Lectins in Quality Control: Calnexin and Calreticulin 2

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2.1 Chaperons

A long-standing enigma has been the role of *N*-linked glycans attached to many proteins in the endoplasmic reticulum (ER) and their co- and posttranslational remodelling along the secretory pathway. Evidence is accumulating that intracellular animal lectins play important roles in quality control and glycoprotein sorting along the secretory pathway. Calnexin and calreticulin in conjunction with associated chaperones promote correct folding and oligomerization of many glycoproteins in the ER. The discovery that one of these glycan modifications, mannose 6-phosphate, serves as a lysosomal targeting signal that is recognized by mannose 6-phosphate receptors has led to the notion that lectins may play more general roles in exocytotic protein trafficking (Chaps. 3–6). In present and subsequent chapters we discuss the role of intracellular lectins in quality control (QC) and their role in understanding the mechanisms underlying protein traffic in the secretory pathway (Chaps. 3–7) (Table 2.1).

In eukaryotic cells, the ER plays an essential role in the synthesis and maturation of a variety of important secretory and membrane proteins. The ER is also considered one of the most important and metabolically relevant sources of cellular Ca^{2+}. The ability of ER to control Ca^{2+} homoeostasis has profound effects on many cell functions. To achieve its function the ER and its lumen contain a characteristic set of resident proteins that are involved in every aspect of ER function. For glycoproteins, the ER possesses a dedicated maturation system, which assists folding and ensures the quality of final products before ER release. A chaperone is a protein that binds transiently to newly synthesized proteins and assists in newly synthesized proteins in folding (Ellgaard and Helenius 2003; Trombetta and Parodi 2003). Essential components of this system include the lectin chaperones calnexin (Cnx) and calreticulin (Crt) and their associated co-chaperone ERp57, a glycoprotein specific thiol-disulfide oxidoreductase. The significance of this system is underscored by the fact that Cnx and Crt interact with practically all glycoproteins investigated to date, and by the debilitating phenotypes revealed in knockout mice deficient in either gene. Compared to other important chaperone systems, such as the Hsp70s, Hsp90s and GroEL/GroES, the principles whereby this system works at the molecular level are relatively poorly understood. However, structural and biochemical data have provided important new insights into this chaperone system and present a solid basis for further mechanistic studies. Both Crt and Cnx act as lectins which recognize CRD and act as molecular chaperones. Both of them bind monoglucosylated proteins and associate with the thiol oxidoreductase ERp57, which is a protein disulfide isomerase (PDI)-like protein resident in ER and promotes disulfide formation/isomerization in glycoproteins. Calreticulin, together with Cnx and ERp57 comprise the so-called "calreticulin/calnexin cycle", which is responsible for QC and folding in newly synthesized (glyco) proteins.

Crt/Cnx pathway is thought to monitor protein conformation through modification of N-linked glycans covalently attached to Asn residues through the activity of glycosyl transferases, glucosidases and chaperones. Folding enzymes such as PDI and ERp57 interact with polypeptides displaying non-native disulfide bonds, effectively reorganizing these covalent cross-links to their native pattern. ERp57, a thiol oxidoreductase that catalyzes disulfide formation in heavy chains of MHC class I molecules, also forms a mixed disulfide with tapasin within the class I peptide loading complex, stabilizing the complex and promoting efficient binding of peptides to class I molecules. ERp57 appears to play a structural rather than catalytic role within the peptide loading complex (Zhang et al. 2009). The PDI can help the formation of disulfide bonds that are the critical structure of protein secondary structure (Jun-Chao et al. 2006).

G.S. Gupta et al., *Animal Lectins: Form, Function and Clinical Applications*,
DOI 10.1007/978-3-7091-1065-2_2,

Table 2.1 Lectins associated in quality control of proteins and the secretory pathway

Lectin	Localization	Sugar specificity	Major function
Calnexin	ER	Gluc + Man	Folding and degradation
Calreticulin	ER	Gluc + Man	Folding and degradation
F-Box Proteins	ER	Man	Degradation
EDEM	ER	Man	Degradation
CD-MPR[a]	LE	Man-6-P	Golgi-to-endosome transport
IGF-II/CI-MPR[b]	LE	Man-6-P	Golgi-to-endosome and plasma membrane-to -endosome transport
ERGIC-53	ERGIC	Man	ER-to-ERGIC transport
VIP36	cis-Golgi/ ERGIC	Man6–9	Retrieval?

[a]CD-MPR = Cation-dependent mannose 6-phosphate receptor
[b]IGF-II/CI-MPR = Insulin-like growth factor-II or cation-independent mannose 6-phosphate/receptor; ER = Endoplasmic reticulum; LE = late endosome

2.1.1 Calnexin

The Calnexin Protein

Calnexin is a 90-kDa integral membrane protein of the ER. It binds Ca^{2+} and functions as a chaperone in the transition of proteins from ER to outer cellular membrane. Calnexin has been cloned from placenta. A subdomain containing four internal repeats binds Ca^{2+} with highest affinity. This sequence is highly conserved when compared to calreticulin, and yeast and plant calnexin homologues. An adjacent subdomain, also highly conserved and containing four internal repeats, failed to bind Ca^{2+}. The carboxyl-terminal, cytosolic domain is highly charged and binds Ca^{2+} with moderate affinity. The calnexin amino-terminal domain (residues 1–253) also binds Ca^{2+}, in contrast to the amino-terminal domain of calreticulin, which is relatively less acidic. A subdomain containing four internal repeats binds Ca^{2+} with the highest affinity. This sequence is highly conserved when compared to Crt, and yeast and plant Cnx homologues. An adjacent subdomain, also highly conserved and containing four internal repeats, failed to bind Ca^{2+}. Cnx cDNA is highly conserved when compared to calreticulin, an Onchocerca surface antigen, and yeast and plant Cnx homologues. Comparison of mouse and rat calnexin sequences reveals very high conservation of sequence identity (93–98%), suggesting that calnexin performs important cellular functions. The gene for human calnexin is located on the distal end of the long arm of human chromosome 5 at 5q35 (Ellgaard et al. 1994; Tjoelker et al. 1994).

Calmegin: A Male Germ Cell Specific Homologue of Calnexin

During mammalian spermatogenesis, many specific molecules are expressed. A 93-kDa male meiotic germ cell-specific antigen (Meg 1) is exclusively expressed in germ cells from pachytene spermatocyte to spermatid stage (Watanabe et al. 1994). A cDNA from mouse testis showed this transcript of 2.3 kb in length and expressed only in testis and not in other somatic tissues or in ovary. The expression of the mRNA was first detected at pachytene spermatocyte stage of male germ cell development. The predicted protein consists of 611 amino acids, including a hydrophobic NH2 terminus characteristic of a signal peptide, two sets of internal repetitive sequences (four repeats of IPDPSAVKPEDWDD and GEWXPPMIPNPXYQ), and a hydrophilic COOH terminus. The deduced amino acid sequence showed 58% homology with dog Cnx and significant partial homology with Crt at repetitive sequence. The name calmegin was proposed for this antigen. Calmegin is a Ca^{2+}-binding protein that is specifically expressed in spermatogenesis (Watanabe et al. 1994). *Calmegin* gene contained GC-rich sequences and potential binding sites for AP2 and Sp1, but lacked TATA sequence. The CAT gene activity was detected exclusively in testes, indicating that the 330 bp calmegin 5′ sequence was sufficient for the testis-specific expression. The existence of testicular nuclear factors specifically bound to the putative promoter sequence was also demonstrated (Watanabe et al. 1995). The human homolog of mouse Calmegin showed 80% identity with the mouse calmegin and strong conservation of two sets of internal repetitive sequences (Ca^{2+} binding motif), and the hydrophilic COOH terminus, which corresponds to the putative ER retention motif. The transcript was 3 kb in length and was expressed exclusively in the testis. Human Calmegin gene was mapped to chromosome 4q28.3–q31.1 (c/r Gupta 2005). Calmegin functions as a chaperone for one or more sperm surface proteins that mediate the interactions between sperm and egg. The defective zona pellucida-adhesion phenotype of sperm from calmegin-deficient mice is reminiscent of certain cases of unexplained infertility in human males (Ikawa et al. 1997). These results suggest that spermatogenic cell endoplasmic reticulum has a unique calcium binding protein, calnexin-t, which appears to be a calnexin variant.

2.1.2 Calnexin Structure

The ER luminal segment of Cnx and its soluble paralog Crt share sequence similarity that is most pronounced in a central segment containing two proline-rich sequence motifs repeated in tandem (David et al. 1993). Motifs 1 and 2, which are repeated three times each in Crt and four times each in Cnx, have consensus sequences of I-DP(D/E) A-KPEDWD(D/E) and G-W-P-IN-P-Y, respectively. In addition, there are three segments of high sequence similarity, A, B, and C, with the last two flanking the repeat motifs (Fig. 2.1 and 2.2). The globular domain contains the oligosaccharide-binding site with amino acids that contact

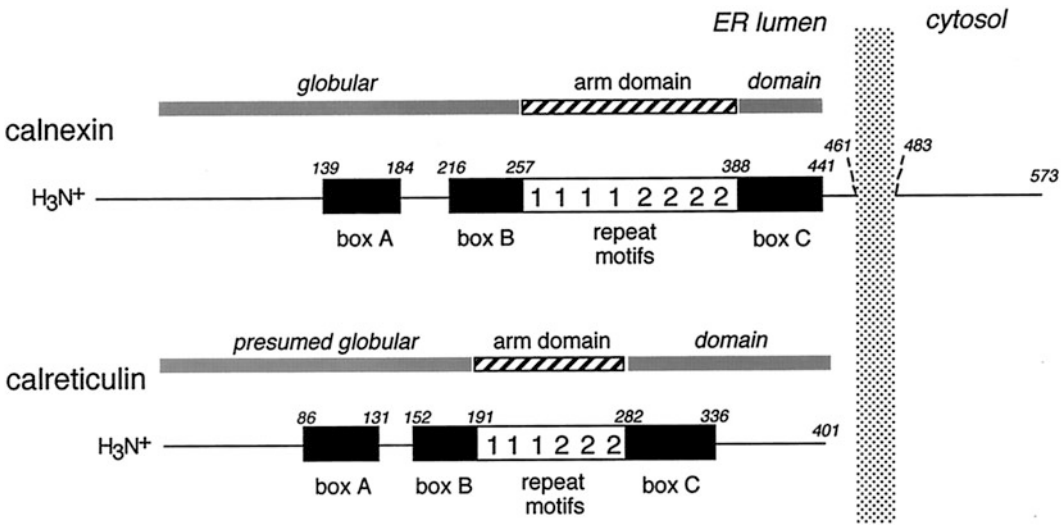

Fig. 2.1 Comparison of the linear sequences of CNX and CRT. Regions of sequence similarity are represented by *large rectangles*. The *white rectangles* correspond to segments with highest identity which are comprised of two sequence motifs repeated in tandem (indicated by the *numbers 1* and *2*). *Black rectangles* represent segments that share substantial sequence identity, and they are termed *boxes A*, *B*, and *C* to facilitate discussion. Two domains identified in the x-ray crystal structure of CNX are shown: an arm domain that corresponds to the repeat motifs (*hatched bar*) and a globular domain (*gray bar*). An arm domain has also been identified in CRT (*hatched bar*) which corresponds to the repeat motifs. The structure of the remainder of the molecule has not been solved but is presumed to form a globular domain analogous to that of CNX (*gray bar*) (Reprinted by permission from Leach et al. 2002 © The American Society for Biochemistry and Molecular Biology)

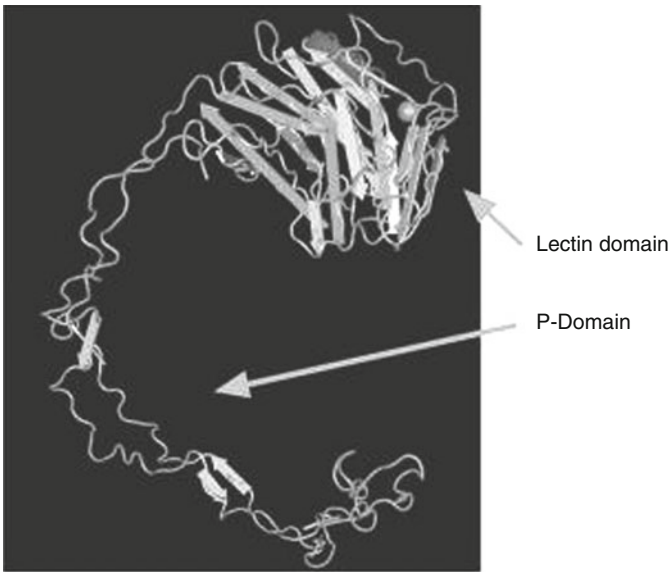

Fig. 2.2 Domain organization of calnaxin (Adapted with permission from Schrag et al. 2001 © Elsevier)

the terminal glucose residue. Each motif-1-repeat is paired with a motif-2-repeat on the opposite strand.

The lectin domain confers specific binding to glycoproteins bearing Asn-linked oligosaccharides of the form Glc1Man5–9GlcNAc2 (Leach et al. 2002; Ware et al. 1995). A 140-Å hairpin loop forms the arm domain, the tip of which includes the binding site for a thiol oxidoreductase, ERp57 (Leach et al. 2002; Pollock et al. 2004). In vitro studies have shown that the recruitment of a reduced glycoprotein to Cnx-ERp57 complex greatly enhances oxidative folding relative to ERp57 alone (Zapun et al. 1998). Both the globular and arm domains have been shown to bind Ca^{2+} but the crystal structure revealed only a single bound Ca^{2+} within the globular domain (Schrag et al. 2001). Although the complete structure of Crt is yet to be solved, ~39% overall sequence identity to Cnx, combined with conserved oligosaccharide binding specificity (Vassilakos et al. 1998), a shorter but similar arm domain structure as determined by NMR (Ellgaard et al. 2001a), and conserved ERp57 association properties (Frickel et al. 2002) all suggest a similar overall structure for the two chaperones. Ca^{2+} also binds to the globular and arm domains of Crt, which suggests that this ion is important for stabilizing both chaperones (Brockmeier and Williams 2006; Li et al. 2001).

2.1.3 Calnexin Binds High-Mannose-Type Oligosaccharides

In ER and in early secretory pathway, where repertoire of oligosaccharide structures is still small, the glycans play a pivotal role in protein folding, oligomerization, quality control, sorting, and transport. They are used as universal "tags" that allow specific lectins and modifying enzymes to establish order among the diversity of maturing glycoproteins (Yamashita et al. 1999). High-mannose-type oligosaccharides have been shown to play important roles in protein

quality control (QC). Several intracellular proteins, such as lectins, chaperones and glycan-processing enzymes, are involved in this process. These include Cnx/Crt, UDP-glucose:glycoprotein glucosyltransferase (UGGT), cargo receptors (such as VIP36 and ERGIC-53), mannosidase-like proteins (e.g. EDEM and Htm1p) and ubiquitin ligase (Fbs). They are thought to recognize high-mannose-type glycans with subtly different structures, although the precise specificities are yet to be clarified. Calnexin binds mostly with N-glycosylated proteins. Soluble Cnx binds specifically with Glc1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoprotein. Findings suggested that once complexes between calnexin and glycoproteins are formed, oligosaccharide binding does not contribute significantly to the overall interaction (Leach et al. 2002; Ware et al. 1995).

