Acta Med. Nagasaki 48 : 85－92

Review Article

A Novel Regulatory Mechanism of Apoptosis by Calreticulin, a Molecular Chaperone in the Endoplasmic Reticulum

Yoshito IHARA

Department of Biochemistry and Molecular Biology in Disease, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences

Calreticulin (CRT) is a Ca^{2+}-binding lectin-like molecular chaperone of the lumen of the endoplasmic reticulum. Recently, CRT has been revealed to be a multi-functional molecule related with glycoprotein maturation and chaperone function, Ca^{2+} homeostasis, cell adhesion, cell signaling, transcriptional regulation, and nuclear transporting mechanisms. CRT is also essential for cardiac and neural development in mice, suggesting an importance in the regulation of cell survival and death during development.

To examine the role of CRT in cardiac differentiation, we established CRT-overexpressing myocardiac H9c2 cells, and investigated the effect of the overexpression on cardiac differentiation in vitro. We found that the cells transfected with the CRT gene were highly susceptible to apoptosis compared with controls. In the gene-transfected cells, Akt/protein kinase B signaling was significantly suppressed during the differentiation. Furthermore, PP2A, a Ser/Thr protein phosphatase, was significantly upregulated implying a suppression of Akt signaling due to dephosphorylation of Akt by the upregulated PP2A via the regulation of Ca^{2+} homeostasis. Thus, overexpression of CRT promotes differentiation-dependent apoptosis in H9c2 cells by suppressing the Akt signaling pathway. Our findings demonstrate a novel mechanism in which cytoplasmic Akt signaling is modulated to cause apoptosis by a resident protein of the endoplasmic reticulum, CRT.

ACTA MEDICA NAGASAKIENSIA 48 : 85－92, 2003

Address Correspondence: Yoshito Ihara, M.D., Ph.D.
Department of Biochemistry and Molecular Biology in Disease, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
Recipient of Tsunoo Scientific Award, Nagasaki University School of Medicine, 2003
TEL: +81-95-849-7099, FAX: +81-95-849-7100
E-mail: y-ihara@net.nagasaki-u.ac.jp

Key Words: *calreticulin, endoplasmic reticulum, molecular chaperone, calcium, akt/protein kinase B, apoptosis*

Introduction

Apoptosis or programmed cell death is an important physiological process that plays a pivotal role in development and tissue homeostasis. However, apoptosis is also involved in a variety of pathological processes and conditions.[1] Numerous diseases involve too much apoptosis (e.g., neurodegenerative diseases, Parkinson's disease, Alzheimer's disease, spinal muscular atrophy, AIDS) or too little apoptosis (e.g., cancer or autoimmune diseases [type I diabetes, encephalomyelitis]). Some toxins and forms of cellular stress can also trigger apoptosis (e.g., oxidative stress, alcohol, radiation).

During the past decade research into the mechanism of apoptosis has made extensive progress. The research has revealed that apoptosis is regulated by several important molecular mechanisms involving caspases and their substrates, the Bcl-2 family and mitochondrial functions, death ligands and receptors such as the Fas receptor.[2] Although DNA damage in the nucleus and the ligation of plasma-membrane death receptors have long been recognized as initial triggers of apoptosis, other organelles including the endoplasmic reticulum (ER), lysosome, and Golgi apparatus are also major points of integration of pro-apoptotic signaling.[3]

The ER senses local stress through chaperones, Ca^{2+}-binding proteins and Ca^{2+}-releasing channels, which might transmit Ca^{2+} responses to mitochondria. The ER contains several Bcl-2-related proteins[4, 5], and Bcl-2 has been reported to express an anti-apoptotic effect at the ER.[3]

In this review, I focus on calreticulin (CRT), a unique multi-functional protein in the ER, and describe and discuss our novel finding that CRT plays a

critical role in the regulation of apoptosis in myocardiac H9c2 cells.

CRT, a Ca^{2+}-binding protein

CRT is a Ca^{2+}-binding molecular chaperone in the ER.[6, 7] CRT was first isolated as a Ca^{2+}-binding protein by Ostward and MacLennan in 1974.[8] Its cDNA was then cloned in 1989 by Smith and Koch[9] and Fliegel et al.[10] CRT is a 46 kDa protein with a cleavable N-terminal amino acid signal sequence and the C-terminal sequence Lys-Asp-Glu-Leu (KDEL), a retrieval signal in the ER. With these specific amino acid sequences, CRT is localized in the ER. It is a highly conserved protein with over 90% amino acid identity in mammals including human, rabbit, rat and mouse.[6] It is also known that calnexin (CNX), another membrane-binding homologue of CRT, shares the chaperone function in the ER.[11, 12] Based on structural and functional studies, CRT can be divided into three distinct domains; N-terminal [N], proline-rich [P], and C-terminal [C] (Fig. 1).[6] The globular N-domain of CRT has been modeled based on crystallographic data for CNX.[13] The proline-rich P-domain shows a characteristic structure with an extended and curved arm connected to a globular N-domain. The domain contains three sets of two characteristic repeats 1 (-Ileu-X-Asp-Pro-Asp/Glu-Ala-X-Lys-Pro-Glu-Asp-Trp-Asp-Asp/Glu-) and 2 (-Gly-X-Trp-X-X-Pro-X-Ileu-Asn-X-Pro-X-Y-). The N-terminal region encompassing the N and P-domain of CRT interacts with misfolded proteins and glycoproteins, binds ATP, Zn^{2+} and Ca^{2+} with high affinity and low capacity, and is likely to be involved in chaperone function of the protein.[14, 15] The C-domain binds Ca^{2+} with high capacity and plays a role in Ca^{2+} storage in the ER in vivo[6], though no structural information is available at present. The CRT gene is upregulated by ER stress such as unfolded protein responses[16] and deprivation of Ca^{2+} in the ER.[17]

