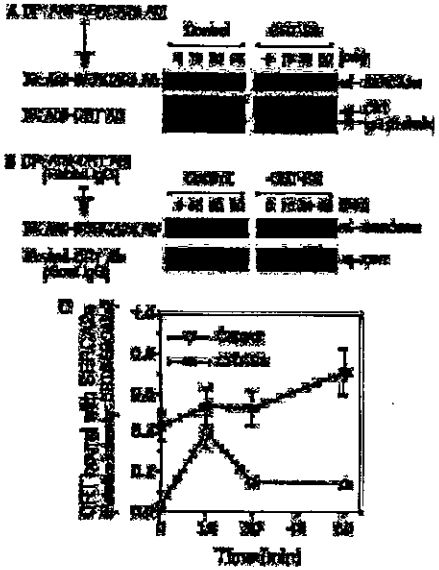

Fig. 3. The interaction between CRT and SERCA2a is increased in CRT-overexpressing H9c2 cells under oxidative stress. Control and gene-transfected (CRT-S8) cells were treated with 75 μM $H_2O_2$ for the periods indicated. (A) SERCA2a was immunoprecipitated with the specific antibody, then the immunoprecipitates were characterized by SDS-PAGE followed by immunoblot analysis using the anti-SERCA2 and CRT antibodies. (B) CRT was immunoprecipitated with the anti-CRT (C-terminal) antibody, then the immunoprecipitates were characterized by SDS-PAGE followed by immunoblot analysis using the anti-SERCA2 and CRT (N-terminal) antibodies. (C) The protein band intensity for SERCA2a and CRT in A was quantified by densitometry and the relative ratio of CRT bound to SERCA2a was expressed. Each value represents the mean ± SD of four independent experiments.

indicated that SERCA2a transiently interacted with CRT under oxidative stress. In CRT-overexpressing cells, SERCA2a was coimmunoprecipitated with anti-CRT antibody, and the interaction increased slightly during 60-min treatment with $H_2O_2$. The results were consistent with the data in Fig. 3A. Taken together, these results indicate that CRT transiently interacts with SERCA2a under oxidative stress with $H_2O_2$, but the interaction is more enhanced and sustained in the CRT-overexpressing cells than controls (Fig. 3C).

### *SERCA2a is degraded via a proteasome-dependent pathway in CRT-overexpressing H9c2 cells under oxidative stress with $H_2O_2$*

Next to investigate how the $H_2O_2$-induced degradation of SERCA2a was accelerated in CRT-overexpressing cells, the effect of protease inhibitors on the degradation profile was investigated. In Fig. 4, the effect of lactacystin, a specific inhibitor for proteasomes, on the degradation of SERCA2a was examined by immunoblot analysis in the gene-transfected cells during oxidative stress. In the presence of lactacystin (50 μM), the degradation of SERCA2a was suppressed during oxidative stress. In contrast, leupeptin (120 μM), an inhibitor for lysosomal cysteine and trypsin-like proteases [27], showed no effect on the degradation of SERCA2a during the stress. A lysosomal cysteine protease inhibitor, E64, also showed no effect on the degradation of SERCA2a during the stress (data not shown). Collectively, the results indicate that, in the CRT-overexpressing cells, SERCA2a is degraded by oxidative stress through a proteasome-dependent pathway.

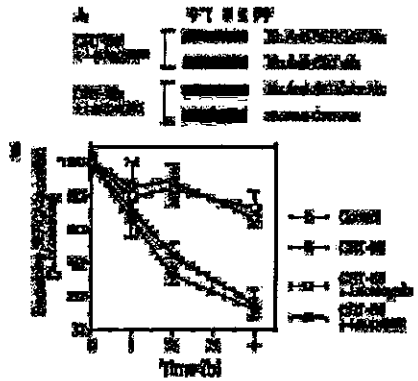

Fig. 4. Overexpression of CRT promotes proteasome-dependent degradation of SERCA2a in H9c2 cells under oxidative stress with $H_2O_2$. (A) CRT-overexpressing (CRT-S8) cells were treated with 75 μM $H_2O_2$ for the periods indicated in the presence of lactacystin (50 μM) or leupeptin (120 μM). Degradation of SERCA2a was characterized by immunoblot analysis as described in Fig. 2A. (B) Results of immunoblot analysis with the anti-SERCA2 antibody from three independent experiments for $H_2O_2$-treated control and gene-transfected (CRT-S8) cells in the presence or absence of lactacystin or leupeptin were quantified by densitometry. The average amount of SERCA2a remaining at each time point was expressed as a percentage of the initial amount of SERCA2a present in the untreated control and gene-transfected cells. Each value represents the mean ± SD of three independent experiments.

