The Interplay between Calcium and the in Vitro Lectin and Chaperone Activities of Calreticulin[†]

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ABSTRACT: The ER resident protein calreticulin fulfills at least two important roles. It works as a chaperone preventing Golgi exit of non-native protein species and enhancing protein folding efficiency in either *N*-glycan-dependent, lectin chaperone, or classical chaperone, *N*-glycan-independent, modes and is one of the main calcium buffers in the cell. This last feature is independent from the lectin chaperone properties of the protein as this last activity is also observed in a CRT fragment lacking calcium buffer capacity. Here we study the interplay between calcium and the lectin and chaperone activities of CRT. The affinity of CRT for monoglucosylated glycans measured in solution by equilibrium dialysis and fluorescence anisotropy was not affected by the absence of calcium. Binding of CRT to monoglucosylated neoglycoproteins displaying either native or molten globule-like conformations was also independent of calcium concentration. Moreover, calcium and monoglucosylated glycans stabilized the CRT structure in an apparent additive, independent manner when the protein was subjected to thermal or urea-induced denaturation. In addition, the ability of CRT to decrease the level of aggregation of a chemically denatured monoglucosylated and nonglycosylated protein was also independent of calcium level.

The ER[1] is one of the most active compartments regarding protein synthesis, second only to the cytoplasm. In many aspects, the ER environment is qualitatively different from the cytoplasm, the redox potential in the ER lumen being more oxidizing and its calcium concentration being at least 2 orders of magnitude higher (*1*). In noncontractile cells, the ER is the main intracellular calcium reservoir, where the calcium concentration fluctuates from 1−5 mM when it is full to 1−50 μM upon opening of the ER channel (*2*). Most of this calcium is not free in the ER lumen but bound to proteins that act as calcium buffers, which bind calcium with low affinity and release it according to the physiological needs of the cell (*3*). The effect of ER calcium fluctuations on protein folding is unclear. While in some cases a particular protein may intrinsically need calcium to attain its native conformation, a more general effect arises from the calcium dependence of several ER chaperones and folding assisting enzymes.

A new level of complexity in protein maturation in the ER arises from *N*-glycosylation, the most frequent and drastic posttranslational modification. *N*-Glycans increase the solubility of folding intermediates, inhibit protein aggregation, and facilitate the acquisition of some elements of secondary structure (*4*). In addition to these structural roles, *N*-glycans carry information about the folding status of glycoproteins (*5*). After the initial *N*-glycan transfer and glucose trimming by the sequential action of glucosidases I and II, species exhibiting non-native conformations in the ER are reglucosylated by the UDP-Glc:glycoprotein glucosyltransferase (UGGT) that transfers a single glucose unit to high mannose-type glycans (*6*). This modification triggers the recognition of folding intermediates by two highly homologous ER-resident lectins, calreticulin (CRT) and calnexin (CNX), the former being a soluble protein in the ER lumen and the latter a membrane-bound species. As a consequence of this interaction, unfolded proteins and partially assembled oligomers are retained in the ER. Interaction of CRT or CNX with monoglucosylated glycoproteins or glycoprotein oligomers not only precludes their exit to the Golgi but also enhances folding efficiency by preventing aggregation. This interaction stops upon acquisition of native tertiary or quaternary structures as properly folded or assembled species are substrates of glucosidase II but not of UGGT. In addition to this lectin chaperone or *N*-glycan-dependent enhancement of glycoprotein folding efficiency in which CRT exclusively interacts with monoglucosylated glycans, it has been reported that CRT may also behave as a classical, *N*-glycan-

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[1] Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonic acid; CI2, chymotrypsin inhibitor 2; CI2(1−*x*)-G1M9, N-terminal fragment of CI2 composed of *x* amino acids chemically glycosylated with $Glc_1Man_9GlcNAc_2$-Asn at Cys7; CNX, calnexin; CRT, calreticulin; ER, endoplasmic reticulum; G1M9N2, $Glc_1Man_9GlcNAc_2$; IgY, immunoglobulin Y from egg yolk; PF-IgY, immunoglobulin Y from egg yolk deglycosylated with peptidyl *N*-glycanase F; PNGase F, peptidyl *N*-glycanase F; M9N2, $Man_9GlcNAc_2$; EGTA, ethylene glycol tetraacetic acid; FITC, fluorescein isothiocyanate; SBA, soybean agglutinin; sulfoSMCC, sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane 1-carboxylate; UGGT, UDP-Glc:glycoprotein glucosyltransferase.