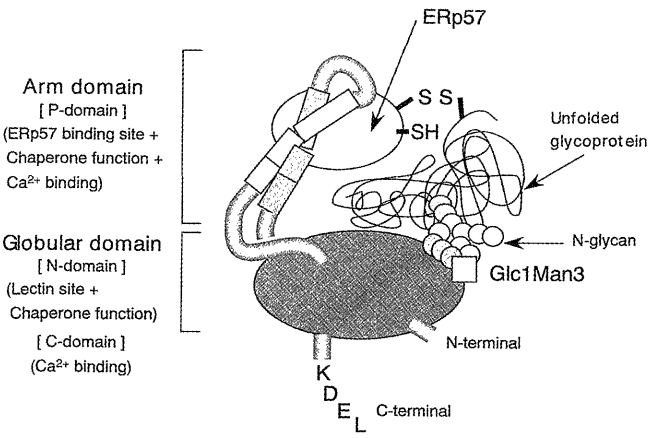

Figure 1. Schematic representation of the CRT structure.

The functions of CRT in the cell

CRT is involved in many biological processes including the regulation of glycoprotein folding, Ca^{2+} homeostasis and intracellular signaling, cell adhesion, gene expression, and nuclear transport.[6, 7]

[1] CRT, a lectin-like molecular chaperone in the ER

Molecular chaperones are the proteins that suppress the aggregation of unfolded proteins and help with the proper folding of misfolded proteins during the synthesis of new proteins or conditions of cellular stress.[18, 19] The molecular chaperone function of CRT was first reported for myeloperoxidase[20] and hemmagulutinin

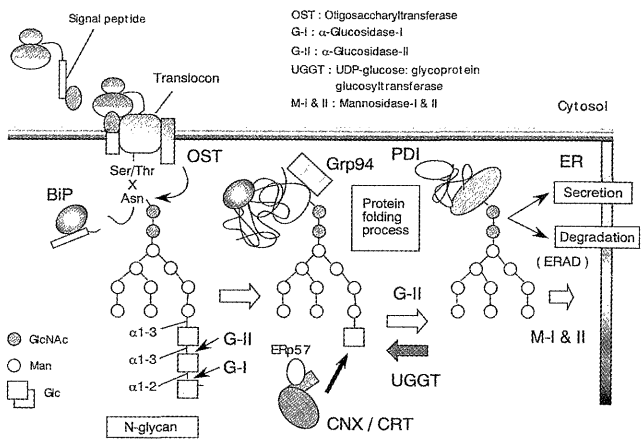

Figure 2. Molecular chaperones and folding of secretory glycoproteins in the ER.
Newly synthesized polypeptides of secretory proteins were transported into the ER through the polypeptide transporter complex, translocon. The polypeptide was properly folded in the ER with the help of molecular chaperones, such as BiP, Grp94, protein disulfide isomerase (PDI), ERp57, CRT, CNX and so forth. In the case of N-glycoproteins, the oligosaccharide of Glc3Man9GlcNAc2 is attached by oligosaccharyltranferase (OST) to the Asn residue contained in the consensus sequence Asn-X-Ser/Thr, of newly synthesized polypeptides. The oligosaccharides were processed with glucosidase-I (G-I) and II (G-II), and then CNX or CRT bound the Glc1Man5-9GlcNAc in the glycoproteins. If the glycoprotein is completely folded, the terminal glucose is removed by G-II and the glycoprotein is released from the CNX/CRT to exit the ER. However, if the glycoprotein is not properly folded, the terminal glucose is once again attached by the action of UDP-Glc: glycoprotein glucosyltransferase (UGGT). Nevertheless, if proper folding is not accomplished, the misfolded glycoprotein is transported to the cytosol for degradation by proteasomes (ER-associated degradation: ERAD).

of influenza virus[21], with various other substrates then identified for CRT-binding.[12] The molecular mechanism of the substrate-binding has been well characterized in the case of CNX, the membrane-bound homologue of CRT[22-28], and is similar to that of CRT[29-32] (Fig. 2). In the biosynthesis of N-glycoproteins in the ER, the oligosaccharide Glc3Man9GlcNAc2 is attached to the Asn residue contained in the consensus sequence Asn-X-Ser/Thr, of newly synthesized polypeptides.[33] The oligosaccharides are processed by glucosidase-I and II, and then CNX or CRT binds the Glc1Man5-9GlcNac in glycoproteins.[25, 29] The N-domain of CRT and CNX is speculated to be the oligosaccharide-binding site (lectin site).[15] If the glycoprotein is completely folded in the ER, the terminal glucose is removed by glucosidase-II and the glycoprotein is released from the CNX/CRT chaperone cycle (Fig. 2). However, if the glycoprotein is not properly folded, the terminal glucose is once again attached by the action of UDP-Glc: glycoprotein glucosyltransferase (UGGT), which discriminates between folded and unfolded substrates.[34] Together, CRT and CNX form a specific chaperone cycle for the biosynthesis of glycoproteins in the ER (Fig. 2).[33] Because of the binding-preference of CNX/CRT to oligosaccharides in the substrates, CNX and CRT are called "lectin-like chaperones". However, protein-protein interaction between CNX/CRT and substrates is suggested.[25, 26, 32] Further investigation is required to clarify the overall CNX/CRT chaperone cycle. In addition to CRT and CNX, the ER contains other molecular chaperones and related enzymes, such as BiP/Grp78, Grp94, ER protein 57 (ERp57), and protein disulfide isomerase (PDI), thought to form a chaperone network in the ER. CRT and CNX function with the help of other chaperones such as ERp57[35] or BiP/GRp78.[28] The binding site for ERp57 has been identified in the P-domain of CRT or CNX (Fig. 1).[14, 15]