2.1.4 Functions of Calnexin

Cnx/Crt as Classical Chaperones of Protein Folding

The polypeptide-binding site on Cnx and Crt permits them to function as classical chaperones capable of recognizing non-native features of protein folding intermediates and suppressing their aggregation. This function was initially uncovered through in vitro experiments that demonstrated that both Cnx and Crt can suppress the aggregation not only of glycoproteins bearing monoglycosylated oligosaccharides but that of nonglycosylated proteins as well (Culina et al. 2004; Rizvi et al. 2004; Thammavongsa et al. 2005). Aggregation suppression ability was enhanced in presence of physiological ER Ca^{2+} concentrations as well as millimolar ATP, the latter causing an increased hydrophobic surface on chaperones (Brockmeier and Williams 2006). Several studies have validated the existence of functional polypeptide-based interactions between either Cnx or Crt and folding glycoproteins in living cells. ER molecular chaperones of the calreticulin/calnexin cycle have overlapping and complementary but not redundant functions. The absence of one chaperone can have devastating effects on the function of the others, compromising overall QC of the secretory pathway and activating unfolded protein response (UPR)-dependent pathways.

A number of proteins have been demonstrated to bind calnexin. The 90-kDa phosphoprotein (p90) of ER formed stable and transient complexes with other cellular proteins, and associated with heavy chain of MHC class I protein. A truncated version of integral membrane glycoprotein Glut 1 (GT_{155}) interacts with calnexin in vitro. Reports highlight the importance of the calnexin cycle in the functional maturation of the g-secretase complex (Hayashi et al. 2009b). γ-secretase is a membrane protein complex that catalyzes intramembrane proteolysis of a variety of substrates including the amyloid precursor protein of Alzheimer disease. Nicastrin (NCT), a single-pass membrane glycoprotein that harbors a large extracellular domain, is an essential component of the γ-secretase complex. Lectin deficient mutants of Cnx were shown to interact with heavy chains of MHC class I molecules in insect cells and to prevent their rapid degradation (Leach and Williams 2004). Similarly, Crt-deficient mutants were found not only to interact with a broad spectrum of newly synthesized proteins and dissociate with normal kinetics, but it was also able to complement all MHC class I biosynthetic defects associated with Crt deficiency (Ireland et al. 2008). Studies are consistent with a model wherein Cnx and Crt associate with folding glycoproteins through both lectin- and polypeptide-based interactions thereby increasing the avidity of association relative to either interaction alone (Stronge et al. 2001). Binding of Cnx or Crt serves to prevent premature release of folding intermediates from the ER and promotes proper folding by suppressing off-pathway aggregation and by providing a privileged environment in which associated ERp57 promotes thiol oxidation and isomerization reactions (Zapun et al. 1998). The Crt is essential for normal Cnx chaperone function. In the absence of Crt, Crt substrates are not "picked up" by Cnx but accumulate in ER lumen, resulting in the activation of unfolded protein response (UPR), which is activated to induce transcription of ER-localized molecular chaperones (Shen et al. 2004). Pancreatic ER kinase (PERK) and Ire1a, UPR-specific protein kinases, and eIF2a are also activated in the absence of Crt (Knee et al. 2003).

Cnx/Crt Cycle

Calnexin binds only those N-glycoproteins which possess GlcNAc2Man9Glc1 oligosaccharides. Oligosaccharides with three sequential glucose residues are added to asparagine residues of the nascent proteins in the ER. The monoglucosylated oligosaccharides that are recognized by Cnx result from trimming of two glucose residues by sequential action of two glucosidases (GLS), I and II. GLS II can also remove third and last glucose residue (Hebert et al. 1995). The ER also contains a uridine diphosphate (UDP)-glucose, glycoprotein transferase (UGGT), which can re-glucosylate chains that have been glucose-trimmed. If the glycoprotein is not properly folded, the UGGT will add the glucose residue back onto the oligosaccharide thus regenerating the glycoprotein's ability to bind to calnexin. The improperly-folded glycoprotein chain thus loiters in the ER, risking the encounter with α-mannosidase (MNS1), which eventually sentences the underperforming glycoprotein to degradation by removing its mannose residue. If the protein is correctly translated, the chance of it being correctly folded before it encounters MNS1 is high. ATP and calcium ions are two of the cofactors involved in substrate binding for calnexin. Together, UGGT and GLS II establish a cycle of de-glucosylation and re-glucosylation.

Importantly, UGGT discriminates between folded and unfolded proteins, adding back a glucose residue to unfolded proteins only. This results in "rebuilding" of the monoglucosylated oligosaccharide on unfolded substrates, enabling them to interact with Crt and/or Cnx again. This de-glucosylation/glucosylation cycle may be repeated several times before a newly synthesized glycoprotein is properly folded (Roth et al. 2003) (Fig. 2.3). The lectin-oligosaccharide interaction is regulated by the availability of the terminal glucose on Glc1Man5–9Glc-NAc2 oligosaccharides. If folding of glycoprotein does not occur promptly, the folding sensor UGGT recognizes non-native conformers and reglucosylates *N*-glycans, thereby allowing re-entry into the chaperone cycle (Caramelo et al. 2004; Ritter et al. 2005; Taylor et al. 2004). Terminally folding-defective glycoproteins are further processed by demannosylation that diverts them from Cnx/Crt cycle into ERAD disposal pathway (Helenius and Aebi 2004). The lectin-binding site of Cnx and Crt is localized to the Ca^{2+} binding P-domain of the protein and the bound Ca^{2+} is essential for the lectin-like function of these proteins. Moreover, while glycoproteins are bound to Cnx and Crt, the disulphide bonds of the substrates are rearranged by the PDI activity associated with ERp57 suggesting that Crt binding to carbohydrates may be a 'signal' to recruit other chaperones to assist in protein folding. It should be emphasized, however, that monoglucosylated high-mannose carbohydrates may not be a prerequisite for substrate binding to Crt. For example, castanospermine and 1-deoxynojirimycin, inhibitors of the glucosidase II, do not affect association between Crt and Factor VIII or between Crt and mucin. Calreticulin also binds directly to PDI, ERp57, perforin, the synthetic peptide KLGFFKR and the DNA-binding domain of steroid receptors, indicating that chaperone function of Crt may involve both protein–protein and protein–carbohydrate interactions (Michalak et al. 1999).

Site of Interaction/Substrate Specificity

Although lectin sites of Cnx and Crt have been well defined through structural and mutagenesis studies (Schrag et al. 2001; Kapoor et al. 2004; Thomson and Williams 2005), less is known about the location and substrate specificity of the polypeptide-binding sites. Deletion mutagenesis of rabbit Crt as well as Cnx suggested that their abilities to suppress the aggregation of nonglycosylated proteins reside primarily within their globular domains (Leach et al. 2002; Xu et al. 2004). Furthermore, in vitro binding experiments with nonglycosylated proteins such as citrate synthase and malate dehydrogenase have indicated that both chaperones interact preferentially with non-native conformers, suggesting that they act as folding sensors in addition to the role provided by UDP-glucose:glycoprotein glucosytransferase (Saito et al. 1999). To characterize the specificity of the polypeptide-binding site of Crt, Sandhu et al. (2007) and Duus et al. (2008) examined a panel of peptides for their binding to Crt using a competitive ELISA. Peptide binding required a minimum peptide length of five residues that were hydrophobic in nature. In another study, a hydrophobic Crt-binding peptide was shown to compete with the ability of Crt to suppress the thermally induced aggregation of a soluble MHC class I molecule (Rizvi et al. 2004). Collectively, these findings indicate the presence of a site on Cnx and Crt that recognizes non-native protein conformers and that, in the case of Crt, exhibits specificity for hydrophobic peptide segments.

Brockmeier et al. (2009) investigated the location and characteristics of the polypeptide-binding function of Cnx under physiological conditions of ER lumen. Using an assay in which soluble ER luminal domain of Cnx (S-Cnx) suppresses the aggregation of nonglycosylated firefly luciferase at 37 °C and 0.4 mM Ca^{2+} (Brockmeier and Williams 2006), Brockmeier et al. showed that this aggregation suppression function resides within the globular lectin domain but is enhanced by the presence of the full-length arm domain. Direct binding experiments revealed a single site of peptide binding in globular domain at a location distinct from the lectin site. The site in globular domain is responsible for aggregation suppression and is capable of binding hydrophobic peptides with μM affinity ($K_D = 0.9$ μM). Furthermore, binding studies with peptides and non-native proteins of increasing size revealed that the arm domain contributes to the aggregation suppression function of S-Cnx not through direct substrate binding but rather by sterically constraining large polypeptide chains.

Cnx as a PACS-2 Cargo Protein

Proteins of Cytosolc Sorting (Pacs): CK2-phosphorylatable acidic clusters are hallmark interacting sequences for proteins of cytosolc sorting (PACS) protein family, which includes PACS-1 and PACS-2. The interaction of these acidic motifs with PACS proteins mediates a variety of intracellular steps that include trafficking between *trans*-Golgi (TGN) network and endosomes, localization to mitochondria and retention in ER. Cargo proteins can usually interact with both PACS-1 and PACS-2. Changing the amount of Cnx on plasma membrane could affect cell surface properties and might have implications on phagocytosis or cell–cell interactions. Hence, the amount of Cnx on plasma membrane could depend on cell type or cellular homeostasis, and it might be the result of regulated intracellular retention (c/r Myhill et al. 2008). Myhill et al. (2008) identified Cnx as a PACS-2 cargo protein on ER. Cnx interacts with PACS-2 using its acidic CK2 motif. Results suggest that the phosphorylation state of the calnexin cytosolic domain and its interaction with PACS-2 sort this chaperone between domains of the ER and the plasma membrane.

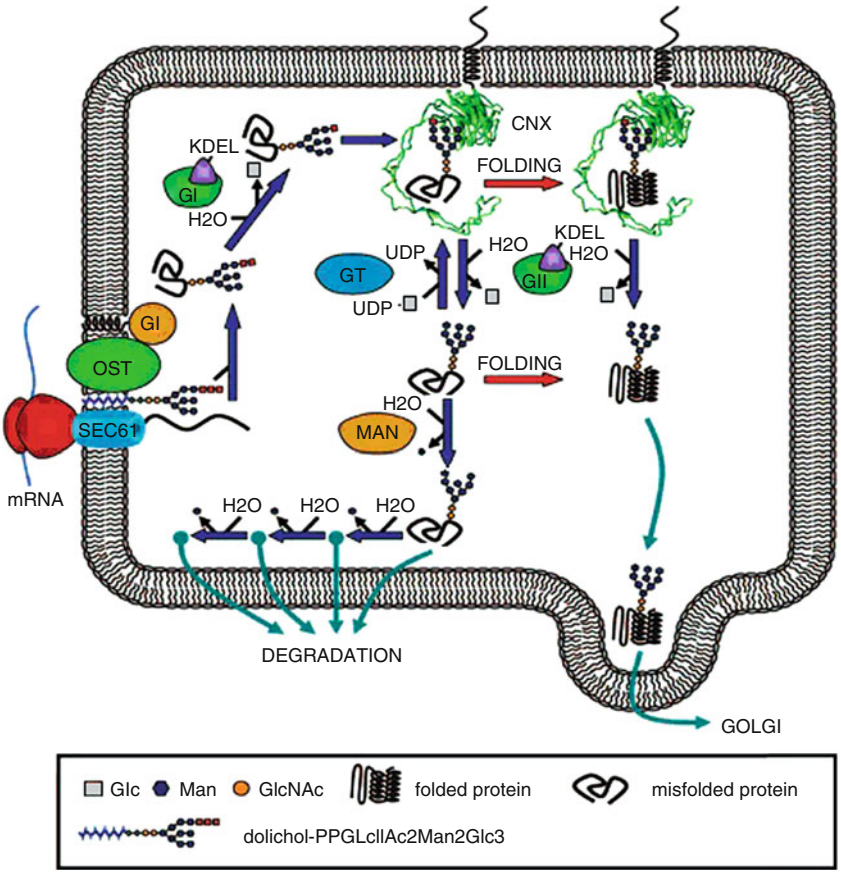

Fig. 2.3 Model proposed for the quality control of glycoprotein folding. Proteins entering the ER are *N*-glycosylated by the oligosaccharyltransferase (*OST*) as they emerge from the translocon. Two glucose units are removed by the sequential action of GI and GII to generate monoglucosylated species that are recognized by Cnx and/or Crt (only Cnx is shown), which is associated with ERp52. The complex between the lectins and folding intermediates/misfolded glycoproteins dissociates upon removal of the last glucose by GII and is reformed by GT activity. Once glycoproteins have acquired their native conformations, either free or complexed with the lectins, GII hydrolyzes the remaining glucose residue and releases the glycoproteins from the lectin anchors. These species are not recognized by GT and are transported to the Golgi. Glycoproteins remaining in misfolded conformations are retrotranslocated to the cytosol, where they are deglycosylated and degraded by the proteasome. One or more mannose residues may be removed during the whole folding process (Reprinted with permission from Caramelo and Parodi 2008 © American Society for Biochemistry and Molecular Biology)

In addition to folding intermediates, ribosomes and SERCA2b, Cnx also interacts with BAP31, an ER cargo receptor that mediates export of transmembrane proteins from the ER and shuttles them to the ER quality control compartment. Thus, Cnx can reach the plasma membrane and can also interact with numerous ER membrane proteins that are found on multiple domains of the ER such as the Mitochondria Associated Membrane (MAM). Although Cnx and other ER chaperones clearly localize to multiple cellular membranes, it is currently not understood whether the cell has mechanisms in place that control the distribution of chaperones between these various locations. Support for the hypothesis of a controlled distribution of ER proteins to specific membrane domains comes from pioneering studies on Cnx (Chevet et al. 1999; Roderick et al. 2000), which showed that PKC, ERK-1 and protein kinase CK2 (CK2) can phosphorylate the Cnx cytosolic domain. Phosphorylation by ERK-1 on serine 583 increases interaction of Cnx with ribosomes, but also interaction with SERCA2b. In addition, CK2 phosphorylation of serines 554 and 564 by CK2 synergizes with ERK-1 phosphorylation of serine 583 to promote interaction with ribosomes (Chevet et al. 1999). Hence, the Cnx phosphorylation state could lead to enrichment on the MAM and the rER, where these Cnx interactors are found. However, it is still unclear what happens to dephosphorylated v that has been demonstrated to exist in vivo (Myhill et al. 2008).