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independent chaperone, enhancing protein folding efficiency through protein−protein interactions (*7*).

Although the main cellular location of CRT is the ER, it has also been detected in the plasma membrane and secretory granules (*8*), reflecting alternative fates of this protein once it has entered the secretory pathway. In the plasma membrane, CRT associates with CD69 (*9*), CD91 (*10*), and CD59 (*11*), to mention just a few targets. In addition, CRT modulates complement activation through its association with C1q (*12*, *13*) and the clearance of viable or apoptotic cells by activating the LDL receptor-related protein on the phagocyte (*14*). CRT is the only ER chaperone that is also localized in secretory granules of cytotoxic T lymphocytes and NK lymphocytes, where it regulates the activity of perforin (*15*−*17*). Probably, CRT can reach the plasma membrane and the secretory granules as a consequence of its inefficient retention in the ER. Most surprising are reports that find CRT in the cytosol and nucleus, in both mammalian and *Trypanosoma cruzi* cells (*18*). For instance, CRT has been implicated in the export of several steroid hormone receptors from the nucleus (*19*−*23*). In addition, CRT has been shown to modulate the stability and translational efficiency of certain mRNAs (*24*−*26*) and has been detected also in association with histones in metaphase chromosomes (*27*). One puzzling issue is the mechanism by which CRT, a typical protein of the secretory pathway, gains access to the nucleus and cytoplasm, since in principle the protein should cross a membrane to reach those locations. Two models have been proposed to explain this process. In the first one, CRT is retrotranslocated from the ER to the cytosol in a mechanism mediated by the C-terminal domain, making CRT the first mammalian protein that undergoes retrotranslocation with a fate other than being degraded by the proteasome (*28*). An alternative model proposes an inefficient translocation of CRT into the ER lumen due to its suboptimal signal peptide (*29*). Both mechanisms are probably active, and their relative importance may vary depending on the particular cell involved as well as on its physiological state.

CRT is divided in three structural domains (*30*). (i) The lectin domain located on the N-terminal half of the protein is predicted to display an antiparallel β-sheet globular fold. (ii) The arm domain forms an extended protruding loop and has been implicated in the lectin chaperone activity of CRT and in the binding to ERp57, a protein disulfide isomerase acting on glycoproteins. (iii) The C-terminal domain comprises the last 60 residues of the protein and is highly enriched in negatively charged amino acids. Besides its role in protein folding quality control, CRT is one of the main calcium buffers in the ER. This activity is located in the C-terminal domain, which can bind approximately 20 calcium ions with a low affinity ($K_d \sim 2$ mM) (*31*). It has been reported that CRT lectin chaperone activity depends on calcium (*32*), but this activity is independent of the calcium buffering capacity of the protein as it is also present in CRT fragments lacking the C-terminal domain (*33*). As extreme ER calcium concentration variations might result in faulty CRT−glycoprotein folding intermediate interactions and thence in glycoprotein aggregation, we have re-examined not only the calcium dependence of the *N*-glycan-dependent, lectin chaperone, activity of CRT but also that of the classical, *N*-glycan-independent one. By using several biophysical approaches here, we show that both CRT chaperone activities are indeed independent of calcium concentrations.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were purchased from Sigma with the exception of sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane 1-carboxylate, which was purchased from Pierce. Rat liver UGGT and UDP-[^{14}C]Glc (300 mCi/mmol) were obtained as previously described (*34*). Full-length mature CRT from rabbit endowed with a His tag at its C-terminus was expressed in *Escherichia coli* and purified according to the method of Vassilakos et al. (*32*). This protein is identical to mature CRT with the addition of the PLEQKLISEEDLNSAVDHHHHHH sequence following the KDEL C-terminal ER retention signal. CRT-ΔC was generated by deleting residues from position 343 to 392 of mature CRT by the inverse PCR method with primers 5′-TTTCATCTGCTTCTCGG-3′ and 5′-GCGGCCGCCGGCCAGGCC-3′. This protein was purified like CRT. Since lectins may carry variable calcium concentrations after purification, to homogenize the cation concentrations of all CRT preparations, prior to all measurements, CRT and CRT-ΔC were submitted to size exclusion chromatography on a Superdex 200 column developed with 10 mM MOPS, 150 mM NaCl, and 10 μM $CaCl_2$ (pH 7.5). This procedure also allowed isolation of monomeric CRT from minor amounts of aggregated CRT formed during the purification procedure. The average molecular weight of CRT was determined on a Precision Detectors PD2010 light-scattering instrument tandemly connected to a high-performance liquid chromatography system and an LKB 2142 differential refractometer. Soybean agglutinin (SBA) was purified from untoasted soybean meal by affinity chromatography on acid-treated Sepharose 6B (*35*). [^{14}C]G1M9N2 was obtained by incubating denatured SBA with purified rat liver UGGT and 1×10^6 cpm of UDP-[^{14}C]Glc (300 mCi/mmol) for 18 h at 37 °C. The labeled *N*-glycan was obtained by treating labeled SBA with Pronase and PNGase F as described below.