[2] CRT, a regulator of Ca^{2+} homeostasis in the ER

The ER is the main reservoir of intracellular Ca^{2+} and plays an important role in Ca^{2+} homeostasis.[36] The concentration of Ca^{2+} in the ER is maintained at approximately 400 μM, which is in contrast with the level of cytoplasmic free Ca^{2+} (-100 nM).[37] CRT has two Ca^{2+}-binding sites and this characteristic contributes to the function of the ER as a Ca^{2+} reservoir. It is known that a high affinity, low capacity (Kd = 1 μM) and a low affinity, high capacity (Kd = 2 mM) Ca^{2+}-binding sites are present in the P and C-domain, respectively.[6] Ca^{2+} is released from the ER by receptors for inositol-1,4,5-trisphosphate and ryanodine, and taken up into the ER by sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase (SERCA). With respect to the regulation of Ca^{2+} level, the involvement of CRT and SERCA2b[38, 39] or the IP3-receptor[40] has been reported. Johnson et al.[39] demonstrated that CRT could bind with SERCA2b through the lectin site of CRT, and suppressed Ca^{2+}-ATPase activity under conditions of a full Ca^{2+} store in the ER. On the other hand, IP3-receptor-dependent Ca^{2+} release was suppressed in CRT-deficient MEF cells, suggesting that CRT is functionally involved in the IP3-receptor pathway. Furthermore, the store-operated Ca^{2+} release from the ER was shown to be suppressed by overexpression of CRT protein.[41-43] These findings indicate that CRT is not only a reservoir of Ca^{2+} but also a regulator of Ca^{2+}-homeostasis in the ER.

[3] Other miscellaneous functions of CRT in and out of the ER

CRT is involved in cell adhesion by affecting integrin-related cell signaling.[44] In CRT-deficient embryonic stem cells, integrin-mediated Ca^{2+} influx was impaired leading to a decrease in cell adhesion to fibronectin and laminin. It is still not clear whether CRT affects integrin directly or indirectly to regulate cell adhesion signaling. Although CRT was reported to bind to the cytoplasmic tail of integrin in vitro[45], it is not known how CRT exits the ER and is transported to the cytosol.

Cell surface expression of CRT has also been reported in various cell types, and may be related with cell adhesion and migration.[7] The removal of the C-terminal ER-retrieval signal (KDEL) by proteolysis may be one mechanism for the extracellular secretion of CRT, but the mechanism involved is still unclear. The cell surface CRT may modulate cell adhesion by binding with extracellular matrix proteins, such as fibrinogen[46], laminin[47], and thrombospondin.[48] Furthermore, it was reported that CRT or its fragments inhibited angiogenesis and suppressed tumor growth.[49] Although, this suggests some potential for use in cancer therapy, the molecular mechanism of CRT actions at the cell surface is not fully understood.

Extracellular CRT is implicated in the pathological processes of autoimmune diseases. Autoantibodies against CRT were found in approximately 40% of patients with systemic lupus erythematosus (SLE)[50], patients with secondary Sjogrens syndrome[51], rheumatoid arthritis[52], celiac disease[53], complete congenital heart block[54], and halothane hepatitis.[55] CRT is known to bind to complement, C1q, and compete with antibodies for binding to C1q and inhibition of C1q-dependent hemolysis.[56] In autoimmune diseases,

impairment of the classical pathway of compliment causes a failure to clear immune complex, resulting in progression of the disease. Therefore, extracellular CRT may contribute to the progression of autoimmune diseases by preventing clearance of immune complex. Extracellular CRT is not only an autoantigen but also an enhancer for various autoimmune diseases, and may be involved in each pathological mechanism. However, it is still controversial whether CRT is exported from necrotic cells or apoptotic cells under pathologic conditions.

Recently, it has been reported that cytosolic CRT functions as an export factor for multiple nuclear hormone receptors, such as steroid hormone, non-steroid hormone, and orphan receptors.[57, 58] This novel function of CRT is consistent with previous findings that CRT suppresses the transactivation of nuclear hormone receptors including androgen receptor[59] and vitamin D[60], and also supports the molecular mechanism. However, the mechanisms by which CRT molecules are transported into, and retained in, the cytosol/nucleus are undefined.

Biological significance of CRT in development

It was shown that CRT is essential for cardiac and neural development in mice.[40, 61] CRT gene knockout mice showed embryonic lethality at day 14.5-16.5 with insufficient development of the heart and neuronal tube. CRT is well expressed in embryonic rat heart but its expression is significantly suppressed after birth.[62] Cardiac development is believed to be regulated cooperatively by a variety of transcription factors (e.g. GATA, MEF2, HAND, COUP-TFII, Nkx2.5, TBX5, NF-ATc, Smad6, Pax3, RXR/RAR, TEF-1, WT-1, etc.).[63] Interestingly, the gene expression of CRT is known to be regulated by a transcription factor, Nkx2.5, that is involved in the regulation of the gene expression for cardiac development.[64] CRT-deficient embryonic cells showed an impaired nuclear import of nuclear factor of activated T cell (NF-AT3), a transcription factor, indicating that CRT functions in cardiac development as a component of the Ca^{2+}/calcineurin/NF-AT/GATA-4 transcription pathway.[40] Actually, cardiac-specific expression of calcineurin reversed the embryonic lethality of CRT-deficient mouse.[65]

It was also reported that CRT transgenic mice suffer a complete heart block and sudden death.[66] In that study, it was described that CRT-dependent cardiac block involves an impairment of both the L-type Ca^{2+} channel and gap junction connexins (Cx40 and Cx43). Also observed was a decrease of phosphorylated Cx43 in CRT transgenic heart, suggesting that the functions of protein kinases are altered via the regulation of Ca^{2+} homeostasis. The study indicates that the overexpression of CRT affects not the morphogenesis but the physiological function of cardiomyoblasts. The overall mechanism of the dephosphorylation of Cx43 in CRT transgenic heart cells is not known but may involve the altered regulation of protein kinase pathways. These studies suggest that CRT plays a vital role in cardiac differentiation and function, though how has not been fully clarified.