However, Calnexin deficiency is not embryonic lethal. This is amazing considering great structural and functional similarities between the Crt and Cnx chaperones. The $Cnx^{-/-}$ animals exhibit impaired motor function and die within the first 5 weeks of life. Studies with Crt and Cnx gene knockout mice indicate that these proteins are unable to compensate for the loss of each other, suggesting they have unique functions. The molecular chaperone function of calreticulin

and calnexin may only partially explain phenotypes of $Cnx^{-/-}$ and $Crt^{-/-}$ mice (Denzel et al. 2002).

2.1.5 Patho-Physiology of Calnexin Deficiency

ER and Oxidative Stresses Are Common Mediators of Apoptosis: The field of the ER stress in mammalian cells has expanded rapidly during the past decade, contributing to understanding of the molecular pathways that allow cells to adapt to perturbations in ER homeostasis. One major mechanism is mediated by molecular ER chaperones which are critical not only for quality control of proteins processed in the ER, but also for regulation of ER signaling in response to ER stress. Proteome analysis of human diploid fibroblasts (HDF) showed that Cnx significantly decreased with aging. Oxidative stress-induced expression of Cnx also attenuated in old HDF compared to young cells (Choi and Kim 2004). Ni and Lee (2007) reviewed the properties and functions of Cnx, Crt, and their role in development and diseases. Many of the new insights are derived from constructed mouse models where the genes encoding the chaperones are genetically altered, providing invaluable tools for examining the physiological involvement of the ER chaperones in vivo. Wei et al. (2008) uncovered that chemical disruption of lysosomal homeostasis in normal cells causes ER stress, suggesting a cross-talk between the lysosomes and the ER. Most importantly, chemical chaperones that alleviate ER and oxidative stresses are also cytoprotective in all forms of LSDs studied. It was proposed that ER and oxidative stresses are common mediators of apoptosis in both neurodegenerative and non-neurodegenerative LSDs. Hepatic ER stress induced by burn injury was associated with compensatory upregulation Cnx and Crt, suggesting that ER calcium store depletion was the primary trigger for induction of ER stress response in mice. Thus, thermal injury causes long-term adaptive and deleterious hepatic function characterized by significant upregulation of ER stress response (Song et al. 2009). Pre-administration of α-tocopherol is protective against oxidative renal tubular damage and subsequent carcinogenesis by ferric nitrilotriacetate (Fe-NTA) in rats. In addition to scavenging effects, α-tocopherol showed significantly beneficial effects in renal protection. Results suggest that α-tocopherol modifies glycoprotein metabolism partially by conferring mild ER stress (Lee et al. 2006).

Calnexin in Apoptosis Induced by ER Stress in *S. pombe*: Stress conditions affecting the functions of the endoplasmic reticulum (ER) cause the accumulation of unfolded proteins. ER stress is counteracted by the unfolded-protein response (UPR). However, under prolonged stress the UPR initiates a proapoptotic response. Mounting evidence indicate that the Cnx is involved in apoptosis caused by ER stress. Overexpression of Cnx in *Schizosaccharomyces pombe* induces cell death with apoptosis markers. Guerin et al. (2008) argue for the conservation of the role of calnexin in apoptosis triggered by ER stress, and validate *S. pombe* as a model to elucidate the mechanisms of Cnx-mediated cell death. The ER is highly sensitive to stresses perturbing the cellular energy levels and ER lipid or glycolipid imbalances or changes in the redox state or Ca^{2+} concentration. Such stresses reduce the protein folding capacity of the ER, which results in the accumulation and aggregation of unfolded proteins, a condition referred to as ER stress. When the capacity of the ER to fold proteins properly is compromised or overwhelmed, a highly conserved UPR signal-transduction pathway is activated. Guerin et al. (2008) further showed that the apoptotic effect of calnexin is counteracted by overexpression of Hmg1/2p, the *S. pombe* homologue of the mammalian antiapoptotic protein HMGB1 (high-mobility group box-1 protein). Interestingly, the overexpression of mammalian Cnx also induced apoptosis in *S. pombe*, suggesting the functional conservation of the role of Cnx in apoptosis. Inositol starvation in *S. pombe* causes cell death with apoptotic features. Observations indicated that Cnx takes part in at least two apoptotic pathways in *S. pombe*, and suggested that the cleavage of Cnx has regulatory roles in apoptotic processes involving Cnx (Guerin et al. 2009).

Calnexin in Biogenesis of Cystic Fibrosis: Deletion of phenylalanine at position 508 (δ F508) in first nucleotide-binding fold of cystic fibrosis transmembrane conductance regulator (CFTR) is the most common mutation in patients with cystic fibrosis. Although retaining functional Cl^- channel activity, this mutant is recognized as abnormal by cellular "quality control" machinery and is retained within ER. This intracellular retention was restricted to the immature (or ER-associated) forms of the CFTR proteins. Study indicated that Cnx retains misfolded or incompletely assembled proteins in ER and thus is likely to contribute to the mislocalization of mutant CFTR (Pind et al. 1994).

Cnx in Rod Opsin Biogenesis: Misfolding mutations in rod opsin are a major cause of the inherited blindness retinitis pigmentosa. A report from *Drosophila* rhodopsin Rh1 suggests the requirement of Cnx for its maturation and correct localization to R1–6 rhabdomeres (Rosenbaum et al. 2006). However, unlike *Drosophila* Rh1, mammalian rod opsin biogenesis does not appear to have an absolute requirement for Cnx. Other chaperones are likely to be more important for mammalian rod opsin biogenesis and quality control (Kosmaoglou and Cheetham 2008). Furthermore, the over-expression of Cnx leads to an increased accumulation of misfolded P23H opsin but not the correctly folded protein.

Finally, the increased levels of Cnx in the presence of the pharmacological chaperone 11-cis-retinal increase the folding efficiency and result in an increase in correct folding of mutant rhodopsin. These results demonstrate that misfolded rather than correctly folded rhodopsin is a substrate for Cnx and that the interaction between Cnx and mutant- misfolded rhodopsin can be targeted to increase the yield of folded mutant protein (Noorwez et al. 2009). Thus, Cnx preferentially associates with misfolded mutant opsins during retinitis pigmentosa.

Congenital Disorders: Autosomal dominant polycystic liver disease (PCLD), a rare progressive disorder, is characterized by an increased liver volume due to many fluid-filled cysts of biliary origin. Disease causing mutations in PRKCSH or SEC63 are found in approximately 25% of the PCLD patients. Hepatocystin is directly involved in the protein folding process by regulating protein binding to Cnx/Crt in the ER. A separate group of genetic diseases affecting protein N-glycosylation in the ER is formed by the congenital disorders of glycosylation (CDG). In distinct subtypes of this autosomal recessive multisystem disease specific liver symptoms have been reported that overlap with PCLD. Recent research revealed novel insights in PCLD disease pathology such as the absence of hepatocystin from cyst epithelia indicating a two-hit model for PCLD (Janssen et al. 2010)

Peripheral Neuropathies: Schwann cell-derived peripheral myelin protein-22 (PMP-22) when mutated or over-expressed causes heritable neuropathies with a unexplained "gain-of-function" ER retention phenotype. Missense point mutations in Gas3/PMP22 are responsible for the peripheral neuropathies Charcot-Marie-Tooth 1A and Dejerine Sottas syndrome. These mutations induce protein misfolding with the consequent accumulation of proteins in ER and the formation of aggresomes. In Trembler-J (Tr-J) sciatic nerves, prolonged association of mutant PMP-22 with Cnx is found. In 293A cells overexpressing PMP-22(Tr-J), Cnx and PMP-22 colocalize in large intracellular structures. Similar intracellular myelin-like figures were also present in Schwann cells of sciatic nerves from homozygous Trembler-J mice with no detectable activation of stress response pathway as deduced from BiP and CHOP expression. Sequestration of Cnx in intracellular myelin-like figures may be relevant to the autosomal dominant Charcot-Marie-Tooth-related neuropathies (Dickson et al. 2002). During folding PMP22 associates with calnexin. Calnexin interacts with the misfolded transmembrane domains of PMP22. The emerging models indicate for a glycan-independent chaperone role for calnexin and for the mechanism of retention of misfolded membrane proteins in the endoplasmic reticulum (Fontanini et al. 2005)

2.2 Calreticulin

2.2.1 General Features

Calreticulin (Crt) was first identified as a Ca^{2+}-binding protein of the muscle sarcoplasmic reticulum in 1974. Calreticulin is a ubiquitous protein, found in a wide range of species and in all nucleated cell types, and has a variety of important biological functions. Since its discovery (Ostwald and MacLennan 1974) from rabbit skeletal muscle it has been cloned in several vertebrates and invertebrates, and also higher plants (Michalak et al. 1999). There is no Crt gene in yeast and prokaryotes whose genomes have been fully sequenced. The human gene for calreticulin contains nine exons and eight introns. The deduced amino acid sequence indicates that calreticulin has a 17 amino acid hydrophobic signal sequence at its N terminus and that mature calreticulin contains 400 amino acids. The structure of calreticulin has been well characterized. It has at least three structural and functional domains.

The genomic configuration of the mouse and human calreticulin gene is shown in Fig. 2.4. The protein is encoded by a single gene (McCauliffe et al. 1992; Waser et al. 1997), and only one species of 1.9 kb mRNA encoding calreticulin has been identified. There is no evidence for alternative splicing of the calreticulin mRNA. The Crt gene consists of nine exons and spans approx. 3.6 kb or 4.6 kb of human or mouse genomic DNA respectively (Fig. 2.4). Human and mouse genes have been localized to chromosomes 19 and 8 respectively (McCauliffe et al. 1992; Rooke et al. 1997). The exon–intron organization of human and mouse genes is almost identical. The nucleotide sequences of the mouse and human gene show greater than 70% identity, with the exception of introns 3 and 6, indicating a strong evolutionary conservation of the gene. In the mouse gene these introns are approximately twice the size of the corresponding introns in the human gene. The promoter of the mouse and human calreticulin genes contain several putative regulatory sites, including AP-1 and AP-2 sites, GC-rich areas, including an Sp1 site, an H4TF-1 site, and four CCAAT sequences (McCauliffe et al. 1992; Waser et al. 1997). AP-2 and H4TF-1 recognition sequences are typically found in genes that are active during cellular proliferation. There is no obvious nuclear factor of activated T-cell (NF-AT) and nuclear factor κB (NF-κB) sites in the calreticulin promoter. Several poly (G) sequences, including GGGNNGGG motifs, are also found in the promoter regions of calreticulin and other ER/sarcoplasmic reticulum (SR) luminal proteins, including glucose-regulated protein 78 (Grp78) and Grp94. These motifs may therefore play a role in regulation of the expression of luminal ER proteins and in ER stress-

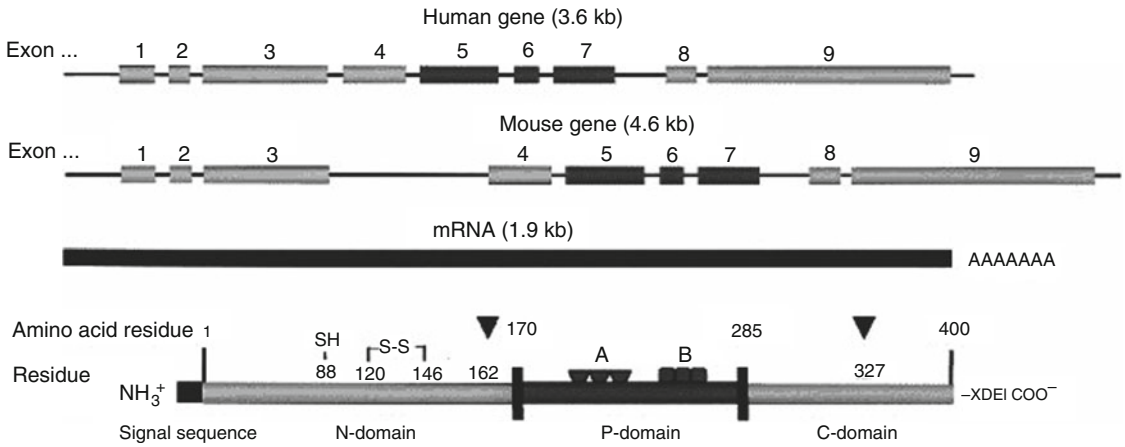

Fig. 2.4 The calreticulin gene: The Figure shows a schematic representation of the genomic configuration of domain structure of calreticulin protein. Structural predictions for calreticulin suggest that the protein has at least three structural and functional domains. Exons encoding the N domain (including the N-terminal signal sequence), and the C domain of calreticulin are in *grey* color while P is *black*. The N, P, and C domains are also presented in same color. The protein contains an N-terminal amino acid signal sequence (*black box*) and a C-terminal KDEL ER retrieval signal. The locations of 3 cysteine residues and the disulphide bridge in the N domain of calreticulin are indicated. The arrows indicate the location of potential glycosylation sites (residues 162 and 327). Repeats A (amino acid sequence PXXIXDPDAXKPEDWDE) and B (amino acid sequence GXWXPPXIXNPXYX) are also indicated (Reprinted with permission from Michalak et al. 1999; Biochem J. 344: 281–97 © The Biochemical Society)

dependent activation of the calreticulin gene (Michalak et al. 1999).

Depletion of Ca^{2+} stores induces severalfold activation of Crt promoter followed by increase in Crt mRNA and protein levels (Waser et al. 1997). Expression of Crt is also activated by bradykinin-dependent Ca^{2+} depletion of intracellular Ca^{2+} stores both in vitro and in vivo (Waser et al. 1997). The calreticulin promoter is activated by Zn^{2+} and heat shock. Expression of calreticulin is also induced by viral infection (Zhu 1996), by amino acid deprivation and in stimulated cytotoxic T-cells, further indicating that the calreticulin gene is activated by a variety of chemical and biological stresses. Since calreticulin has been implicated in a wide variety of cellular processes, the stress-dependent activation of the calreticulin gene may affect numerous biological and pathophysiological conditions (Michalak et al. 1999).

2.2.2 The Protein

Calreticulin is a 46-kDa protein with an N-terminal cleavable amino acid signal sequence and a C-terminal KDEL ER retrieval signal (Fig. 2.4). These specific amino acid sequences are responsible for targeting and retention of Crt in the ER lumen. Depending on species, Crt may have one or more potential N-linked glycosylation sites. The glycosylation pattern of the protein seems to be heterogeneous and does not appear to be a conserved property of the protein. The glycosylation of Crt is more common in plants than in animal cells (Navazio, et al. 1996). Heat shock may trigger glycosylation of calreticulin (Jethmalani, and Henle 1998); however, the functional consequence of this stress-induced glycosylation of the protein is presently not clear. Calreticulin has three cysteine residues, and all of them are located in the N-domain of the protein. Importantly, the location of these amino acid residues is conserved in calreticulin from higher plants to that in humans (Michalak et al. 1999). Two out of three cysteine residues found in the protein form a disulphide bridge (Cys^{120}–Cys^{146}) (Michalak et al. 1999; 2009), which may be important for proper folding of N-terminal region of Crt. ER localization of Crt is specified by two types of targeting signals, an N-terminal hydrophobic sequence that directs insertion into ER and a C-terminal KDEL sequence that is responsible for retention in the ER. Afshar et al. (2005) showed that Crt is fully inserted into ER, undergoes processing by signal peptidase, and subsequently undergoes retrotranslocation to the cytoplasm. C-terminal Ca^{2+} binding domain plays an important role in Crt retrotranslocation. Calreticulin is an ATP-binding protein but it does not contain detectable ATPase activity. Digestion of the protein with trypsin in presence of Mg^{2+} ATP protects the full-length protein. Results indicate that calreticulin may undergo frequent, ion-induced conformation changes, which may affect its function and its ability to interact with other proteins in lumen of ER (Corbett et al. 2000).