Expression, Purification, and Glycosylation of Chymotrypsin Inhibitor 2. CI2(1−61) and CI2(1−64) were purified and chemically glycosylated as described previously (*36*). G1M9N2-Asn was prepared from egg yolk IgY digested with Pronase and purified by Concanavaline A−Sepharose affinity chromatography followed by size exclusion chromatography with a Superdex Peptide column eluted with a 90:10 (by volume) H_2O/ethanol mixture. M9-Asn was purified from Pronase-digested SBA and purified like G1M9N2-Asn. M9N2 and G1M9N2 were obtained by PNGase F digestion of the corresponding glycopeptides and purified with a Superdex Peptide column eluted with a 90:10 (by volume) H_2O/ethanol mixture at a flow rate of 1 mL/min. The glycans eluted from this column were further purified by partition chromatography using a Glyco-Pak N column (Waters) eluted with a linear gradient from CH_3CN/H_2O (85:25, v/v) to H_2O over 120 min at a flow rate of 0.8 mL/min. Fractions containing *N*-glycans were pooled and dried in a SpeedVac.

Spectroscopic Characterization of CRT and CRT-ΔC. The lectin concentration was determined from their absorbance at 280 nm using molar absorption coefficients of 81 485 and 81 360 cm^{-1} M^{-1} for CRT and CRT-ΔC, respectively. CD

spectra were measured with a Jasco J810 spectropolarimeter and intrinsic fluorescence with an Aminco Bowman Series 2 spectrofluorimeter. Lectin was dissolved at a concentration of 1 μM in 10 mM MOPS and 150 mM NaCl (pH 7.5) with the addition of EGTA or calcium chloride.

Labeling of Glycopeptides with FITC. FITC (60 μL) dissolved in DMSO (1 mg/mL) was added to approximately 2 mg of G1M9N2-Asn or M9N2-Asn dissolved in 650 μL of 100 mM KH_2PO_4 (pH 8.0). Excess FITC was quenched after 2 h at room temperature by the addition of 80 μL of 1 M Tris-HCl (pH 8.8). Labeled glycans were purified with a Superdex Peptide column eluted with a H_2O/ethanol mixture (90:10, v/v). The labeling efficiency with respect to the original glycan that was employed was >90%. To obtain Tris-FITC, 60 μL of the DMSO solution of FITC was added to 500 μL of 200 mM Tris-HCl (pH 8.0). The product was purified using a similar procedure after 4 h at room temperature.