The regulation of apoptosis by CRT in myocardiac H9c2 cells

Recently, we investigated the biological role of CRT using rat cardiomyoblast H9c2 cells transfected with the CRT gene.[67] In the present study, we examined the effect of overexpression of CRT on the cardiac differentiation of H9c2 cells. When cultured in a differentiation medium containing 1% FCS and 10 nM RA, the overexpressers showed a decrease in cell number and an increase in DNA double strand breaks, indicating that they were highly susceptible to apoptosis compared with controls. We found that Akt signaling was significantly suppressed in the gene-transfected cells compared with the mock-transfected controls during the differentiation. In control cells, the phosphorylation and activity of Akt showed a gradual decline during the culture. In contrast, the decline was significantly accelerated in the gene-transfected cells. Furthermore, in the transfectants, expression of protein phosphatase 2A (PP2A), a Ser/Thr protein phosphatase, was significantly upregulated in response to the treatment, implying that the suppression of Akt signaling was due to dephosphorylation of Akt caused by the upregulated PP2A expression. Consequently, we conclude that the overexpression of CRT promotes the differentiation-dependent apoptosis of H9c2 cells through suppression of the Akt signaling pathway via upregulation of PP2A expression caused by altered C a^{2+} homeostasis. This is the first report of the Akt-mediated cell survival signaling pathway being modulated by the introduction of the CRT gene.

Apoptosis is regulated by several signaling pathways including stress-activated protein kinases (SAPKs), mitogen-activated protein kinases (MAPKs), and Akt/protein kinase B.[68] The Akt signaling pathway is a well characterized signal for cell survival which leads to the phosphorylation of BAD to dissociate Bcl-2 from its complex, to the expression of Bcl-2 through transactivation of E2F, and to the inhibition of GSK-3

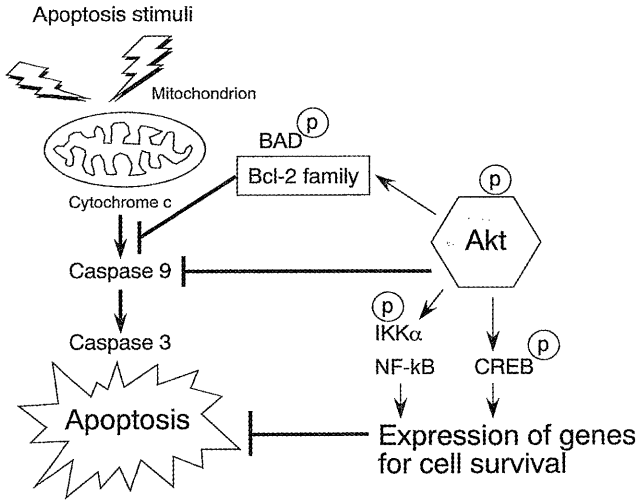

Figure 3. Regulation of cell survival signaling by Akt/protein kinase B.
Akt is a Ser/Thr kinase, the enzyme activity of which is regulated by its own phosphorylated state. Akt signaling leads to the phosphorylation of BAD to dissociate Bcl-2 from its complex, resulting in anti-apoptosis. Akt also phosphorylates caspases 9, resulting in its inactivation. Furthermore, Akt signaling leads to the phosphorylation of IKK α to activate the NFkB pathway, and also of cAMP-responsive element binding protein (CREB). In both cases, the specific genes for cell survival were upregulated to function as anti-apoptosis.

as well as caspases (Fig. 3).[69, 70] Akt is an important cell survival and anti-apoptotic signal in differentiating H9c2 cells. Although Akt is regulated by upstream signals such as phosphatidylinositol 3-kinase (PI3K) and PDK1/PDK2, the upstream signals are not influenced by overexpression of CRT during differentiation in H9c2 cells. To identify other regulators of Akt activity and signaling, we focused on protein phosphatases that could regulate the activity by dephosphorylating phosphoseryl or phosphothreonyl residues of Akt. We found that the expression and activity of PP2A were significantly increased in the gene-transfectants compared with the controls throughout the differentiation. Ser/Thr-specific PP2A is present in most eukaryotic cells and functions in a variety of processes including cell cycle regulation, cell differentiation, and signal transduction.[71, 72] PP2A is known to modulate the activities of several kinases in vitro and in vivo, such as phosphorylase kinase, the MAPKs, the calmodulin-dependent kinase, PKA, Akt/PKB, PKC, p70S6 kinase, the IkB kinase and the Cdks .[73] Akt is inactivated in vitro by PP2A and is activated in cells upon treatment with okadaic acid and calyculin A[73], suggesting that Akt is a putative substrate for PP2A. We also observed that calyculin A inhibited PP2A activity to prevent the differentiation-induced dephosphorylation and inactivation of Akt in cells transfected with the CRT gene. Collectively, these results strongly suggest that PP2A acted as a regulator for dephosphorylation and inactivation of Akt in the transfected H9c2 cells during their differentiation.

In a report by Nakamura et al.[74], overexpression of CRT resulted in increased sensitivity of HeLa cells to both thapsigargin- and staurosporine-induced apoptosis. The authors suggested that overexpression of CRT affects the communication between the ER and mitochondria to increase the sensitivity to apoptosis via the altered Ca^{2+} homeostasis, and this has recently been supported by the study of Arnaudeau et al.[75] Pinton et al.[76] reported that the releasable Ca^{2+} concentration in the ER is important for ceramide-induced apoptosis, and also showed that overexpression of CRT enhanced the ceramide-induced apoptosis in HeLa cells. Furthermore, a necrosis-promoting effect of CRT is also reported in *C. elegance*.[77] These results seem to support our findings that CRT expression positively regulates the apoptotic process under specific cellular conditions such as during cell differentiation. Oyadomari et al.[78], however, reported that the overexpression of CRT actually protects pancreatic β cells from nitric oxide-induced apoptosis, whereas Zhu and Wang[79] found that CRT antisense oligonucleotide down-regulated CRT protein production and significantly increased the sensitivity to calcium ionophore-induced apoptosis. This discrepancy may be due to the different cell types, stress stimuli, and experimental models used, but further investigation is needed into the molecular mechanism of the apoptotic process in each of these experimental models.