Calreticulin consists of various structural and functional domains. The N-domain of calreticulin, together with the central P-domain, is responsible for protein's chaperone function. Studies by Guo et al. (2003) and Michalak et al.

(2002a) with site-specific mutagenesis showed that mutation of a single His^{153} in calreticulin's N-domain destroys the protein's chaperone function. The P-domain of Crt (residues 181–290) contains a proline-rich region, forms an extended-arm structure, and interacts with other chaperones in the lumen of ER. The extended-arm structure is predicted to curve like that in Cnx that is similar to Crt, forming an opening that is likely to accommodate substrate binding, including the carbohydrate-binding site. As a molecular chaperone, Crt binds the monoglucosylated high mannose oligosaccharide (Glc1Man9GlcNAc2) and recognizes the terminal glucose and four internal mannoses in newly synthesized glycoproteins (Michalak et al. 2002b). Changes within ER, such as alterations in concentration of Ca^{2+}, Zn^{2+} or ATP, may affect the formation of these chaperone complexes and thus the ability of Crt to assist in protein folding (Trombetta and Parodi 2003).

Three Structural Domains

Similar to Cnx, Crt promotes the folding of proteins carrying N-linked glycans. Both proteins cooperate with an associated co-chaperone, the thiol-disulfide oxidoreductase ERp52. Three distinct structural domains have been identified in calreticulin: the amino-terminal, globular N-domain; the central P-domain; and the carboxyl-terminal C-domain (Michalak et al. 1999). NMR (Ellgaard et al. 2001), modeling (Michalak et al. 2002), and biochemical studies (Nakamura et al. 2001a) indicate that the globular N-domain and the "extended arm" P-domain of Crt may form a functional protein-folding unit (Michalak et al. 2002). This region of Crt contains a Zn^{2+} binding site and one disulfide bond, and it may also bind ATP (Baksh et al. 1995; Andrin et al. 2000; Corbett et al. 2000).

The exon–intron organization of the Crt gene suggests that the central P-domain of the protein may be encoded by exons 5, 6 and 7, whereas the first four exons and the last two exons may encode the N- and C-domain of the protein respectively (Fig. 2.4). Table 2.1 summarizes functional properties of these domains. The N-terminal part of the protein, encompassing the N- and P-domain of Crt, has the most conserved amino acid sequence (Michalak et al. 1999).

i). N-Domain

The N-terminal half of the molecule is predicted to be a highly folded globular structure containing eight anti-parallel β-strands connected by protein loops. The amino acid sequence of N-domain of Ctr is extremely conserved in all calreticulins. The N-domain binds Zn^{2+} (Baksh et al. 1995b, c; Andrin et al. 2000; Corbett et al. 2000) and it undergoes dramatic conformational changes (Michalak et al. 1999). Chemical modification of Crt has revealed that four histidines located in the N-domain of the protein (His^{25}, His^{82}, His^{128}, and His^{153}) are involved in Zn^{2+} binding (Baksh et al. 1995c). The Zn^{2+}-dependent conformational change in Crt affects its ability to bind to unfolded protein/glycoprotein substrates in vitro (Saito et al. 1999), suggesting that conformational changes in Crt may modify its chaperone function. ER of calreticulin-deficient cells with N-terminal histidine (His^{25}, His^{82}, His^{128}, and His^{153}) indicated that His^{153} chaperone function was impaired. Thus, mutation of a single amino acid residue in Crt has devastating consequences for its chaperone function, and may play a significant role in protein folding disorders (Guo et al. 2003). The N-domain interacts with the DNA-binding domain of the glucocorticoid receptor in vitro (Burns et al. 1994), with rubella virus RNA (Singh and Atreya 1994; Nakhasi et al. 1994), β-integrin and with protein disulphide-isomerase (PDI) and ER protein 57 (ERp57) (Baksh et al. 1995a, Corbett et al. 1999). Interaction of this region of Crt with PDI inhibits PDI chaperone function (Baksh et al. 1995a), but enhances ERp57 activity (Zapun et al. 1998). These protein–protein interactions are regulated by Ca^{2+} binding to the C-domain of Crt (Corbett et al. 1999). The N-domain of Crt also inhibits proliferation of endothelial cells and suppresses angiogenesis (Pike et al. 1998).

ii). P-Domain

The P-domain of Crt 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) (Fig. 2.5a). While the C-domain is responsible for the low-affinity and high-capacity Ca^{2+} binding, the P-domain of Crt binds Ca^{2+} with high affinity. The repeats may be essential for high-affinity Ca^{2+} binding of Crt (Baksh and Michalak 1991; Tjoelker et al. 1994) that interact with PDI (Corbett et al. 1999), NK2 homeobox 1 (Perrone et al. 1999), and perforin (Andrin et al. 1998; Fraser et al. 1998), a component of the cytotoxic T-cell granules. More importantly, repeats A and B are critical for the lectin-like chaperone activity of Crt (Vassilakos et al. 1998). The P-domain is one of the most interesting and unique regions of Crt because of its lectin-like activity and amino acid sequence similarities to other Ca^{2+}-binding chaperones, including Cnx (Bergeron et al. 1994), calmegin (Watanabe et al. 1994) and CALNUC, a Golgi Ca^{2+}-binding protein (Lin et al. 1998). However, the C-domain is less conserved than other domains of Crt (Michalak et al. 1999). Four amino acid residues (Glu^{239}, Asp^{241}, GLu^{243}, and Trp^{244}) at the tip of the 'extended arm' of P-domain are critical in chaperone function of Crt (Martin et al. 2006). Martin et al. (2006) focused studies on two cysteine residues (Cys^{88} and Cys^{120}), which form a disulfide bridge in N-terminal domain of Crt, a tryptophan residue located in CRD (Trp^{302}), and on certain residues located at the tip of "hairpin-like" P-domain of Crt (Glu^{238}, Glu^{239},

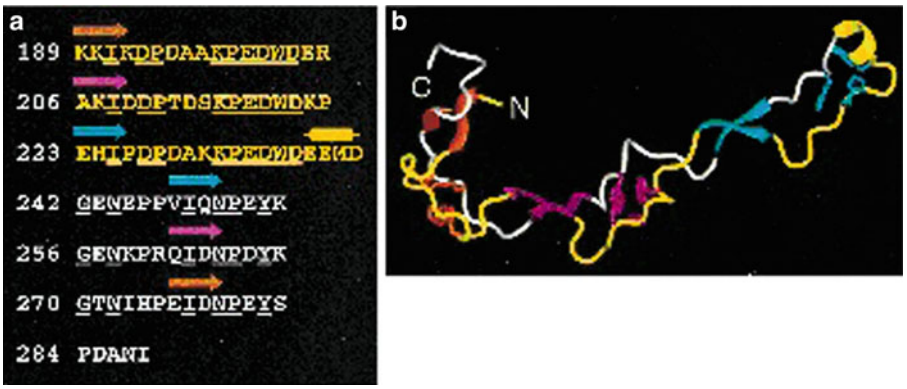

Fig. 2.5 (**a**). Alignment of the sequence repeats in rat Crt (189–288). The three 17-residue type 1 repeats are shown in yellow, and the three 14-residue type 2 repeats are white. The positions of the strands in three short antiparallel β-sheets are indicated by *orange*, *red*, and *blue arrows* above the sequence. The position of a helical turn, which includes a two-residue insert between the third and fourth repeat, is shown by a *yellow* cylinder above the sequence. Residues conserved in all three repeats of one type are underlined. (**b**). Bundles of the 20 energy-minimized conformers used to represent the NMR structure of Crt(189–288). (a) Superposition for best fit of the backbone atoms N, Ca, and C9 of the residues 219–258. (b) Superposition for best fit of the backbone atoms N, Ca, and C9 of the residues 189–209 and 262–284. In each drawing the polypeptide segments used for the superposition are colored *yellow*, and the remaining residues are *white*. (c) Cartoon of the conformer from *a* for which the *white* region is on the extreme *left*. The β-sheets and the helical turn on the extreme *right* are represented by ribbons and colored as in Fig. 4a. The same color code is used for the three associated hydrophobic clusters. The polypeptide segments that connect the β-strands are drawn as thin cylindrical rods, which are *yellow* for the type 1 repeats and white for the type 2 repeat (Reprinted with permission from Ellgaard et al. 2001a © National Academy of Sciences, USA)

Asp^{241}, Glu^{243}, and Trp^{244}). It was revealed that bradykinin-dependent Ca^{2+} release from ER was rescued by wild-type Crt and by Glu^{238}, Glu^{239}, Asp^{241}, and Glu^{243} mutants. Other amino acids mutants under study rescued the Crt-deficient phenotype only partially (~40%), or did not rescue it at all. Thus, amino acid residues Glu^{239}, Asp^{241}, Glu^{243}, and Trp^{244} at the hairpin tip of P-domain are critical in the formation of a complex between ERp57 and Crt. Although the Glu^{239}, Asp^{241}, and Glu^{243} mutants did not bind ERp57 efficiently, they fully restored bradykinin dependent Ca^{2+} release in $crt^{-/-}$ fibroblast cells.

The central, proline-rich P-domain of Crt, comprising residues 189–288, contains three copies of each of two repeat sequences (types 1 and 2), which are arranged in a characteristic '111222' pattern. The central proline-rich P-domain of Crt (189–288) shows an extended hairpin topology, with three short anti-parallel β-sheets, three small hydrophobic clusters, and one helical turn at the tip of the hairpin. The loop at the bottom of the hairpin consists of residues 227–247, and is closed by an anti-parallel β-sheet of residues 224–226 and 248–250. Two additional β-sheets contain residues 207–209 and 262–264, and 190–192 and 276–278. The 17-residue spacing of the β-strands in N-terminal part of hairpin and 14-residue spacing in the C-terminal part reflect the length of type 1 and type 2 sequence repeats. As a consequence of this topology the peptide segments separating the β-strands in the N-terminal part of the hairpin are likely to form bulges to accommodate the extra residues. Further, the residues 225–251 at the tip of Crt P-domain are involved in direct contacts with ERp52. The Crt P-domain fragment Crt(221–256) constitutes an autonomous folding unit, and has a structure highly similar to that of corresponding region in Crt(189–288). Of the 36 residues present in Crt(221–256), 32 form a well-structured core, making this fragment one of the smallest known natural sequences to form a stable non-helical fold in absence of disulfide bonds or tightly bound metal ions. Crt(221–256) comprises all the residues of intact P-domain that were shown to interact with ERp52.

The NMR structure of the rat calreticulin P-domain, comprising residues 189–288, Crt(189–288), shows a hairpin fold that involves the entire polypeptide chain, has the two chain ends in close spatial proximity, and does not fold back on itself. This globally extended structure is stabilized by three antiparallel β-sheets, with the β-strands comprising the residues 189–192 and 276–279, 206–209 and 262–265, and 223–226 and 248–251, respectively. The hairpin loop of residues 227–247 and the two connecting regions between the β-sheets contain a hydrophobic cluster, where each of the three clusters includes two highly conserved tryptophyl residues, one from each strand of the hairpin. The three β-sheets and the three hydrophobic clusters form a repeating pattern of interactions across the hairpin that reflects the periodicity of the amino acid sequence, which consists of three 17-residue repeats followed by three 14-residue repeats. Within the global hairpin fold there are two well-ordered subdomains comprising the residues 219–258, and 189–209 and 262–284, respectively. These are separated by

a poorly ordered linker region, so that the relative orientation of the two subdomains cannot be precisely described. The structure for Crt(189–288) provides an additional basis for functional studies of the abundant endoplasmic reticulum chaperone calreticulin (Ellgaard et al. 2001).

iii). C-Domain

The C-terminal region of Crt (C-domain) is highly acidic and terminates with the KDEL ER retrieval sequence (Fig. 2.1). The C-domain of Crt is susceptible to proteolytic cleavage and that the N- and P-domains form a proteolytically stable tight association. The C-domain of Crt binds over 25 mol of Ca^{2+}/mol of protein (Baksh and Michalak 1991), binds to blood-clotting factors (Kuwabara et al. 1995) and inhibits injury-induced restenosis (Dai et al. 1997). Ca^{2+} sensitivity, confined to the C-terminal part of the protein (C-domain), suggests that the C-domain of Crt may play a role of Ca^{2+} 'sensor' in ER lumen. Ca^{2+} binding to C-domain of Crt plays a regulatory role in the control of Crt interaction with PDI, ERp57 and perhaps other chaperones (Corbett et al. 1999). A modified form of calreticulin lacking the C-terminal hexapeptide including KDEL ER retention sequon has been isolated. Such a truncation may point to a mechanism that allows escape of Crt from ER (Hojrup and Roepstorff 2001). A transcription-based reporter assay revealed an important role for C-domain in Crt retrotranslocation (Afshar et al. 2005). At present, no structural information is available for C-domain which is involved in Ca^{2+} storage in the lumen of ER (Nakamura et al. 2001b).

A study by Jin et al. (2009) shows that only one of the three forms of the ER folding helper Crt of the plant Arabidopsis thaliana interacts with a mutated form of BRI1, the plasma membrane leucine-rich-repeat kinase receptor for brassinosteroids, plant-specific hormones playing important roles in plant growth. Arabidopsis CRT1 and CRT2 are very similar and have homologs in nonplant organisms, but the BRI1-interacting CRT3 seems to be a plant-specific form, with orthologs in higher and lower plant species (Persson et al. 2003). Gene coexpression analysis indicates that CRT3 can be grouped with stress resistance genes, whereas CRT1 and CRT2 are coexpressed mainly with other folding helpers. The important observations by Jin et al. (2009) point to plant-specific functional divergence in CRT family.