## Discussion

It is known that the transport of $Ca^{2+}$ by SERCA can be inhibited by superoxide and $H_2O_2$ in smooth muscle cells [15,16]. It seems that SERCA can be inhibited both by oxidation of its sulfhydryl residues and by a direct attack of oxidants on the ATP-binding site [17]. In the present study, we focused on SERCA2a function in the CRT-overexpressing cells under oxidative stress, because SERCA is an ER/SR resident protein which is highly susceptible to peroxide stress [16]. We found that in vitro activities of SERCA2a and $^{45}Ca^{2+}$ uptake were both suppressed by $H_2O_2$ in the microsomes from CRT-overexpressing cells compared with controls. Moreover, the degradation of SERCA2a due to oxidative stress was apparently enhanced in the cells overexpressing CRT. These indicate that the $H_2O_2$-induced inactivation and degradation of SERCA2a were accelerated by overexpression of CRT, although precise mechanism for acceleration is not known. Actually, CRT transiently interacts with SERCA2a in control cells treated with $H_2O_2$, suggesting some chaperone function of CRT for SERCA2a under the stress. In the CRT-overexpressing cells, the interaction is rather enhanced during the stress with $H_2O_2$, and then the trapped SERCA2a undergoes degradation. Although the interaction was detected in the CRT-overexpressing cells, it did not solely enhance the inactivation of SERCA2a under the non-stressed condition. Together, these suggest that, under oxidative stress, prolonged complex formation between unfolded SERCA2a and CRT may be a prerequisite for the inactivation and degradation of SERCA2a.



SERCA is composed of three homologous proteins, SERCA1, SERCA2, and SERCA3 [12]. SERCA2 has two splicing variants, SERCA2a and SERCA2b, which are specifically expressed in cardiac muscle and non-muscle tissues, respectively. Hoch et al. [28] detected a cardiac isotype SERCA2a in H9c2 cells. We also confirmed that isolated SERCA had no N-glycan by immunoblot analysis to determine the sensitivity to endo H and N-glycanase (data not shown), and concluded that the non-glycosylated form of SERCA2a was expressed in H9c2 cells. CRT is known to be a lectin chaperone which mainly functions in the folding and maturation of glycoproteins via monoglucosylated high mannose type N-glycans [2]. However, the interaction between SERCA2a and CRT under the stress may not depend on the oligosaccharides–lectin interaction, which is reported in many cases of chaperone-based interaction with CRT [3]. A peptide-based interaction of CRT with non-glycosylated proteins is reported in protein disulfide isomerase [29], ERp57 [30], and non-glycosylated peptides both in vitro and in vivo [31]. Furthermore, CRT has been shown to discriminate in its binding between native and non-native conformations of non-glycosylated proteins in vitro [32–34]. In this respect, the present study is another in vivo example of a peptide-based interaction of CRT with unfolded non-glycosylated proteins.

In the ER, misfolded proteins are translocated to the cytosol and degraded by the ubiquitin/26S proteasome pathway (ERAD: ER-associated protein degradation) [35]. After forming a complex with CRT and other chaperones, SERCA2a underwent degradation via the proteasome pathway in gene-transfected cells under oxidative stress. In ERAD, misfolded proteins are usually degraded by 26S proteasomes after polyubiquitination [35]. We also investigated the ubiquitination of SERCA2a under stress with $H_2O_2$, but could not find any increase in the polyubiquitination of immunoprecipitated SERCA2a and associated proteins under the conditions (data not shown). This may not be compatible with the typical ERAD of misfolded proteins of the ER. However, under oxidative stress, oxidized proteins are mainly degraded by 20S proteasomes, not by the ubiquitin/26S proteasome pathway [26]. These results suggest that the $H_2O_2$-dependent degradation of SERCA2a which was associated with CRT is not simply explained by a typical ERAD process.

We have shown that overexpression of CRT promotes apoptosis during cardiac differentiation in H9c2 cells [11]. In that study, we showed that Akt signaling was suppressed in H9c2 cells overexpressing CRT via an increase in the $[Ca^{2+}]_i$. In addition, we have recently reported that cAMP response element-dependent transcriptional upregulation of the protein phosphatase 2Acα gene is involved in the inactivation of Akt leading to the enhancement of oxidant-induced apoptosis in H9c2 cells under conditions in which the elevation of $[Ca^{2+}]_i$ is prolonged [36]. These suggest that overexpression of CRT modulates myocardiac functions via the alteration of $Ca^{2+}$ homeostasis in H9c2 cells. In previous reports, overexpression of CRT led to an increase in the intracellular store of $Ca^{2+}$ [2]. CRT also appears to modulate store-operated $Ca^{2+}$ influx [2,37]. However, the precise mechanism behind the altered $Ca^{2+}$ homeostasis caused by overexpression of CRT in H9c2 cells was not clear. For the differentiation of cardiomyocytes, the importance of the intracellular generation of reactive oxygen species is implicated [38]. In this respect, the altered $Ca^{2+}$ homeostasis in the CRT-overexpressing H9c2 cells during the differentiation may be related to a similar mechanism via the regulation of SERCA2a in the cells exposed to oxidative stress, although further investigation is required.

In conclusion, this study indicates that: (1) in myocardiac cells overexpressing CRT, SERCA2a is inactivated by oxidative stress, in accordance with the formation of a complex with CRT; (2) the prolonged formation of the complex promotes degradation of SERCA2a via proteasomes in the CRT-overexpressing cells under oxidative stress. These results suggest that overexpression of CRT modulates the oxidative stress response of myocardiac cells by changing the $Ca^{2+}$ homeostasis via the regulation of SERCA2a.

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