Previous study indicates that an enhanced expression of CRT increases the Ca^{2+} storage capacity of the ER.[6] CRT also appears to control store operated Ca^{2+} influx[41, 42, 80, 81] and to alter Ca^{2+} transport by SERCA2b.[39] Very recently, Scorrano et al.[5] has reported that Ca^{2+} reserved in the ER acts as an important gateway for apoptosis via its influence on mitochondrial Ca^{2+} homeostasis. Overexpression of CRT also influences the mitochondrial Ca^{2+} homeostasis.[75] Together, the enhanced susceptibility to apoptosis in CRT-overexpressing H9c2 cells may also be due to the modulation of mitochondrial Ca^{2+} homeostasis by the altered Ca^{2+} responses in the ER overexpressing CRT.

In our study, the intracellular concentration of free Ca^{2+} was higher in the CRT gene-transfectants than in the controls throughout the differentiation. To elucidate whether altered Ca^{2+} homeostasis could affect PP2Aca expression and Akt signaling, the effect of Ca^{2+} modulators on the expression and signaling was tested. The results showed that the PP2Ac α expression

increased to suppress the Akt signaling on treatment with thapsigargin and ionomycin, which increased the level of intracellular Ca^{2+}. Furthermore, upon treatment with BAPTA-AM, which decreases intracellular Ca^{2+} levels, PP2Ac α expression decreased to enhance the Akt signaling. These results strongly suggest that PP2Ac α gene expression is controlled by intracellular Ca^{2+} levels and homeostasis. The gene structure and regulation of PP2A has been elucidated in humans and rats. In both PP2Ac α genes, the promoter region is rich in GC and lacks TATA and CCAAT sequences, consistent with a housekeeping gene.[82, 83] The PP2Ac α gene contains several Sp1 binding sites and a potential cAMP-responsive element (CRE). CRE is regulated by a Ca^{2+}-regulated transcription factor, CRE binding protein (CREB), through Ca^{2+}/calmodulin dependent kinases (CaMKs)[84], but no Ca^{2+}-dependent regulation of Sp1 function is known. In H9c2 cells, expression of the PP2Ac α gene is upregulated by an increase in cytoplasmic free Ca^{2+} induced by thapsigargin through the activation of CRE (Yasuoka and Ihara, unpublished data). Although elevations in Ca^{2+} act as a signal, a prolonged increase in the concentration of Ca^{2+} can be lethal.[85] Moreover, transcription factors are activated differently by the amplitude and duration of the response to Ca^{2+}.[86] Therefore, overexpression of CRT may affect the transcriptional regulation of PP2Ac α mainly via the regulation of Ca^{2+} homeostasis in H9c2 cells.

CRT functions as a molecular chaperone in the ER. This chaperone function may also contribute to the differentiation-induced apoptosis of H9c2 cells overexpressing CRT by affecting the ER stress signaling pathway. When the ER is under stress, resident kinases such as IRE1 and PERK are activated to produce stress signals through a change in the luminal environment, for example, an accumulation of unfolded proteins in the ER.[87] Urano et al.[88] demonstrated that IRE1 activates c-Jun amino terminal kinase (JNK) in response to ER stress. This strongly suggests that endogenous signals initiated in the ER modulate cytoplasmic signal transduction cascades. Nakagawa et al.[89] reported that caspase-12 is activated in the ER specifically in response to ER stress. Therefore, it is also possible that a pathway containing caspase-12 is involved in the differentiation-induced apoptosis of H9c2 cells overexpressing CRT. Recently, we have found that overexpression of CRT promotes apoptosis in H9c2 cells under oxidative stress due to H_2O_2, and that the apoptosis is triggered by an increase of cytoplasmic free Ca^{2+} through enhancement of the inactivation and degradation of SERCA2a via increased interaction with CRT under the stress (Ihara et al., unpublished results). The enhanced chaperone-like interaction between CRT and SERCA2a under stress may be part of the cause of altered Ca^{2+} homeostasis and subsequent activation of signaling cascade of apoptosis. Finally, a working hypothesis for the mechanism of apoptosis regulated by CRT is indicated in Fig. 4.

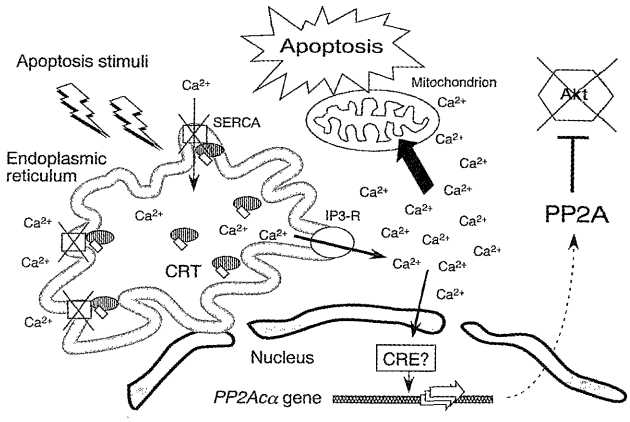

Figure 4. Working hypothesis for the apoptosis in CRT-overexpressing H9c2 cells.
In CRT-overexpressing cells, Ca^{2+} homeostasis in the ER is changed to increase cytosolic free Ca^{2+} and load Ca^{2+} into mitochondria under stress conditions such as oxidative stress. This causes the activation of Ca^{2+}-dependent transcription of specific genes such as the PP2Ac α gene. Upregulation of PP2A expression leads to the suppression of Akt signaling, resulting in an increase in susceptibility to apoptosis. Alternatively, Ca^{2+} overload in the mitochondria also triggers apoptosis.