2.2.3 Cellular Localization of Calreticulin

Numerous studies confirmed ER localization of the protein in many diverse species, including plants. Besides its main location in ER (Opas et al. 1996), Crt has been found to reside in the nuclear envelope, the spindle apparatus of the dividing cells (Denecke et al. 1995), the cell surface (Gardai et al. 2005), and the plasmodesmata (Laporte et al. 2003; Chen et al. 2005), indicating that Crt is essential for normal cell function**.** The protein has also been localized to the cytoplasmic granules of the cytotoxic T-cell (Andrin et al. 1998; Fraser et al. 1998; Dupuis et al. 1993), sperm acrosomes (Nakamura et al. 1993), tick saliva (Jaworski et al. 1996), the cell surface (Arosa et al. 1999; Basu and Srivastava 1999), and it may even be secreted in the bloodstream. However, Crt has also been found outside the ER, such as within the secretory granules of cytotoxic lymphocytes, the cell surface of melanoma cells and virus-infected fibroblasts, and the cytosol and nucleus of several cell types (Arosa et al. 1999). Given its lectin-like properties, Crt is considered to be an ER chaperone involved in the assembly and folding of nascent glycoproteins. Surprisingly, there is a considerable controversy concerning cellular localization of Crt (Michalak et al. 1999). It was proposed that this may be due to the direct interaction between Crt and the DNA-binding domain of steroid receptors (Burns et al. 1994; Dedhar et al. 1994) and the cytoplasmic tail of α-integrin (Dedhar 1994). For calreticulin to bind to these molecules, the protein would have to be present in the nucleus and/or cytosol. However, to date there have been no reports on the identification of calreticulin or calreticulin-like protein in the cytosol. Calreticulin-like immunoreactivity was detected in the nucleus of some cells (Opas et al. 1996; Dedhar et al. 1994), in squamous carcinoma cell nuclei in response to ionizing radiation (Ramsamooj et al. 1995) or in nucleus of dexamethasone-treated LM(TK^-) cells (Roderick et al. 1997). However studies on the biosynthesis of MHC class I molecules have never reported associations of ER chaperones with MHC class I molecules outside the ER and Golgi compartments (c/r Arosa et al. 1999). In human peripheral blood T lymphocytes calreticulin is expressed at the cell surface, where it is physically associated with a pool of unfolded MHC class I molecules. Michalak et al. (1996) indicated that Crt is not a nuclear resident protein, and identification of the protein in the nucleus (Opas et al. 1996) was likely an artifact of immunostaining. There is also accumulating evidence for diverse roles for Crt localized outside ER, including reports suggesting important roles for Crt localized to the outer cell surface of a variety of cell types, in the cytosol, and in the extracellular matrix (ECM). Moreover, the addition of exogenous Crt rescues numerous Crt -driven functions, such as adhesion, migration, phagocytosis, and immunoregulatory functions of Crt-null cells.

2.2.4 Functions of Calreticulin

Calreticulin Is a Multi-Process Molecule

Calreticulin (also referred as calregulin, CRP55, CaBP3, mobilferrin and calsequestrin-like protein) is a multifunctional protein involved in many biological processes that include the regulation of Ca^{2+} homeostasis (Michalak et al. 1999), intercellular or intracellular signaling, gene expression (Johnson et al. 2001), glycoprotein folding (Helenius and Aebi 2004), and nuclear transport (Holaska et al. 2001). Overexpression of Crt enhanced apoptosis in myocardiac H9c2 cells under conditions inductive to differentiation with retinoic acid (Kageyama et al. 2002) or under oxidative stress (Ihara et al. 2006). Moreover, Crt regulates p53 function to induce apoptosis by affecting the rate of degradation and nuclear localization of p53 (Mesaeli and Phillipson 2004). Michalak et al. (1999, 2009) focussed on calreticulin, as a ER luminal Ca^{2+}-binding chaperone implicated in playing a role in many cellular functions, including lectin-like chaperoning, Ca^{2+} storage and signaling, regulation of gene expression, cell adhesion and autoimmunity. Several excellent reviews have been published concerning the structure and function of Crt in animals (Bedard et al. 2005; Johnson et al. 2001; Michalak et al. 1999, 2009) and in plants (Jia et al. 2009)

Calreticulin binds Ca^{2+} in the lumen of ER with high capacity and also participates in the folding of newly synthesized proteins and glycoproteins. Hence, it is an ER luminal Ca^{2+}-buffering chaperone. The protein is involved in regulation of intracellular Ca^{2+} homoeostasis and ER Ca^{2+} capacity. The protein impacts on store-operated Ca^{2+} influx and influences Ca^{2+}-dependent transcriptional pathways during embryonic development. It is a component of the calreticulin/calnexin pathway (Ellgaard et al. 1999). Both Crt and Cnx proteins cooperate with an associated co-chaperone, the thiol-disulfide oxidoreductase ERp57, which catalyzes the formation of disulfide bonds in Cnx and Crt-bound glycoprotein substrates. Calreticulin has been implicated to participate in many (perhaps too many) cellular functions. This strongly exemplifies the central role that the ER plays in a variety of cellular functions. It is not surprising, therefore, that any changes in calreticulin expression and function have profound effects on many cellular functions. There is also accumulating evidence for diverse roles for Crt localized outside the ER, including roles for Crt localized to the outer cell surface of a variety of cell types, in the cytosol, and in the extracellular matrix. Furthermore, the addition of exogenous Crt rescues numerous Crt-driven functions, such as adhesion, migration, phagocytosis, and immunoregulatory functions of Crt-null cells. Nonetheless, it has become clear that Crt is a multicompartmental protein that regulates a wide array of cellular responses important in physiological and pathological processes, such as wound healing, the immune response, fibrosis, and cancer (Gold et al. 2010). Notwithstanding, there is a widespread agreement that calreticulin performs two major functions in the ER lumen: (1) chaperoning and (2) regulation of Ca^{2+} homoeostasis.

In a soluble form, and in association with the homologous membrane bound protein Cnx, Crt binds to glycoproteins, preventing aggregation and allowing the proteins to attain their correct folding conformation (Bedard et al. 2005). Calreticulin also plays an important role in maintenance of cellular calcium homeostasis (Michalak et al. 1992). By regulating the amount of free and bound calcium within the lumen of the endoplasmic reticulum, calreticulin affects many different cellular functions, including cell shape, adhesion and motility (Bedard et al. 2005). Other activities that influence the various roles that this multi-functional protein plays include its capacity to bind to zinc (Baksh et al. 1995b, c) and a number of hormone receptors (Burns et al. 1994; Dedhar et al. 1994).

Calreticulin as a Chaperone

Calreticulin forms part of the quality control systems of ER for newly synthesized proteins.similar to calnexin. Both chaperones participate in the ‘quality-control’ process during the synthesis of a variety of molecules, including ion channels, surface receptors, integrins, MHC class I molecules, and transporters. Signicant progress has been made in understanding how Crt/Cnx act jointly with other ER chaperones and assisting proteins, in correct folding of proteins in ER. Molecular chaperones prevent the aggregation of partially folded proteins, increase the yield of correctly folded proteins and assembly, and also increase the rate of correctly folded intermediates by recruiting other folding enzymes. Researches document that Crt functions as a lectin-like molecular chaperone for many proteins (c/r Michalak et al. 1999, 2009).

Correct folding of a protein is determined in large part by the sequence of the protein, but it is also assisted by interaction with enzymes and chaperones of the ER. Calreticulin, calnexin, and ERp57 are among the endoplasmic chaperones that interact with partially folded glycoproteins and determine if the proteins are to be released from ER to be expressed, or alternatively, if they are to be sent to proteosome for degradation (Bedard et al. 2005; Michalak et al. 2009). Calnexin and calreticulin share many substrates and may form a link of lectin-like chaperones handing over the glycoproteins from one to the other to ensure proper folding (Bass et al. 1998; Helenius and Aebi 2004; Van Leeuwen et al. 1996a, b; Wada et al. 1995). Proposed functions for calreticulin range from chaperoning in ER to antithrombotic effects at the cell surface, and from the regulation of Ca^{2+} signaling to the modulation of gene expression

Table 2.2 Putative functions of calreticulin domains[a]

(a) Structural features and function	P-domain	C-domain
Calreticulin CRT ensuring proper protein folding and preventing aggregation. Proceeded by the N-terminal signal sequence targeting the protein to the ER lumen; highly conserved amino acid sequence; potential phosphorylation site; potential glycosylation site (bovine proteins); putative autokinase activity; inhibits PDI activity; suppresses tumors; inhibits angiogenesis	The P-domain contains the lectin site and a high-affinity Ca-binding region and is proline-rich; the lectin site recognizes N-linked oligosaccharide processing intermediates of glycoproteins and prolonged interaction with misfolded proteins initiates rejection and subsequent direction to the proteasome for degradation. CRT also engages in direct protein–protein interactions. CRT amino acid sequence shows similarity to calnexin, calmegin and CANLUC.	The acidic carboxy-terminal C-domain contains the high-capacity, low-affinity Ca^{2+}, binding sequence and terminates in a KDEL (lysine, aspartic acid, glutamic acid, leucine) sequence for ER retrieval; Putative glycosylation site; antithrombotic activity; prevents restenosis Ca^{2+} 'sensor' of calreticulin–protein interactions. CRT was shown to exist outside the ER by retrotranslocation to the cytoplasm through C-domain (Afshar et al. 2005).
(b) Ion binding		
binds Zn^{2+}	High-affinity Ca^{2+}-binding site	High-capacity Ca^{2+}-binding site
(c) Molecules binding		
Binds to DNA-binding domain of steroid receptor; binds to α-subunit of integrin; binds rubella RNA; interacts with PDI and ERp57; weak interactions with perforin. CRT directly binds to the identical amino-acid sequence, GFFKR, in the alpha integrin cytoplasmic tails. The binding site in the N-terminus of CRT that binds heparin-binding domain I, to mediate TSP-1 signaling, has been localized to amino-acid residues 19–36	Binds to a set of ER proteins; strong interactions with PDI; strong interactions with perforin; lectin-like chaperone site. Cell surface CRT binds to the carbohydrate constituent (mannose) of the cell adhesion and basement membrane protein, laminin, important in cell migration through integrin binding	Binds a set of ER proteins; binds factor IX and factor X; binds to cell surface

[a]Michalak et al. (1999; Gold et al. (2006)

and cellular adhesion (Table.2.2). Two major functions of calreticulin in the ER lumen, ie, chaperoning and regulation of Ca^{2+} homeostasis, were intensively investigated and well characterized.

The assembly of MHC class I molecules is one of the most widely studied examples of protein folding in ER. It is also one of the most unusual cases of glycoprotein quality control involving ERp57 and chaperones Cnx/Crt. Pulse-chase experiments showed that Crt was associated with several proteins ER in T lymphocytes and suggested that it was expressed at the cell surface. The cell surface 46-kDa protein co-precipitated with Crt is unfolded MHC-I. Results show that after T cell activation, significant amounts of Crt are expressed on T cell surface, where they are found in physical association with a pool of β2-free MHC class I molecules (Arosa et al. 1999). Calreticulin promotes folding of HLA class I molecules to a state at which they spontaneously acquire peptide binding capacity. However, it does not induce or maintain a peptide-receptive state of class I-binding site, which is likely to be promoted by one or several other components of class I loading complexes (Culina et al. 2004).

MHC class I molecules consist of two non-covalently linked subunits, an integral membrane glycoprotein (β chain), a small soluble protein [β_2-microglobulin (β_2-m)], and a peptide of eight to eleven residues. The multi-step assembly of MHC class-I heavy chain with β_2-m and peptide is facilitated by these ER-resident proteins and further tailored by the involvement of a peptide transporter, aminopeptidases, and the chaperone-like molecule tapasin (Wearsch and Cresswell 2008). In mouse and human, β chains associate with calnexin soon after its synthesis via interactions with both immature glycans and with residues in the transmembrane domain of β chains. In human chain, β_2-m binding to β chain displaces Cnx, and the resulting β chain–β_2-m heterodimer binds Crt. MHC class I expression and transport to the cell surface are not changed in Cnx-deficient cells, suggesting that Cnx is not essential for MHC class I synthesis and transport. These findings indicate, albeit indirectly, that Crt can function in the absence of Cnx. Wearsch and Cresswell (2008) presented the roles of these general and class I-specific ER proteins in facilitating the optimal assembly of MHC class I molecules with high affinity peptides for antigen presentation. Bass et al. (1998) demonstrated a chaperone function for Cnx/Crt in human insulin receptor (HIR) folding in vivo and also provided evidence that folding efficiency and homo-dimerization are counter balanced.

ER Luminal Ca^{2+} and Calreticulin Function

Ca^{2+} is released from the ER and taken up to the ER lumen. Ca^{2+} storage capacity of ER lumen is enhanced by Ca^{2+}-binding chaperones. These include calreticulin, Grp94, immunoglobulin-heavy-chain-binding protein (BiP; Grp78), PDI, ERp72 and ER/calcistorin. Reduction of $[Ca^{2+}]_{ER}$ (ER Ca^{2+}-depletion conditions) leads to accumulation of misfolded

proteins, activation of expression of ER chaperones and ER–nucleus and ER–plasma membrane 'signaling'. Ca^{2+} depletion inhibits ER–Golgi trafficking, blocks transport of molecules across the nuclear pore and affects chaperone function. Clearly, changes of the ER luminal $[Ca^{2+}]_{ER}$ have profound effects at multiple cellular sites, including the structure and function of the ER luminal Ca^{2+}-binding chaperones (Michalak et al. 1999).

Several studies of mammalian Crts have elucidated a number of key physiological functions, including the regulation of Ca^{2+} homeostasis and Ca^{2+}-dependent signal pathways (Michalak et al. 2002b; Gelebart et al. 2005), and integrin-dependent Ca^{2+} signaling at the extra-ER sites in mammalian cells (Coppolino et al. 1997; Krause and Michalak, 1997), and molecular chaperone activity in the folding of many proteins (Denecke et al. 1995; Williams, 2006). Ca^{2+} is a universal signaling molecule in the cell cytosol and can affects several processes in ER lumen, including modulation of chaperone–substrate and protein–protein interactions. For example, binding of carbohydrate to calreticulin and calnexin occurs at high $[Ca^{2+}]_{ER}$ and it is inhibited at low $[Ca^{2+}]_{ER}$ similar to Ca^{2+} depletion in stores. Several steps during chaprone action of both Cnx and Crt are regulated by Ca^{2+}. Studies suggest that C-domain of Crt plays a role of Ca^{2+} 'sensor' in the ER lumen. Association of Crt with Grp94, Grp78 and Cnx, maturation of thyroglobulin and apolipoprotein B may be regulated by Ca^{2+} binding.. The structure of Crt provides a unique feature enabling it to perform several functions in the ER lumen, while responding to continuous fluctuations of the free $[Ca^{2+}]_{ER}$. Calreticulin also affects Ca^{2+} homoeostasis (see below), and, in one case, the protein may even be taking advantage of its chaperone, lectin-like activity to modulate Ca^{2+} fluxes across the ER membrane (Leach et al. 2002; Michalak et al. 1999).