Binding Measurements. FITC-modified glycans (0.05 μM) or Tris-FITC (0.05 μM) was dissolved in 10 mM MOPS and 150 mM NaCl (pH 7.5) with the alternative addition of 10 mM EGTA, 10 μM $CaCl_2$, or 1 mM $CaCl_2$ in the presence of the indicated concentrations of CRT or CRT-ΔC. The fluorescence anisotropy was measured after a 2 h incubation in an Aminco Bowman Series 2 spectrofluorimeter. Glycoprotein binding was assessed with 1 or 3 μM G1M9N2-Asn-FITC and 1.1 or 3 μM lectin after incubation for 2 h with the indicated concentrations of glycosylated CI2. In both experiments, excitation and emission wavelengths of 485 and 516 nm were employed with slits of 8 and 4 nm, respectively. Binding of [^{14}C]G1M9N2 to immobilized CRT was conducted as described previously (*32*). Briefly, 500 μg of CRT was immobilized on 400 mg of Ni^{2+}-loaded IMAC matrix in 1 mL of 30 mM MOPS, 150 mM NaCl, and 10 mM $CaCl_2$ (pH 7.5). Unbound CRT was removed by washing samples four times with 1 mL of the same buffer, and three-quarters of the resin was equilibrated with 30 mM MOPS, 150 mM NaCl, and 10 mM EGTA buffer. Both portions were incubated for 30 min at 30 °C. The EGTA-containing sample was divided in three equal parts, and two portions were washed twice with 1 mL of 30 mM MOPS and 150 mM NaCl containing either 10 mM $CaCl_2$ or 10 mM $MgCl_2$. Approximately 9000 cpm of [^{14}C]G1M9N2 was added to each sample and incubated for 3 min at room temperature in the presence of 300 μL of 30 mM MOPS and 150 mM NaCl containing alternatively 10 mM $CaCl_2$, 10 mM EGTA, or 10 mM $MgCl_2$. Samples were cooled in an ice bath and centrifuged at 14 000 rpm for 1 min, and the resin was washed three times with 1 mL of buffer containing EGTA, $CaCl_2$, or $MgCl_2$ (10 mM). Radioactivity associated with the resin was measured by liquid scintillation. In parallel, the same procedure was performed with Ni^{2+}-loaded IMAC matrix without CRT as a control of specificity.

Chaperone Activity. IgY (9.5 mg/mL) purified from egg yolk was denatured with 6 M guanidinium hydrochloride and 40 mM DTT in 100 mM Tris-HCl (pH 6.8). In parallel,