Conclusion

When overexpressed, CRT modulates Akt signaling to promote differentiation-induced apoptosis in H9c2 cells. CRT is essential for cardiac development, and its expression is strictly downregulated in mature cardiomyocytes. As CRT functions to promote apoptosis, it may have some important physiological function in the process of cardiogenesis. Although further investigation of the correlation between CRT expression and apoptotic signals is required, our recent study has revealed a novel pathway of cellular signaling for apoptosis and its regulation via a change in the ER luminal environment and Ca^{2+} homeostasis.

Acknowledgements

I thank Drs. T. Kondo and K. Kageyama for fruitful collaboration in the present study.

References

1. Lawen A: Apoptosis-an introduction. *Bioessays* 25: 888-896, 2003.
2. Zimmermann KC, Bonzon C, and Green DR: The machinery of programmed cell death. *Pharmacol Therapeutics* 92: 57-70, 2001.
3. Ferri KF and Kroemer G: Organelle-specific initiation of cell death pathways. *Nature Cell Biol* 3: E255-E263, 2001.
4. Zong W-X, Li C, Hatzivassiliou G, et al: Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. *J Cell Biol* 162: 59-69, 2003.
5. Scorrano L, Oakes SA, Opferman JT, et al: Bax and Bak regulation of endoplasmic reticulum Ca^{2+}: A control point for apoptosis. *Science* 300: 135-139, 2003.
6. Michalak M, Corbett EF, Mesaeli N, Nakamura K, and Opas M: calreticulin: one protein, one gene, many functions. *Biochem J* 344: 281-292, 1999.
7. Johnson S, Michalak M, Opas M, and Eggleton P: Ins and outs of calreticulin: from the ER lumen to the extracellular space. *Trends Cell Biol* 11: 122-129, 2001.
8. Ostwald TJ and MacLennan DH: Isolation of a high affinity calcium binding protein from sarcoplasmic reticulum. *J Biol Chem* 249: 974-979, 1974.
9. Smith MJ and Koch GL: Multiple zones in the sequence of calreticulin (CRP55, calregulin, HACBP), a major calcium binding ER/SR protein. *EMBO J* 8: 3581-3586, 1989.
10. Fliegel L, Burns K, MacLennan DH, Reithmeier RA, and Michalak M: Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. *J Biol Chem* 264: 21522-21528, 1989.
11. Williams DB: Calnexin: a molecular chaperone with a taste for carbohydrate. *Biochem Cell Biol* 73: 123-132, 1995.
12. Helenius A, Trombetta ES, Hebert DN, and Simons JF: Calnexin, calreticulin and the folding of glycoproteins. *Trends Cell Biol* 7: 193-200, 1997.
13. Schrag JD, Bergeron JJ , Li Y, et al: The structure of calnexin, and ER chaperone involved in quality control of protein folding. *Mol Cell* 8: 633-644, 2001.
14. Ellgaard L and Helenius A: ER quality control: towards an understanding at the molecular level. *Curr Opin Cell Biol* 13: 431-437, 2001.
15. Schrag JD, Procopio DO, Cygler M, Thomas DY, and Bergeron JJ: Lectin control of protein folding and sorting in the secretory pathway. *Trends Biochem Sci* 28: 49-57, 2003.
16. Yoshida H, Haze K, Yanagi H, Yura T, and Mori K: Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. *J Biol Chem* 273: 33741-33749, 1998.
17. Waser M, Mesaeli N, Spencer C, and Michalak M: Regulation of calreticulin gene expression by calcium. *J Cell Biol* 138: 547-557, 1997.
18. Hartl FU: Molecular chaperones in cellular protein folding. *Nature* 381: 571-580, 1996.
19. Ellgaard L and Helenius A: Quality control in the endoplasmic reticulum. *Nature Rev Mol Cell Biol* 4: 181-191, 2003.
20. Nauseef WM, McCormick SJ, and Clark RA: Calreticulin functions as a molecular chaperone in the biosynthesis of myeloperoxidase. *J Biol Chem* 270: 4741-4747, 1995.
21. Peterson JR, Ora A, Van PN, and Helenius A: Transient, lectin-like association of calreticulin with folding intermediates of cellular and viral glycoproteins. *Mol Biol Cell* 9: 1173-1184, 1995.
22. Ou W-J, Cameron PH, Thomas DY, and Bergeron JJM: Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature* 364: 771-776, 1993.
23. Hammond C, Braakman I, and Helenius A: Role of N-linked oligosaccharides, glucose trimming and calnexin during glycoprotein folding in the endoplasmic reticulum. *Proc Natl Acad Sci USA* 91: 913-917, 1994.
