-/-}$ mice are rescued by expression of constitutively active calcineurin in the heart (Guo et al. 2002). Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase (Rusnak and Mertz 2000). It is a heterotetramer containing A and B subunits. Calcineurin-A is the catalytic subunit (Rusnak and Mertz 2000). A constitutively-active form of calcineurin (activated-calcineurin) can be generated by deletion of its C-terminal region (Rusnak and Mertz 2000). Cardiac expression of activated-calcineurin reversed the embryonic lethality seen in calreticulin-deficient mice and these mice exhibit severe postnatal pathology and die 7-35 days after birth (Guo et al. 2002). The rescue mice have severe growth retardation and metabolic problems (Guo et al. 2002). For example, they have elevated levels of both cholesterol and triacyglyceroles (TAG) (Guo et al. 2002). The underlying cause of the metabolic aberrations in these mice is not understood. *crt*$^{-/-}$ mice serum lipids may be elevated because of compromised Ca^{2+} release from the ER and impaired function of ER associated molecules involved in lipid synthesis and uptake. Many metabolic processes rely of ER function therefore it is critical to understand a role of calreticulin and ER in postnatal metabolism and pathology.

In sharp contrast, calnexin deficiency is not embryonic lethal (Denzel et al. 2002). This is amazing considering great structural and functional similarities between the two chaperones. *cnx*$^{-/-}$ animals exhibit impaired motor function and die within the first 5 weeks of life (Denzel et al. 2002). *cnx*$^{-/-}$ mice are smaller than their siblings, develop sever motor problems of lower limb and difficulties with maintaining a proper balance.

Studies with calreticulin and calnexin gene knockout mice indicate that these proteins are unable to compensate for the loss of each other suggesting they must have unique functions (Denzel et al. 2002, Mesaeli et al. 1999, Nakamura et al. 2001). One function of calreticulin that cannot be compensated by calnexin is its role in modulation of Ca^{2+} homeostasis (Arnaudeau et al. 2002, Nakamura et al. 2001). We created viable *crt*$^{-/-}$ and *cnx*$^{-/-}$ cell lines indicating that in mammalian cell culture calreticulin and calnexin (and the calreticulin/calnexin cycle) are not essential for cell survival (Mesaeli et al. 1999, Scott and Dawson 1995). Deletion of glucosidase II in mammalian cells and glucosidase II and UGGT, key components of the calreticulin/calnexin cycle, in *S. pombe* has no serious consequences on cellular function (D'Alessio et al. 1999).

Yet, calnexin deficiency is lethal in *S. pombe* (Parlati et al. 1995a), *S. cervisiae* lacks most of the calnexin/calreticulin components (Parlati et al. 1995b). Calnexin and calreticulin deficiency is not lethal but it affects phagocytosis in *Dictyostelium* (Muller-Taubenberger et al. 2001) and promotes necrotic cell death in *C. elegans* (Xu et al. 2001). In summary, these findings support the hypothesis that calreticulin and calnexin are multifunctional proteins. Molecular chaperone function of calreticulin and calnexin may only partially explain phenotypes of *cnx*$^{-/-}$ and *crt*$^{-/-}$ mice.

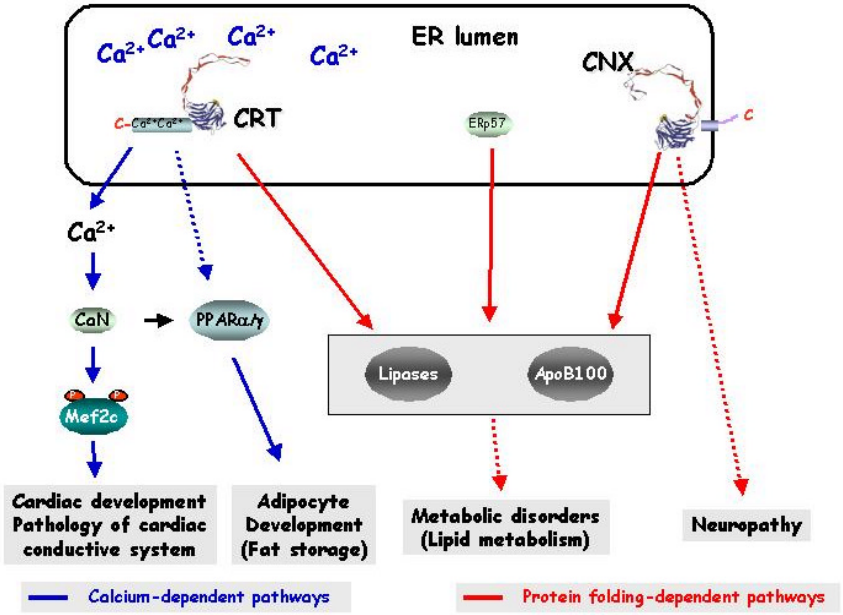

Fig. 3. **A model of role of ER chaperones in development and metabolism.** Schematic representation of relationship between calreticulin in the ER lumen, calnexin and developmental/metabolic pathways. Calreticulin affects many aspects of cardiac development and influences formation of adipose tissue. These effects are due to calreticulin function as regulator of Ca^{2+} homeostasis (blue). Calreticulin, ERp57 and calnexin also affects energy metabolism, especially lipid metabolism in mice. These effects of ER chaperones are likely due their role in protein folding (red).

ER QUALITY CONTROL AND LIPID METABOLISM

Analysis of chaperone-deficient mouse models revealed an important role of the ER quality control in regulation of energy metabolism. Calreticulin deficient mice die from a lesion in cardiac development. Animals rescued with activated-calcineurin are born alive but exhibit serious metabolism problem (Guo et al. 2002). Furthermore, these animals have no significant adipose tissue indicting that calreticulin affect lipid metabolism and lipid storage after birth. In the absence of calreticulin there is a large increase in circulating lipids and lipoproteins (Guo et al. 2002). Preliminary experiments indicate that calreticulin's role in modulation of Ca^{2+} homeostasis affect adipogenesis but its chaperone function may modulate lipid synthesis and, ultimately fat metabolism (Fig. 3). In contrast low expression of ERp57 (heterozygote animals) or calnexin deficiency result in decrease levels of circulating lipoprotein particles and reduced level of serum cholesterol. These animals have also reduced level of ApoB100 suggesting that ERp57 and calnexin may be involved in folding and secretion of this lipoprotein. Chaperone function of ERp57 and calnexin may, therefore, play critical role in regulating lipid metabolism (Fig. 3). Future studies on the relationship between ER quality control and its chaperones should help us better understand the role of protein folding in the control and regulation of energy metabolism.

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