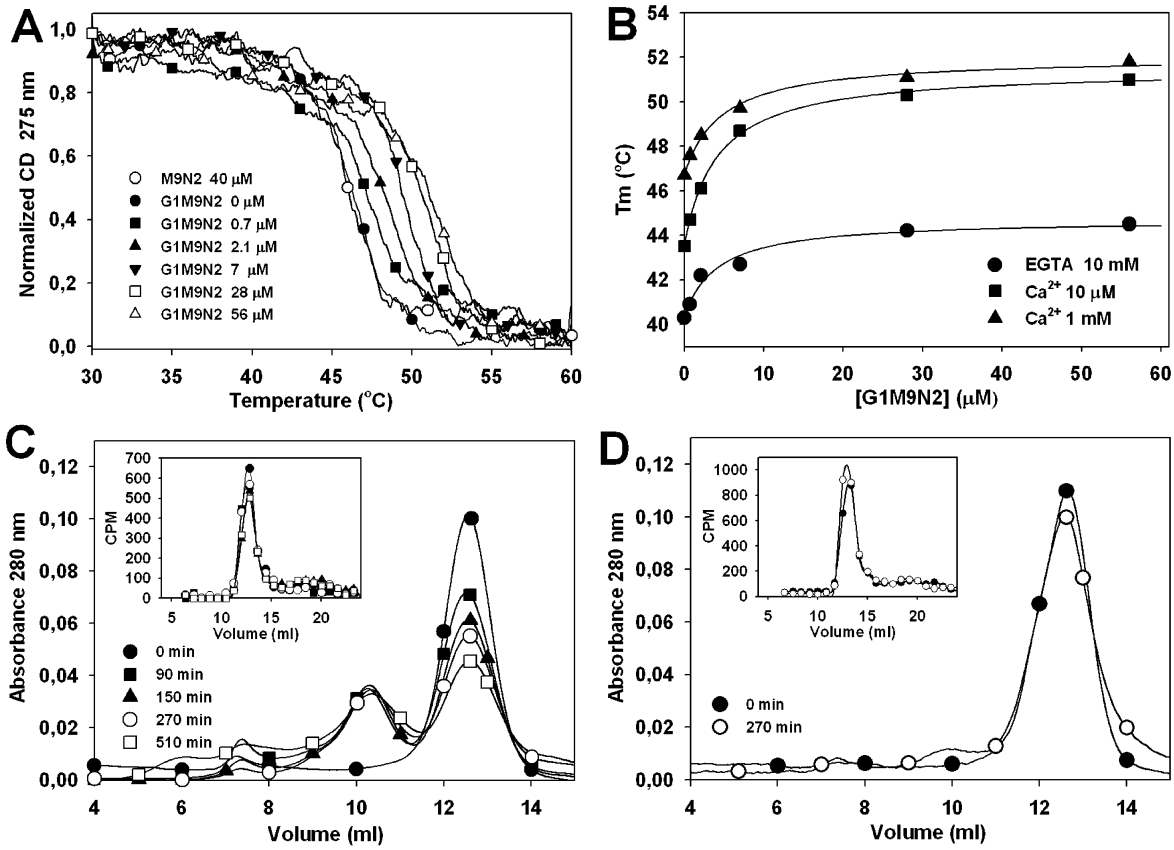

FIGURE 1: Effect of calcium and *N*-glycans on the thermal stability of CRT. (A) Thermal unfolding of CRT at 1 mM Ca^{2+} followed by CD at 275 nm with the addition of M9N2 or G1M9N2. (B) Melting temperatures of CRT measured as in panel A with an increasing concentration of G1M9N2. (C and D) Oligomerization of CRT at 37 °C in the presence of 10 mM EGTA (C) or 10 μM Ca^{2+} (D) followed by size exclusion chromatography in a Superdex 200 column. CRT was incubated for the times indicated in the figure prior to its injection into the column. The inset shows the binding of 5000 cpm of [^{14}C]G1M9N2 measured from the radioactivity that eluted from the column. The radioactivity peak eluted under both conditions at the same volume as monomeric CRT.