Calreticulin and Regulation of Ca^{2+} Homoeostasis: Calreticulin has two Ca^{2+}-binding sites: a high-affinity, low-capacity site ($K_d = 1$ μM; $B_{max} = 1$ mol of Ca^{2+}/mol of protein) in the P-domain and a low-affinity high-capacity site ($K_d = 2$ mM; $B_{max} = 25$ mol of Ca^{2+}/mol of protein) in the C-domain. Overexpression of Crt in a variety of cells does not affect the cytoplasmic $[Ca^{2+}]$; however, it does result in an increased amount of intracellularly stored Ca^{2+}. Interestingly, Ca^{2+}-storage capacity of the ER is not changed in the calreticulin-deficient embryonic stem cells (ES) or mouse embryonic fibroblasts (MEF) (Michalak et al. 1999). It is reported that changes in intracellular Ca^{2+} homeostasis modulate the rate of apoptosis as in molecular chaperones and radiation-induced apoptosis of gliomas (Brondani et al. 2004). Using human glioma cell lines, overexpression of Crt modulated radiosensitivity of human glioblastoma cells by suppressing Akt/protein kinase B signaling for cell survival via alterations of cell Ca^{2+} homeostasis. The level of CRT was higher in neuroglioma H4 cells than in glioblastoma cells (U251MG and T98G), and was well correlated with the sensitivity to γ-irradiation (Okunaga et al. 2006).

Calreticulin Functions Outside ER

There is considerable evidence to indicate that Crt is found outside ER, although how the protein relocates from ER to outside of the ER remains unclear. Functions of Crt outside the ER include modulation of cell adhesion, integrin-dependent Ca^{2+} signaling, and steroid-sensitive gene expression as well as mRNA destabilization both in vitro and in vivo. One major controversy in Crt research field concerns the mechanisms involved in Crt-dependent modulation of functions outside ER (Yokoyama and Hirata 2005). Outside ER Crt is known to modulate nuclear-hormone receptor-mediated gene expression (Burns et al. 1994; Michalak et al. 1996), control of cell adhesion (Opas et al. 1996; Fadel et al. 1999; Fadel et al. 2001; Goicoechea et al. 2002), and integrin-dependent Ca^{2+} signaling in vitro and in vivo (Coppolino et al. 1997; Michalak et al. 1999). It is also involved in blood function and development (Kuwabara et al. 1995; Andrin et al. 1998; Mesaeli et al. 1999). In addition, Crt appears to play a role in the immune system (Guo et al. 2002) and apoptosis. For example, Crt-dependent shaping of Ca^{2+} signaling was found to be a critical contributor to the modulation of the T cell adaptive immune response (Porcellini et al. 2006). Surface Crt mediates muramyl dipeptide-induced RK13 cell apoptosis through activating the apoptotic pathway (Chen et al. 2005). One major controversy in the calreticulin field concerns the mechanisms involved in Crt-dependent modulation of functions outside ER.

During apoptosis, both CRT expression and the concentration of nitric oxide (NO) are increased. By using S-nitroso-l-cysteine-ethyl-ester, an intracellular NO donor and inhibitor of APLT, phosphotidylserine (PS) and CRT externalization occurred together in an S-nitrosothiol-dependent and caspase-independent manner. Furthermore, the CRT and PS are relocated as punctate clusters on the cell surface. Thus, CRT induced nitrosylation and its externalization with PS could explain how CRT acts as a bridging molecule during apoptotic cell clearance (Tarr et al. 2010b).

Calreticulin and Cell Adhesiveness: Calreticulin may be involved in integrin function and cell adhesion. Crt binds to KXFF($^{K}/_{R}$)R synthetic peptide, a region corresponding to conserved amino acid sequence found in C-terminal tail of β-subunit of integrin. It was suggested that Crt may bind to the C-terminal cytoplasmic tail of β-integrin and modulate its function (Coppolino et al. 1997; Leung-Hagesteijn et al. 1994). Differential adhesiveness correlates inversely with

the expression level of mRNA and protein for the focal contact-associated cytoskeletal protein, vinculin, and the calreticulin. Furthermore, an inverse relationship exists between the level of calreticulin and the level of total cellular phosphotyrosine (Tyr(P)) such that the cells underexpressing calreticulin display a dramatic increase in the abundance of total cellular Tyr(P). This suggests that the effects of calreticulin on cell adhesiveness may involve modulation of the activities of protein-tyrosine kinases or phosphatases (Fadel et al. 1999). In addition, Crt can regulate cell adhesion indirectly from the ER lumen via modulation of gene expression of adhesion-related molecules such as vinculin and β-catenin (Opas et al. 1996; Fadel et al. 1999, 2001). It has also been shown that calreticulin associates transiently with the cytoplasmic domains of integrin α subunits during spreading and that this interaction can influence integrin-mediated cell adhesion to extracellular matrix (Coppolino et al. 1997; Leung-Hagesteijn et al. 1994; Goicoechea et al. 2002). Calreticulin serves as a cytosolic activator of integrin and a signal transducer between integrins and Ca^{2+} channels on the cell surface (Kwon et al. 2000).

Calreticulin can also modulate cell adhesion from the cell surface and mediate cell spreading on glycosylated laminin (Goicoechea et al. 2002) and thrombospondin-induced focal adhesion disassembly (Goicoechea et al. 2000; Pallero et al. 2008). Thrombospondin (TSP) is a member of a group of extracellular matrix proteins that exist in both soluble and extracellular matrix forms and regulates cellular adhesion (Goicoechea et al. 2002). When exposed to cells in its soluble form, thrombospondin has primarily anti-adhesive effects characterized by a reorganization of stress fibers and loss of focal adhesion plaques. A 19-amino acid sequence (aa 17–35) in the N-terminal heparin-binding domain of thrombospondin, referred to as the hep I peptide, has been shown to be sufficient for focal adhesion disassembly. Since, calreticulin can modulate cell adhesion from the cell surface and mediate thrombospondin-induced focal adhesion disassembly (Goicoechea et al. 2000), it was suggested that interactions between calreticulin and thrombospondin are Zn^{2+}- and Ca^{2+}-dependent and involve the RWIESKHKSDFGKFVLSS sequence in the N-terminal region of the N-domain of calreticulin (Goicoechea et al. 2002). TSP binding to Crt-LRP1 signals resistance to anoikis (Pallero et al. 2008).

Calreticulin Affects β-Catenin-Associated Pathways: It was shown that differential adhesiveness correlates inversely with the expression level of mRNA and protein for cytoskeletal protein, vinculin, and the Crt. Calreticulin has been shown to be important in cell adhesion. Furthermore, an inverse relationship existed between the level of Crt and the level of total cellular phosphotyrosine (Tyr(P)) such that cells underexpressing Crt display a dramatic increase in the abundance of total cellular Tyr(P). In either cell type, spatial distributions of Crt and Tyr(P) are complementary, with the former being confined to ER and the latter being found outside of it. Among proteins that are dephosphorylated in cells that over-express Crt is β-catenin, a structural component of cadherin-dependent adhesion complex and a part of the Wnt signaling pathway. To investigate the mechanisms behind Crt dependent modulation of cell adhesiveness, Fadel et al. (2001), using mouse L fibroblasts differentially expressing Crt, showed that stable over-expression of ER-targeted Crt correlates with an increased adhesiveness in transformed fibroblasts, such that their cohesion resembles that of epithelial cells in culture. Fadel et al. (2001) suggest that the changes in cell adhesiveness may be due to Crt-mediated effects on a signaling pathway from the ER, which impinges on Wnt signaling pathway via cadherin/catenin protein system and involves changes in the activity of protein-tyrosine kinases and/or phosphatases. Results suggest that calreticulin may play a role in a signaling pathway from ER, involving protein-tyrosine kinases and/or phosphatases This suggests that the effects of Crt on cell adhesiveness may involve modulation of the activities of protein-tyrosine kinases or phosphatases (Fadel et al. 1999, 2001). Protein phosphorylation/dephosphorylation of tyrosine is a major mechanism for regulation of cell adhesion. Although the mechanism(s) are still elusive, it is conceivable that the effects of Crt overexpression on cell adhesion may be due to Crt effects on a signaling pathway, which includes the vinculin/catenin–cadherin protein system and may involve changes in activity of tyrosine kinases and/or phosphatases. A direct implication of this for cell–substratum interactions is that calreticulin effects may target primarily focal-contact-mediated adhesion (Michalak et al. 1999).

Wound Repair: Crt was originally shown to be the biologically active component of a hyaluronic acid isolate from fetal sheep skin that accelerated wound healing in animal experimental models of cutaneous repair. Nanney et al. (2008) showed roles for exogenous Crt in both cutaneous wound healing and diverse processes associated with repair. Topical application of Crt to porcine excisional wounds enhanced the rate of wound re-epithelialization. The in vitro bioactivities provide mechanistic support for the positive biological effects of Crt observed on both the epidermis and dermis of wounds in vivo, underscoring a significant role for Crt in the repair of cutaneous wounds (Nanney et al. 2008).

Interaction of Calreticulin with C1q: C1q immobilized on a hydrophobic surface, exposed to heat-treatment or bound to Igs showed a strong, rapid and specific binding of Crt.

When both proteins were present in equal amounts in solution, no interaction could be demonstrated. Binding between C1q and Crt could be inhibited by serum amyloid P component. Results suggest Crt as a potential receptor for an altered conformation of C1q as occurs during binding to Igs. Thus, the chaperone and protein-scavenging function of Crt may extend from the ER to the topologically equivalent cell surface, where it may contribute to the elimination of immune complexes and apoptotic cells (Steinø et al. 2004).

Calreticulin in Signal Transduction: As indicated earlier, Crt is also found on cell surface of many cell types where it serves as a mediator of adhesion and as a regulator of the immune response. Calreticulin, present on the extracellular surface of mouse egg plasma membrane, is increased in perivitelline space after egg activation. The extracellular Crt appears to be secreted by vesicles in the egg cortex that are distinct from cortical granules. An anticalreticulin antibody binds to extracellular Crt on live eggs and inhibits sperm-egg binding but not fusion. In addition, engagement of cell surface Crt by incubation of mouse eggs in the presence of anticalreticulin antibodies results in alterations in the localization of cortical actin and the resumption of meiosis as indicated by alterations in chromatin configuration, decreases in cdc2/cyclin B1 and MAP kinase activities, and pronuclear formation. These events occur in absence of any observable alterations in intercellular calcium. These studies suggest that Crt functionally interacts with the egg cytoskeleton and can mediate transmembrane signaling linked to cell cycle resumption. Evidences suggest a role for Crt as a lectin that may be involved in signal transduction events during or after sperm-egg interactions at fertilization (Tutuncu et al. 2004).

Lessons from Calreticulin-Deficient Mice

The calreticulin-deficient mouse, created by the homologous-recombination, is embryonically lethal at 14.5–16.5 days *post coitus*. Calreticulin-deficient embryos most likely die from a lesion in cardiac development. In the adult, calreticulin is expressed mainly in non-muscle and smooth-muscle cells, and is only a minor component of the skeletal and cardiac muscle. However, the calreticulin gene is activated during cardiac development, concomitant with an elevated expression of the protein, which decreases sharply in the newborn heart. Therefore it was not surprising that calreticulin-deficient mice die from heart failure. Calreticulin may in general must play an important role in the formation of heart (Michalak et al. 2002a).

Calreticulin knockout ($Crt^{-/-}$) mice die at the embryonic stage due to impaired heart development, making it impossible to study mature $Crt^{-/-}$ CTLs and NK cells. To specifically investigate the role of Crt in CTL lytic function, Sipione et al. (2005) generated CTL lines from splenocytes derived from these mice and showed that in absence of Crt, CTL cytotoxicity is impaired. Sipione et al. (2005) suggest that Crt is dispensable for the cytolytic activity of granzymes and perforin, but it is required for efficient CTL-target cell interaction and for the formation of the death synapse. It was proposed that Crt may be involved in the mechanisms underlying target recognition by CTLs and/or formation of the death synapse. Calreticulin-deficient ES cells had impaired integrin-mediated adhesion, supporting the observation that changes in expression of calreticulin affect cell adhesion.

Impaired p53 Expression in Crt-Deficient Cells: The tumor suppressor protein, p53 is a transcription factor that not only activates expression of genes containing the p53 binding site but also can repress the expression of some genes lacking this binding site. Overexpression of wild-type p53 leads to apoptosis and cell cycle arrest. The level of Crt has been correlated with the rate of apoptosis. Crt-deficient cells ($crt^{-/-}$) demonstrated that Crt function is required for the stability and localization of the p53 protein. The observed changes in p53 in the $crt^{-/-}$ cells are due to the nuclear accumulation of Mdm2 (murine double minute gene). These results, lead us to conclude that Crt regulates p53 function by affecting its rate of degradation and nuclear localization (Mesaeli and Phillipson 2004).

Calreticulin and Steroid-Sensitive Gene Expression

Calreticulin is also found in nucleus, which suggests that it may play a role in transcriptional regulation. Calreticulin binds to the DNA-binding domain of steroid receptors and transcription factors containing the amino acid sequence KXFF($^{K}/_{R}$)R and prevents their interaction with DNA in vitro (Michalak et al. 1999). With the exception of the peroxisome-proliferator-activated receptor ('PPAR')–retinoid X heterodimers (Winrow et al. 1995), transcriptional activation by glucocorticoid, androgen, retinoic acid and vitamin D_3 receptors in vivo is modulated in cells overexpressing calreticulin (Burns et al. 1994; 1997; Dedhar et al. 1994; Desai et al. 1996; Michalak et al. 1996; St-Arnaud et al. 1995; Sela-Brown et al. 1998 Wheeler et al. 1995).

Outside ER, Crt appears to be affected by varios factors. The mRNA levels of Crt increased as a function of time after UV irradiation in transformed human keratinocytes (HaCaT cells) (Szegedi et al. 2001). A developmentally expressed cytosolic, trophoblast-specific, high Mr 57-kDa Ca-binding protein (CaBP) plays an important role in regulating and/or shuttling cytosolic Ca and represents the primary mechanism

in fetal Ca homeostasis. The full-length cDNA of the mouse CaBP shows significant homology to Crt. In addition, the action of parathyroid hormone related protein (PTHrP) on placental trophoblast Ca transport is likely to involve the regulation of CaBP expression to handle the increasing Ca requirements of developing fetus (Hershberger and Tuan 1998).

Calreticulin binds to the synthetic peptide KLGFFKR, which is almost identical to an amino acid sequence in the DNA-binding domain of the superfamily of nuclear receptors. The amino terminus of calreticulin interacts with the DNA-binding domain of the glucocorticoid receptor and prevents the receptor from binding to its specific glucocorticoid response element. Calreticulin can inhibit the binding of androgen receptor to its hormone-responsive DNA element and can inhibit androgen receptor and retinoic acid receptor transcriptional activities in vivo, as well as retinoic acid-induced neuronal differentiation.