24. Hebert DN, Foellmer B, and Helenius A: Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell* 81: 425-433, 1995.
25. Ware FE, Vassilakos A, Peterson PA, et al: The molecular chaperone calnexin binds Gla1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoproteins. *J Biol Chem* 270: 4697-4704, 1995.
26. Ihara Y, Cohen-Doyle MF, Saito Y, and Williams DB: Calnexin discriminates between protein conformational states and functions as a molecular chaperone in vitro. *Mol Cell* 4: 331-341, 1999.
27. Danilczyk UG and Williams DB: The lectin chaperone calnexin utilizes polypeptide-based interactions to associate with many of its substrates in vivo. *J Biol Chem* 276: 25532-25540, 2001.
28. Stronge VS, Saito Y, Ihara Y, and Williams DB: Relationship between calnexin and BiP in suppressing aggregation and promoting refolding of protein and glycoprotein substrates. *J Biol Chem* 276: 39779-39787, 2001.
29. Spiro RG, Zhu Q, Bhoryoo V, and Soling HD: Definition of the lectin-like properties of the molecular chaperone, calreticulin, and demonstration of its copurification with endomannosidase from rat liver Golgi. *J Biol Chem* 271: 11588-11594, 1996.
30. Wada I, Imai S, Kai M, Sakane F, and Kanoh H: Chaperone function of calreticulin when expressed in the endoplasmic reticulum as the membrane-anchored and soluble forms. *J Biol Chem* 270: 20298-20304, 1995.
31. Vassilakos A, Michalak M, Lehrman M, and Williams DB: Oligosaccharide binding characteristics of the molecular chaperone calnexin and calreticulin. *Biochemistry* 37: 3480-3490, 1998.
32. Saito Y, Ihara Y, Leach MR, Cohen-Doyle MF, and Williams DB: Calreticulin functions in vitro as a molecular chaperone for both glycosylated and unglycosylated proteins. *EMBO J* 18: 6718-6729, 1999.
33. Helenius A and Aebei K: Intracellular functions of N-linked glycans. *Science* 291: 2364-2369, 2001.
34. Parodi AJ: Protein glucosylation and its role in protein folding. *Annu Rev Biochem* 69: 69-93, 2000.
35. High S, Lecomte FJL, Russel SJ, Abell BM, Oliver JD: Glycoprotein folding in the endoplasmic reticulum: a tale of three chaperones? *FEBS Lett* 476: 38-41, 2000.
36. Berridge MJ: The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium* 32: 235-249, 2002.
37. Miyawaki A, Llopis J, Heim R, et al: Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* 388: 882-887, 1997.
38. Camacho P and Lechleiter JD: Calreticulin inhibits repetitive intracellular Ca^{2+} waves. *Cell* 82: 765-771, 1995.
39. John LM, Lechleiter JD, and Camacho P: Differential modulation of SERCA2 isoforms by calreticulin. *J Cell Biol* 142: 963-973, 1998.
40. Mesaeli N, Nakamura K, Zvaritch E, et al: Calreticulin is essential for cardiac development. *J Cell Biol* 144: 857-868, 1999.
41. Bastianutto C, Clementi E, Codazzi F, et al: Overexpression of calreticulin increases the Ca^{2+} capacity of rapidly exchanging Ca^{2+} stores and reveals aspects of their lumenal microenvironment and function. *J Cell Biol* 130: 847-855, 1995.
42. Mery L, Mesaeli N, Michalak M, et al: Overexpression of calreticulin increases intracellular Ca^{2+} storage and decreases store-operated Ca^{2+} influx. *J Biol Chem* 271: 9332-9339, 1996.
43. Llewelyn Roderick H, Llewelyn DH, Campbell AK, and Kendall JM: Role of calreticulin in regulating intracellular Ca^{2+} storage and capacitative Ca^{2+} entry in HeLa cells. *Cell Calcium* 24: 253-262, 1998.
44. Coppolino MG, Woodside MJ, Demaurex N, et al: Calreticulin is essential for integrin-mediated calcium signaling and cell adhesion. *Nature* 386: 843-847, 1997.
45. Rojiani MV, Finlay BB, Gray V, and Dedhar S: In vitro interaction of a polypeptide homologous to human Ro/SS-A antigen (calreticulin) with a highly conserved amino acid sequence in the cytoplasmic domain of integrin a subunits. *Biochemistry* 30: 9859-9866, 1991.
46. Gray AJ, Park PW, Broekelmann TJ, et al: The mitogenic effects