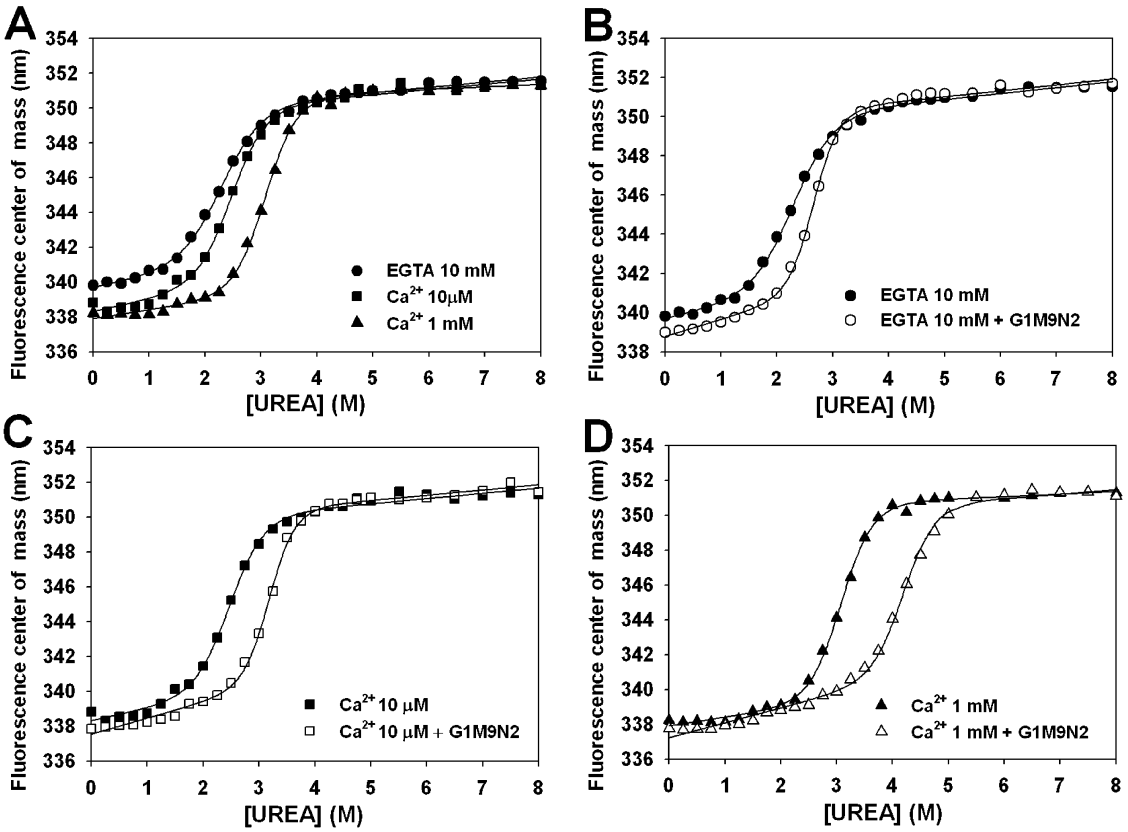

FIGURE 2: Effect of binding of calcium and *N*-glycan on urea-induced unfolding of CRT. (A) Urea-induced unfolding of 1 μM CRT at 25 °C followed by the tryptophan fluorescence center of mass. (B−D) Effect of 10 μM G1M9N2 in the urea-induced unfolding of 1 μM CRT in the presence of 10 mM EGTA (B), 10 μM Ca^{2+} (C), and 1 mM Ca^{2+} (D).

Table 1: Effect of Calcium and G1M9N2 on the Urea-Induced Unfolding of CRT

	10 μM G1M9N2	$\Delta G(H_2O)$ (kcal/mol)	M (kcal mol^{-1} M^{-1})	$[urea]_{50}$ (M)
10 mM EGTA	−	3.7	1.6	2.32
	+	6.9	2.6	2.67
10 μM Ca^{2+}	−	4.7	1.9	2.48
	+	8.1	2.6	3.19
1 mM Ca^{2+}	−	6.5	2.1	3.09
	+	8.8	2.1	4.21

IgY was deglycosylated with PNGase F following the instructions of the manufacturer (New England Biolabs). Complete deglycosylation was confirmed by SDS−PAGE. Deglycosylated IgY (PF-IgY) was denatured in the same buffer as IgY at a final concentration of 3.2 mg/mL. Aggregation was started by diluting 7 μL of IgY or 22 μL of PF-IgY in 3 mL of 10 mM Tris-HCl and 150 mM NaCl (pH 7.6) with the addition of calcium or EGTA as indicated for panel B or C of Figure 5, with or without CRT. The final concentration of both substrates was 0.5 μM monomeric H and L chain. The lectin concentrations were 0.5 and 3 μM for IgY and PF-IgY, respectively. Samples were constantly stirred at 37 °C for IgY or 31 °C for PF-IgY, and the optical density at 360 nm was followed in a Jasco V-550 spectrophotometer.

Protein Stability. Chemical denaturation was achieved by incubating 1 μM CRT for 18 h in urea containing 10 mM MOPS, 150 mM NaCl (pH 7.5), and the indicated concentrations of EGTA, $CaCl_2$, and G1M9N2. The added glycan did not affect the concentration of free calcium in solution, as measured from the effect of 30 μM G1M9N2 on the concentration of free calcium using calcium green 5N as a probe (data not shown). Fluorescence spectra were determined with the excitation wavelength set at 290 nm, and spectra were recorded from 300 to 430 nm. Excitation and emission band passes were both at 4 nm. The fluorescence spectra center of mass values were used to describe the conformational states. Thermal unfolding was followed by CD at 275 nm with a band pass of 4 nm and a temperature gradient of 0.5 °C/min. For CRT aggregation experiments, 1 mL of 1 μM CRT was incubated at 37 °C in 10 mM MOPS and 150 mM NaCl (pH 7.5) with the addition of 10 mM EGTA or 10 μM $CaCl_2$. At indicated times, 5000 cpm of [^{14}C]G1M9N2 was added to 100 μL aliquots of CRT and loaded onto a Superdex 200 column eluted with the same buffer at a flow rate of 0.8 mL/min. Protein elution was followed by monitoring the absorbance at 280 nm, and glycan was detected by liquid scintillation counting of 800 μL fractions sampled at the end of the column.