Crt and Glucocorticoid Receptor Pathways: Calreticulin play an important role in the regulation of glucocorticoid-sensitive pathway of expression of the hepatocytes specific genes during development (Burns et al. 1997). ER Crt but not cytosolic Crt is responsible for inhibition of glucocorticoid receptor-mediated gene expression. These effects are specific to Crt, since over-expression of ER lumenal proteins (BiP, ERp72, or calsequestrin) had no effect on glucocorticoid-sensitive gene expression. The N domain of Crt binds to the DNA binding domain of glucocorticoid receptor in vitro. However, the N + P domain of Crt, when synthesized without ER signal sequence, does not inhibit glucocorticoid receptor function in vivo Michalak et al. (1996) suggest that Crt and glucocorticoid receptor may not interact in vivo and that the Crt-dependent modulation of the glucocorticoid receptor function may therefore be due to a Crt-dependent signaling from the ER. Wnt signaling pathway is a multicomponent cascade involving interaction of several proteins and found to be important for development and function of various cells and tissues. There is increasing evidence that Wnt/beta-catenin pathway constitutes also one of the essential molecular mechanisms controlling the metabolic aspects of osteoblastic cells. However, in bone, glucocorticoids (GCs) have been reported to weaken Wnt signaling. Calreticulin, known to bind the DNA binding domain of glucocorticoid receptor (GR), was found to be involved in the GR-mediated down-regulation of Wnt signaling. Furthermore, GR and β-catenin were shown to exist in same immunocomplex, while interaction between Crt and beta-catenin was observed only in the presence of GR as a mediator molecule. In addition, the GR mutant lacking Crt binding ability impaired the complex formation between beta-catenin and Crt. Together with GR, β-catenin could thus be co-transported from the nucleus in a Crt-dependent way (Olkku and Mahonen A, 2009).

Overexpression of calreticulin in mouse L fibroblasts inhibits glucocorticoid-response-mediated transcriptional activation of a glucocorticoid-sensitive reporter gene and of the endogenous, glucocorticoid-sensitive gene encoding cytochrome P450. This indicates that calreticulin may be important in gene transcription, regulating the glucocorticoid receptor and perhaps other members of the super-family of nuclear receptors (Burns et al. 1994). Thus, calreticulin can act as an important modulator of the regulation of gene transcription by nuclear hormone receptors. These are surprising findings, since calreticulin is an ER-resident protein and steroid receptors are found in the cytoplasm or in the nucleus. What could be a physiological or pathophysiological relevance of calreticulin (ER)-dependent modulation of gene expression? Up-regulation of the calreticulin gene may correlate with increased resistance to steroids. For example, calreticulin is one of the androgen-sensitive genes in prostate cancer (Zhu et al. 1998; Zhu and Wang 1999; Wang et al. 1997). Steroid-dependent regulation of expression of calreticulin may affect differential sensitivity of patients to steroid therapies.

Over-expression of calreticulin and calsequestrin impairs cardiac function, leading to premature death. Calreticulin is vital for embryonic development, but also impairs glucocorticoid action. Glucocorticoid overexposure during late fetal life causes intra-uterine growth retardation and programmed hypertension in adulthood. In view of the known associations between cardiac calreticulin overexpression and impaired cardiac function, targeted up-regulation of calreticulin may contribute to the increased risk of adult heart disease introduced as a result of prenatal overexposure to glucocorticoids (Langdown et al. 2003; Michalak et al. 2002a). The neural cell adhesion molecule, N-CAM inhibits the proliferation of rat astrocytes both in vitro and in vivo. Exposure of astrocytes to N-CAM in vitro, the levels of mRNAs for glutamine synthetase and calreticulin increased while mRNA levels for N-CAM decreased. Glutamine synthetase and calreticulin are known to be involved in glucocorticoid receptor pathways. Inhibition of rat cortical astrocyte proliferation in culture by dexamethasone, corticosterone, and aldosterone suggests that astrocyte proliferation is in part regulated by alterations in glucocorticoid receptor pathways, which may involve Crt (Crossin et al. 1997).

Regulation by Androgens: Calreticulin is an intracellular protein in prostatic epithelial cells. Its expression in prostate is much higher than that in seminal vesicles, heart, brain, muscle, kidney, and liver. The expression of Crt in prostate is conserved evolutionarily. After castration, Crt mRNA and

protein are down-regulated in the prostate and seminal vesicles and restored by androgen replacement. Because Crt is a major intracellular Ca^{2+}-binding protein with 1 high-affinity and 25 low-affinity Ca binding sites, observations suggest that Crt is a promising candidate that mediates androgen regulation of intracellular Ca^{2+} levels and/or signals in prostatic epithelial cells (Zhu et al. 1998). As expected, androgen protects androgen-sensitive LNCaP but not androgen-insensitive PC-3 cells from cytotoxic intracellular Ca^{2+} overload induced by Ca^{2+} ionophore A23182. Observations suggest that Crt mediates androgen regulation of the sensitivity to Ca^{2+} ionophore-induced apoptosis in LNCaP cells (Zhu and Wang, 1999; Meehan et al. 2004).

Estrogens: While estrogens are mitogenic in breast cancer cells, the presence of estrogen receptor α (ERα) clinically indicates a favorable prognosis in breast carcinoma. Calreticulin that could interact with amino acids 206–211 of ERα reversed hormone-independent ERα inhibition of invasion. However, since Crt alone also inhibited invasion, it was proposed that this protein probably prevents ERα interaction with another unidentified invasion-regulating factor. The inhibitor role of the unliganded ER was also suggested in three ERα-positive cell lines, where ERα content was inversely correlated with cell migration. It was concluded that ERα protects against cancer invasion in its unliganded form, probably by protein-protein interactions with the N-terminal zinc finger region, and after hormone binding by activation of specific gene transcription (Platet et al. 2000).

Neonatal treatment with diethylstilbestrol (DES) leads to disruption of spermatogenesis in adult animals after apparently normal testicular development during puberty indicating aberrant androgen action in DES-exposed adult hamsters. Analyses revealed that mRNA levels for AR-responsive genes calreticulin, SEC-23B, and ornithine decarboxylase were significantly decreased in DES-exposed animals and that neonatal DES exposure impairs the action of androgens on target organs in male hamsters (Karri et al. 2004).

Crt Enhances Transcriptional Activity of TTF-1: Calreticulin binds to thyroid transcription factor-1 (TTF-1), a homeodomain-containing protein implicated in the differentiation of lung and thyroid. The interaction between calreticulin and TTF-1 appears to have functional significance because it results in increased transcriptional stimulation of TTF-1-dependent promoters. Calreticulin binds to the TTF-1 homeodomain and promotes its folding, suggesting that the mechanism involved in stimulation of transcriptional activity is an increase of the steady-state concentration of active TTF-1 protein in the cell. It was also demonstrated that calreticulin mRNA levels in thyroid cells are under strict control by the thyroid-stimulating hormone, thus implicating calreticulin in the modulation of thyroid gene expression by thyroid-stimulating hormone (Perrone et al. 1999). The thyroid hormone receptor α1 (TRα) directly interacts with calreticulin, and point to the intriguing possibility that TRα follows a cooperative export pathway in which both calreticulin and CRM1 (Exportin) play a role in facilitating efficient translocation of TRα from the nucleus to cytoplasm (Grespin et al. 2008).

The increased level of calreticulin in activated T-cells suggests that the protein may be part of the Ca^{2+}-dependent transduction pathway(s) in stimulated T-cells, including activation of NF-AT. Therefore stress-dependent stimulation of the T-cells results in the activation of both the NF-AT and calreticulin pathways. Development of the heart as an organ must inflict a tremendous stress on cardiomyocytes. Therefore it is tempting to speculate that similar stress-induced signaling pathways are essential during cardiac development and the activation of the immune system.

Destabilization of 3′-Untranslated Region of mRNA by Crt

Angiotensin II is hypertrophic for cultured adult rat aortic vascular smooth muscle cells (VSMC), whereas platelet-derived growth factor and serum are hyperplastic. Hyperplastic and hypertrophic growth are accompanied by similar changes in protein expression, suggesting that both types of growth require up-regulation of the protein synthesis and folding machinery such as calreticulin and HSPs (Patton et al. 1995). Angiotensin II plays a central role in cardiovascular homeostasis and downregulates type 1 angiotensin II (AT_1) receptor, which appears to involve several mechanisms. The AT1 receptor plays a pivotal role in the pathogenesis of hypertension and atherosclerosis. AT1 receptor expression is regulated posttranscriptionally via destabilization of AT1 receptor mRNA by mRNA binding proteins. Nickening et al. discovered that mRNA binding protein, Crt, binds to the cognate sequence bases 2175–2195 within the 3′ untranslated region of the AT_1 receptor mRNA (Nickenig et al. 2002). Angiotensin II stimulation, which causes destabilization of AT_1 receptor mRNA, causes phosphorylation of Crt. This region comprises aAUUUUA hexamer and is considerably AU-rich. Phosphorylation of Crt is essential for binding of the AT1 receptor mRNA. Findings imply an important role of serine dephosphorylation and tyrosine phosphorylation on Crt mediated AT1 receptor mRNA stability in VSMC (Mueller et al. 2008).

Glucose transport in mammalian cells is mediated by a family of structurally related glycoproteins, the glucose transporters (GLUTs) (McGowan et al. 1995). Totary-Jain et al. report that calreticulin destabilized GLUT-1 mRNA expression in primary bovine aortic endothelial cells and smooth muscle cells under high glucose conditions

(Totary-Jain et al. 2005). They identified Crt as a specific destabilizing trans-acting factor that binds to a 10-nucleotide cis-acting element (CAE 2181–2190) in the 3′-untranslated region of GLUT-1 mRNA. CAE 2181–2190–Crt complex, which is formed in VSMCs and endothelial cells exposed to hyperglycemic conditions, renders GLUT-1 mRNA susceptible to degradation. RNA–protein interactions have been shown to influence many processes, including translation, RNA stability, mRNA transport and localization, splicing, and polyadenylation (Qi and Pekala, 1999, Yokoyama and Hirata 2005).

Calreticulin binds to antibodies in certain sera of systemic lupus and Sjogren patients that contain anti-Ro/SSA antibodies. Systemic lupus erythematosus is associated with increased autoantibody titers against Crt, but Crt is not a Ro/SS-A antigen. Earlier papers referred to Crt as an Ro/SS-A antigen, but this was later disproven. Increased autoantibody titer against human Crt is found in infants with complete congenital heart block of both the IgG and IgM classes.

2.2.5 Structure-Function Relationships in Calnexin and Calreticulin

The three-dimensional structure of the lumenal domain of the lectin-like chaperone calnexin determined to 2.9 A resolution reveals an extended 140 A arm inserted into a beta sandwich structure characteristic of legume lectins. The extended arm is curved, forming an opening, which likely accommodates specific substrates. The glucose-binding site of calnexin is located on the surface of the globular domain, facing the extended arm. The arm is composed of tandem repeats of two proline-rich sequence motifs which interact with one another in a head-to-tail fashion. Identification of the ligand binding site establishes calnexin as a monovalent lectin, providing insight into the mechanism by which the calnexin family of chaperones interacts with monoglucosylated glycoproteins. The globular domain of calnexin also contains a Ca2+ binding site and one disulfide bond. Another disulfide bond is located near the tip of the extended arm (Schrag et al. 2001). A model of calreticulin 3D structure predicts that the N-domain of calreticulin is globular and contains a glucose binding site and a disulfide bridge. The P-domain is also predicted to contain the unusual extended arm structure identified in calnexin. The globular N-domain together with the extended arm P-domain of calreticulin and calnexin may form a functional "protein-folding module". The C-terminal region of calreticulin, which is highly acidic, binds Ca2+ with high capacity and is involved in Ca^{2+} storage in the lumen of the ER in vivo (Nakamura et al. 2001b). Ca^{2+} binding to calreticulin and, consequently, changes in the ER Ca^{2+} storage capacity, affect the protein's chaperone function and thereby influence the "quality control" of the secretory pathway. Calreticulin interacts in a Ca^{2+}-dependent manner with other ER chaperones, modulating their function (Corbett et al. 2000).

Lectin-Deficient Crt Retains Full Functionality as a Chaperone: Calreticulin uses both a lectin site specific for $Glc_1Man_{5\text{-}9}GlcNAc_2$ oligosaccharides and a polypeptide binding site to interact with nascent glycoproteins. The latter mode of substrate recognition is controversial. To examine the relevance of polypeptide binding to protein folding in living cells, in Crt-deficient mutants, class I molecules exhibit inefficient loading of peptide ligands, reduced cell surface expression and aberrantly rapid export from ER. It suggested that Crt can use nonlectin-based modes of substrate interaction to effect its chaperone and quality control functions on class I molecules in living cells. Furthermore, lectin-deficient Crt bound to a similar spectrum of client proteins as wild-type Crt and dissociated with similar kinetics, suggesting that lectin-independent interactions are common place in cells that may be regulated during client protein maturation (Ireland et al. 2008).

2.2.6 Pathophysiological Implications of Calreticulin

Cardiovascular System

It is interesting that high glucose augments calreticulin expression in vascular smooth muscle cells and endothelial cells (Qi and Pekala 1999), although the mechanism and patho-physiological significance of these findings in the vascular cells has not yet been investigated. Dai et al. (1997) documented a profound inhibitory effect of intravenous administration of calreticulin on intimal hyperplasia in rat iliofemoral arteries after balloon injury in vivo. Because calreticulin can be found in extracellular locations including the blood, and it has been associated with regulation of immune responses, calreticulin has also been implicated in a number of pathological processes. The calreticulin gene knock-out study also indicates that the protein plays a role in the development of the heart (Gelebart et al. 2005; Michalak et al. 2008). It is shown that carticulin is upregulated in the heart during the middle stages of embryogenesis, whereas it is expressed at a low level after birth. Further studies are required to unravel the pathophysiological roles of calreticulin in the pathogenesis of various diseases.

Calreticulin and Cardiac Pathology: Calreticulin deficiency is embryonic lethal because it causes lesions during cardiac development (Mesaeli et al. 1999). However, overexpression of the protein in developing and postnatal heart leads to bradycardia, complete heart block and sudden death.

Ultrastructural evidence indicates that the deficiency associated with the absence of calreticulin in the heart may be due to a defect in the development of the contractile apparatus and/or a defect in development of the conductive system as well as a metabolic abnormality. Michalak et al. (2004) postulate that calreticulin and endoplasmic reticulum plays an important role in cardiac development and postnatal pathologies (Michalak et al. 2004).

Calreticulin-Deficient Mouse: Cells isolated from $Crt^{-/-}$ embryos have impaired agonist-induced Ca^{2+} release (Nakamura et al. 2001a), inhibited nuclear import of the transcription factors NF-ATc1, Mef2c and p53, modified sensitivity to apoptosis, compromised function of calnexin, and activated unfolded proteins response (UPR) 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 and exhibit severe postnatal pathology with death 7–35 days after birth (Guo et al. 2002). Calreticulin-deficient animals that have been rescued with cardiac expression of calcineurin go on to develop severe metabolic problems in cholesterol, lipid, and carbohydrate metabolism. The underlying cause of the metabolic aberrations in these mice is not understood but it indicates that many metabolic processes rely on ER function. Calreticulin expression is high in embryonic heart and declines sharply after birth, probably due to transcriptional control of the calreticulin gene. High expression of calreticulin in the heart of transgenic mice results in early postnatal death. Animals over-expressing calreticulin in the heart develop bradycardia associated with sinus node dysfunction, complete cardiac block, and death due to intractable heart failure. This indicates that calreticulin plays a role in the pathology of the heart's conductive system (Nakamura et al. 2001b).