of the B beta chain of fibrinogen are mediated through cell surface calreticulin. *J Biol Chem* 270: 26602-26606, 1995.
47. White TK, Zhu Q, and Tanzer ML: Cell surface calreticulin is a putative mannoside lectin which triggers mouse melanoma cell spreading. *J Biol Chem* 270: 15926-15929, 1995.
48. Goicoechea S, Orr AW, Pallero MA, Eggleton P, and Murphy-Ullrich JE: Thrombospondin mediates focal adhesion disassembly through interactions with cell surface calreticulin. *J Biol Chem* 275: 36358-36368, 2000.
49. Pike SE, Yao L, Jones KD, et al: Vasostatin, a calreticulin fragment, inhibits angiogenesis and suppresses tumor growth. *J Exp Med* 188: 2349-2356, 1998.
50. Boehm J, Orth T, Van Nguyen P, and Soling HD: Systemic lupus erythematosus is associated with increased auto-antibody titers against calreticulin and grp94, but calreticulin is not the Ro/SS-A antigen. *Eur J Clin Invest* 24: 248-257, 1994.
51. Eggleton P, Ward FJ, Johnson S, et al: Fine specificity of autoantibodies to calreticulin: epitope mapping and characterization. *Clin Exp Immunol* 120: 384-391.
52. Verreck FA, Elfelink D, Vermeulen CJ, et al: DR4Dw4/DR53 molecules contain a peptide from the autoantigen calreticulin. *Tissue Antigens* 45: 270-275, 1995.
53. Tuckoval L, Karska K, Walters JR, et al: Anti-gliadin antibodies in patients with celiac disease cross-react with enterocytes and human calreticulin. *Clin Immunol Immunopathol* 85: 289-296, 1997.
54. Orth T, Dorner T, Meyer Zum Buschenfelde KH, and Mayet WJ: Complete congenital heart block is associated with increased autoantibodies against calreticulin. *Eur J Clin Invest* 26: 205-215, 1996.
55. Gut J, Christen U, and Huwyler J: Mechanism of halothane toxicity: novel insights. *Pharmacol Ther* 58: 133-155, 1993.
56. Kovacs H, Campbell ID, Strong P, et al: Evidence that C1q binds specifically to CH2-like immunoglobulin g motifs present in the autoantigen calreticulin and interferes with complement activation. *Biochemistry* 37: 17865-17874, 1998.
57. Holaska JM, Black BE, Love DC, et al: Calreticulin is a receptor for nuclear export. *J Cell Biol* 152: 127-140, 2001.
58. Black BE, Holaska JM, Rastinejad F, and Paschal BM: DNA binding domains in diverse nuclear receptors function as nuclear export signals. *Curr Biol* 11: 1749-1758, 2001.
59. Burns K, Duggan B, Atkinson EA, et al: Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature* 367: 476-480, 1994.
60. Dedhar S, Rennie PS, Shago M, et al: Inhibition of nuclear hormone receptor activity by calreticulin. *Nature* 367: 480-483, 1994.
61. Rauch F, Prud'homme J, Arabian A, Dedhar S, and St-Arnaud R: Heart, brain, and body wall defects in mice lacking calreticulin. *Exp Cell Res* 256: 105-111, 2000.
62. Imanaka-Yoshida K, Amitani A, Ioshii SO, et al: Alteration of expression and distribution of the Ca^{2+}-storing proteins in endo/sarcoplasmic reticulum during differentiation of rat cardiomyocytes. *J Mol Cell Cardiol* 28: 553-562, 1996.
63. Srivastava D and Olson EN: A genetic blueprint for cardiac development. *Nature* 407: 221-226, 2000.
64. Guo L, Lynch J, Nakamura K, et al: COUP-TF1 antagonizes Nkx2.5-mediated activation of the calreticulin gene during cardiac development. *J Biol Chem* 276: 2797-2801, 2001.
65. Guo L, Nakamura K, Lynch J, et al: Cardiac-specific expression of calcineurin reverses embryonic lethality in calreticulin-deficient mouse. *J Biol Chem* 277: 50776-50779, 2002.
66. Nakamura K, Robertson M, Liu G, et al: Complete heart block and sudden death in mice overexpressing calreticulin. *J Clin Invest* 107: 1245-1253, 2001.
67. Kageyama K, Ihara Y, Goto S, et al: Overexpression of calreticulin modulates protein kinase B/Akt signaling to promote apoptosis during cardiac differentiation of cardiomyoblast H9c2 cells. *J Biol Chem* 277: 19255-19264, 2002.
68. Ballif BA and Blenis J: Molecular mechanism mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Differentiation* 12: 397-408, 2001.
69. Coffer PJ, Jin J, and Woodgett JR: Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase mediator. *Biochem J* 335: 1-13, 1998.
70. Nicholson KM and Anderson NG: The protein kinase B/Akt signaling in human malignancy. *Cell Signaling* 14: 381-395, 2002.
71. Janssens V and Goris J: Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signaling. *Biochem J* 353: 417-439, 2001.
72. Sontag E: Protein phosphatase 2A: the Trojan horse of cellular signaling. *Cell Signaling* 13: 7-16, 2001.
73. Millward TA, Zolnierowicz S, and Hemmings BA: Regulation of protein kinase cascades by protein phosphate 2A. *Trends Biochem Sci* 24: 186-191, 1999.
74. Nakamura K, Bossy-Wetzel E, Burns K, et al: Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. *J Cell Biol* 150: 731-740, 2000.
75. Arnaudeau S, Frieden M, Nakamura K, et al: Calreticulin differentially modulates calcium aptake and release in the endoplasmic reticulum and mitochondria. *J Biol Chem* 277: 46696-46705, 2002.
76. Pinton P, Ferrari D, Rapizzi E, et al: The Ca^{2+} concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action. *EMBO J* 20: 2690-2701, 2001.
77. Xu K, Tavernarakis N, and Driscoll M: Necrotic cell death in C. elegans requires the function of calreticulin and regulators of Ca^{2+} release from the endoplasmic reticulum. *Neuron* 31: 957-971, 2001.
78. Oyadomari S, Takeda K, Takiguchi M, et al: Nitric oxide-induced apoptosis in pancreatic b cells is mediated by the endoplasmic reticulum stress pathway. *Proc Natl Acad Sci USA* 98: 10845-10850, 2001.
79. Zhu N and Wang Z: Calreticulin expression is associated with androgen regulation of the sensitivity to calcium ionophore-induced apoptosis in LNCaP prostate cancer cells. *Cancer Res* 59: 1896-1902, 1999.
80. Fasolato C, Pizzo P, and Pozzan T: Delayed activation of the stored-operated calcium current induced by calreticulin overexpression in RBL-1 cells. *Mol Biol Cell* 9: 1513-1522, 1998.
81. Xu W, Longo FJ, Wintermantel MR, et al: Calreticulin modulates capacitative Ca^{2+} influx by controlling the extent of inositol 1,4,5-triphosphate-induced Ca^{2+} store depletion. *J Biol Chem* 275: 36676-36682, 2000.
82. Khew-Goodall Y, Mayer RE, Maurer F, Stone SR, and Hemmings BA: Structure and transcriptional regulation of protein phosphatase 2A catalytic subunit genes. *Biochemistry* 30: 89-97, 1991.
83. Kitagawa Y, Shima H, Sasaki K, and Nagao M: Identification of the promoter region of the rat protein phosphatase 2Aa gene. *Biochim Biophys Acta* 1089: 339-344, 1991.
84. West AE, Chen WG, Dalva MB, et al: Calcium regulation of neuronal gene expression. *Proc Natl Acad Sci USA* 98: 11024-11031, 2001.
85. Berridge MJ, Bootman MD, and Lipp P: Calcium-a life and death signal. *Nature* 395: 645-648, 1998.
86. Dolmetsch RE, Lewis RS, Goodnow CC, and Healy JI: Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature* 386: 855-858, 1997.
87. Kaufman RJ: Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 13: 1211-1233, 1999.
88. Urano F, Wang X, Bertolotti A, et al: Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287: 664-666, 2000.
89. Nakagawa T, Zhu H, Morishima N, et al: Caspase-12 mediates endoplasmic reticulum-specific apoptosis and cytotoxicity by amyloid β. *Nature* 403: 98-103, 2000.