Deletion of Crt gene leads to defects in the heart and the formation of omphaloceal. These defects could both be due to changes in the extracellular matrix composition. Matrix metalloproteinases (MMP)-2 and MMP-9 are two of the MMPs which are essential for cardiovascular remodeling and development. Wu et al. (2007) demonstrated that there is a significant decrease in the MMP-9 and increase in the MMP-2 activity and expression in $Crt^{-/-}$ deficient cells, and a significant increase in the expression of membrane type-1 matrix metalloproteinase (MT1-MMP).

Human Pregnancy and Pre-Eclampsia

Evidence indicates that pre-eclampsia involves widespread activation of maternal endothelial cells. Calreticulin has been shown to have both pro- and anti-inflammatory effects in vitro and in whole animals. In normal human pregnancy and in pre-eclampsia, there was a significant increase (5-fold) in calreticulin in plasma in term pregnant women compared with women who were not pregnant. Results indicate that calreticulin is increased in peripheral maternal blood early in pregnancy and remains elevated throughout normal gestation and that there is a further increase in calreticulin in pre-eclampsia (Gu et al. 2008). Calreticulin can be released into extracellular environment in some circumstances. For example, there is a tenfold increase in Crt in the blood of patients with systemic lupus erythematosus (Eggleton et al. 1997). The sources and roles of extracellular Crt are not clear. Nevertheless, evidence indicates that extracellular as well as intracellular Crt can also affect many cellular functions including adhesion, migration and proliferation (Bedard et al. 2005). In particular, its effects on vascular endothelial cells may be relevant to the normal pregnancy and pre-eclampsia.

Crt Interacts with HIV Envelope Protein

Calreticulin binds in vitro to a number of proteins isolated from ER. In cells expressing recombinant HIV envelope glycoprotein, gp160 bound transiently to calreticulin. The binding kinetics of calnexin and calreticulin to gp160 were very similar. Data suggested that most of the gp160 associated with calreticulin was also bound to calnexin but that only a portion of gp160 associated with calnexin was also bound to calreticulin (Otteken and Moss 1996).

Despite ER targeting and retention signals, calreticulin is also located within the nucleus where its presence increases due to its interaction with glucocorticoid receptors (Roderick et al. 1997). Therefore, Crt can inhibit steroid-regulated gene transcription by preventing receptor binding to DNA. Over-expression of Crt gene in B16 mouse melanoma cells resulted in a decrease in retinoic acid (RA)-stimulated reporter gene expression. Purified Crt inhibited the binding of endogenous RAR to a β-RA response element oligonucleotide, only if added prior to the addition of oligonucleotide. Cyclic AMP increased the expression of Crt. Cyclic AMP may act to antagonize RA action by both decreasing RAR expression and stimulating Crt levels (Desai et al. 1996).

Crt Is a Human Rheumatic Disease-Associated Autoantigen

Reports indicate that Crt is a human rheumatic disease-associated autoantigen. This protein shares an intimate relationship with Ro/SS-A autoantigen complex. Calcium ionophore, heat shock, and heavy metals such as zinc and cadmium are consistently found to increase Crt transcriptional activities in A431 cells (a human epidermoid squamous carcinoma cell line) under transient transfection conditions. Studies suggest that Crt is regulated at transcriptional level, and like some other LE-related autoantigens, Crt appears to function as a heat shock/stress-response gene (Nguyen et al. 1996). CRT was present at higher

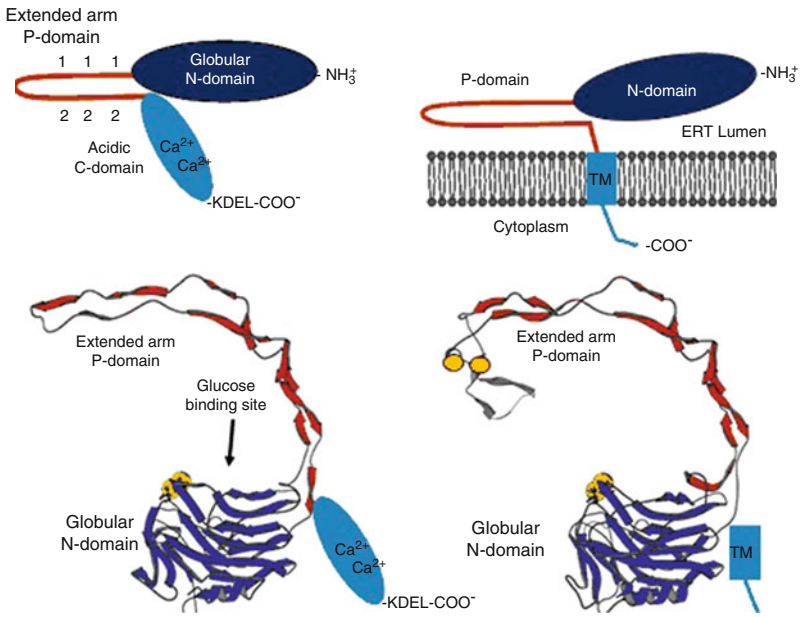

Fig 2.6 Structural models of calnexin and of calreticulin: *Left* panel shows schematic representation of calreticulin domains. *Right* panel shows a model of calnexin based on crystallographic studies of Cnx (1JHN). *Yellow* balls represent cysteines, which form an S-S bridge. Putative glucose-binding site is indicated (Adapted with permission from Schrag et al. 2001 © Elsevier and with permission from Michalak et al. 1999 Biochem J. 344: 281–97 © The Biochemical Society)

concentrations in the plasma and synovial fluid of RA patients. CRT had the capacity to bind directly to FasL, and inhibiting FasL- mediated apoptosis of Jurkat T cells, and might play a role in inhibiting apoptosis of inflammatory T cells in RA (Tarr et al. 2010a).

2.2.7 Similarities and Differences Between Cnx and Crt

Cnx performs the same service for soluble proteins as does calreticulin. Both proteins, Cnx and Crt, have the function of binding to oligosaccharides containing terminal glucose residues, thereby targeting them for degradation. Structural studies suggest that both proteins consist of a globular domain and an extended arm domain comprised of two sequence motifs repeated in tandem (Fig. 2.6). The primary lectin site of Cnx and Crt resides within the globular domain, but the results also point to a much weaker secondary site within the arm domain, which lacks specificity for monoglucosylated oligosaccharides. For both proteins, a site of interaction with ERp57 is centered on the arm domain, which retains ~50% of binding compared with full-length controls. This site is in addition to a Zn^{2+}-dependent site located within the globular domain of both proteins. Finally, calnexin and calreticulin suppress the aggregation of unfolded proteins via a polypeptide binding site located within their globular domains but require the arm domain for full chaperone function (Leach et al. 2002).

In normal cellular function, trimming of glucose residues off the core oligosaccharide added during N-linked glycosylation is a part of protein processing. If "overseer" enzymes note that residues are misfolded, proteins within the RER will re-add glucose residues so that other Calreticulin/Calnexin can bind to these proteins and prevent them from being exported from ER to Golgi. This leads these aberrantly folded proteins down a path whereby they are targeted for degradation.

Calnexin and Calreticulin at Mitochondrial Membranes: ER chaperones, particularly Ca^{2+}-binding chaperones (Cnx, Crt, and BiP), were also found to be compartmentalized at mitochondrial membranes (MM) (Hayashi and Su 2007; Myhill et al. 2008). Under physiological conditions, these chaperones serve as high-capacity Ca^{2+}-binding proteins at ER (Hendershot 2004). Calreticulin provides up to 45% of the Ca^{2+}-buffering capacity for a pool of the IP3-sensitive Ca^{2+} inside the ER (Bastianutto et al. 1995). The compart-mentalized chaperones at the MAM therefore serve as high-capacity Ca^{2+} pools in the ER. In addition, independent of its Ca^{2+}-buffering capacity in the ER, calreticulin inhibits IP3 receptor-mediated Ca^{2+} signaling by using its high-affinity-low-capacity Ca^{2+}-binding domain (Camacho and Lechleiter 1995). Further, calreticulin regulates the activity of Ca^{2+}-ATPase, providing dynamic control of ER Ca^{2+} homeostasis (Li and Camacho 2004). Calnexin

can also regulate the activity of Ca^{2+}-ATPase via a direct protein-protein interaction (Roderick et al. 2000). In addition, the activity and action of calnexin and calreticulin are regulated by other chaperones or proteins most likely occurring at the MAM of the ER (Hayashi et al. 2009a).

Cnx/Crt in MHC Class I Assembly Pathway: MHC class I molecules are ligands for T-cell receptors of $CD8^+$ T cells and inhibitory receptors of natural killer cells. Assembly of the heavy chain, light chain, and peptide components of MHC class I molecules occurs in ER. The folding and assembly of class I molecules is assisted by molecular chaperones and folding catalysts that comprise the general ER quality control system which also monitors the integrity of the process, disposing of misfolded class I molecules through ER associated degradation (ERAD). Fu et al. (2009) showed that reduced class I expression in Crt deficient cells can be restored by direct delivery of peptides into the ER or by incubation at low temperature.

Crt deficient cells exhibited a TAP-deficient phenotype in terms of class I assembly, without loss of TAP expression or functionality. In the absence of Crt, ERp57 is up-regulated, which indicates that they collaborate with each other in class I antigen processing. Specific assembly factors and generic ER chaperones, collectively called the MHC class I peptide loading complex (PLC), are required for MHC class I assembly. Calreticulin has an important role within the PLC and induces MHC class I cell surface expression. Interactions with ERp57 and substrate glycans are important for the recruitment of calreticulin into the PLC and for its functional activities in MHC class I assembly. The glycan and ERp57 binding sites of calreticulin contribute directly or indirectly to complexes between calreticulin and the MHC class I assembly factor tapasin and are important for maintaining steady-state levels of both tapasin and MHC class I heavy chains. The generic polypeptide binding sites per se are insufficient for stable recruitment of calreticulin to PLC substrates in cells. However, such binding sites could contribute to substrate stabilization in a step that follows the glycan and ERp57-dependent recruitment of calreticulin to the PLC (Del Cid et al. 2010).

Association of HLA Class I Antigen Abnormalities with Disease Progression in Malignancies: MHC class I molecules are crucial in presenting antigenic peptide epitopes to cytotoxic T lymphocytes. Proper assembly of MHC class I molecules is dependent on several cofactors, e.g. chaperones Cnx and Crt residing in ER. Lectin deficient mutants of Cnx were shown to interact with heavy chains of major MHC class I molecules in insect cells and to prevent their rapid degradation. Similarly, lectin-deficient Crt was found not only to interact with a broad spectrum of newly synthesized proteins and dissociate with normal kinetics, but it was also able to complement all MHC class I biosynthetic defects associated with Crt deficiency. MHC class Ia downregulation has been repeatedly described on melanoma cells and is thought to be involved in failure of immune system to control tumor progression. Alterations in the expression of chaperones Cnx/Crt may have important implications for MHC class I assembly, peptide loading, and presentation on the tumor cell surface and thus may contribute to immune escape phenotype of tumor cells. Metastatic melanoma lesions exhibited significant downregulation of Cnx as compared to primary melanoma lesions. In contrast, Crt was expressed in melanoma cells of primary as well as of metastatic lesions. Data suggest that chaperone-downregulation, particularly Cnx-downregulation, may contribute to the metastatic phenotype of melanoma cells in vivo. Consistently, conserved chaperone expression in metastatic melanoma lesions may be a useful criterion for selection of patients for treatment with T cell-based immunotherapies (Dissemond et al. 2004). However, mutant human cells lacking Cnx, infected with recombinant vaccinia viruses encoding mouse MHC class I molecules, K^d, K^b, K^k, D^d, D^b, and L^d, indicated that Cnx is not required for the efficient assembly of MHC class I molecules with TAP-dependent or independent peptides (Prasad et al. 1998; Mehta et al. 2008). The IFN-γ inducible proteasome subunits LMP2 and LMP7, TAP1, TAP2, Cnx, Crt, ERp57, and tapasin are strongly expressed in the cytoplasm of normal prostate cells, whereas HLA class I heavy chain (HC) and β_2-microglobulin are expressed on cell surface. Most of antigen processing machinery (APM) components was downregulated in a substantial number of prostate cancers. Thus HLA class I APM component abnormalities are mainly due to regulatory mechanisms, play a role in the clinical course of prostate cancer and on the outcome of T cell-based immunotherapies (Dissemond et al. 2004; Seliger et al. 2010).

2.2.8 Calreticulin in Invertebrates

Crt is highly conserved in eukaryotic cells, which is indicated by sequence analysis on the deduced amino acids of the known Crt cDNA clones from several mammalian species and other organisms including nematode, fruit fly (Smith, 1992), marine snail (Kennedy et al. 1992), clawed frog (Treves et al. 1992), rainbow trout (Stephen et al. 2004), and Cotesia rubecula (Zhang et al. 2006). Kennedy et al. (1992) identified Crt in Aplysia where it was enriched in presynaptic varicosities. The steady-state level of Crt mRNA in Aplysia sensory neurons increases during the maintenance phase of long-term sensitization. This mRNA increase in expression late, some time after training, is consistent with

the idea that long-term neuromodulatory changes underlying sensitization may depend on a cascade of gene expression in which the induction of early regulatory genes leads to the expression of late effector genes (Kennedy et al. 1992).

A human Ro/SS-A (Ro) autoantigen of 60-kDa, homologous to Crt and Aplysia "memory molecule" has a molecular mass, isoelectric point, and significant amino acid sequence similar to Aplysia californica snail neuronal protein 407 (McCauliffe et al. 1990). These homologies suggest that this Ro protein has a very basic cellular function(s) which may in part involve calcium binding (McCauliffe et al. 1990). The Ro autoantigens consist of at least four immunologically distinct proteins which are recognized by autoantibodies typically found in sera from patients with primary Sjogren's syndrome and in subsets of patients with lupus erythematosus. The mouse cDNA-encoded amino acid sequence was found to be 94% homologous to the human Ro sequence and is 100% homologous to murine calreticulin, a high affinity calcium-binding protein which resides in the endoplasmic and sarcoplasmic reticulum. The amino acid sequence of rabbit Crt is 92% homologous to both murine Crt and human Ro. Onchocerca volvulus and Drosophila melanogaster also have molecules that are highly homologous to human Ro.

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http://www.springer.com/978-3-7091-1064-5

Animal Lectins: Form, Function and Clinical Applications
Gupta, G.S.
2012, LIV, 1108 p. In 2 volumes, not available separately., Hardcover
ISBN: 978-3-7091-1064-5