RESULTS

Glycan and Calcium Independently Stabilize CRT. The interplay between *N*-glycan and calcium was first explored through their combined effect on the CRT thermal and chemical stabilities. The CRT melting temperature was elevated from 40.1 °C at 10 mM EGTA to 46.8 °C in the presence of 1 mM calcium (Figure 1B), showing that, as previously described (*37*, *38*), calcium increases CRT stability. The addition of $Glc_1Man_9GlcNAc_2$ (G1M9N2) further stabilized CRT at all calcium concentrations that were

employed (Figure 1A,B), showing a saturable dependence with *N*-glycan concentration. The specificity of this behavior can be assessed from the fact that $Man_9GlcNAc_2$ (M9N2) had no effect on CRT thermal stability (Figure 1A).

Calcium depletion has been shown to promote the formation of oligomeric forms of CRT that exhibit peptide binding properties (*39*). We studied the lectin capacity of this species by a size exclusion chromatography binding assay with [^{14}C]-G1M9N2. Purified recombinant CRT elutes as a single peak in a Superdex 200 column with an average molecular mass of approximately 180 kDa when compared with the molecular mass standard of globular proteins. This anomalous behavior is not surprising since CRT is a highly asymmetric molecule. To verify the aggregation state of this protein, we measured its size by static light scattering and found an average molecular mass of 56 kDa, in agreement with the expected size of a monomeric species (49.5 kDa). Long-time incubations with 10 mM EGTA promoted the appearance of heavier species at 37 °C (but not at 25 °C), but only the monomeric form of CRT retained the capacity to bind *N*-glycans (Figure 1C and inset). This observation indicates that nonfunctional CRT is present in the oligomerized forms, at least from the point of view of its lectin activity. No change with time in either CRT oligomerization or its lectin capacity was observed when the experiment was carried out with 10 μM calcium (Figure 1D and inset), indicating that severe calcium depletion conditions are needed for in vitro oligomerization. Finally, we measured the effect of calcium and G1M9N2 on CRT urea-induced denaturation. Consistent with the previous experiment, calcium also stabilized CRT in this assay (Figure 2A). On the other hand, the addition of G1M9N2 stabilized the complex at all calcium concentrations that were assayed (Figure 2B−D and Table 1). These experiments support the idea that *N*-glycan−CRT interaction occurs regardless of the calcium concentration, since even at 10 mM EGTA the *N*-glycan exerted a stabilizing effect, and suggest that calcium and *N*-glycan contribute to CRT stability in an apparently additive, independent manner.

CRT Lectin Activity Does Not Depend on Calcium Concentration. CRT's calcium requirement for binding monoglucosylated *N*-glycans was originally asserted by a binding assay using the immobilized protein (*32*), a result that was reproduced here. Briefly, CRT endowed with a His tag at its C-terminal end was bound to a chelating matrix, and binding of [^{14}C]G1M9N2 was assessed from the radioactivity associated with the matrix. This parameter diminished when 10 mM EGTA was added to the medium, and subsequent calcium re-addition rendered a partial recovery of the label (Figure 3A). No radioactivity was associated with the matrix in the absence of CRT, thus ascertaining the specificity of the assay. Interestingly, addition of 10 mM magnesium to the EGTA-treated sample rendered a recovery of binding capacity similar to that observed with calcium. In addition, SDS−PAGE of the supernatant and the matrix of the sample treated with EGTA revealed that part of the protein can be displaced from the nickel-loaded column, although most of the protein remained bound (not shown). Unexpectedly, a different outcome was obtained in an equilibrium dialysis experiment performed in parallel with the same ligands (Figure 3B). The amounts of ligand bound to CRT in this assay were remarkably similar

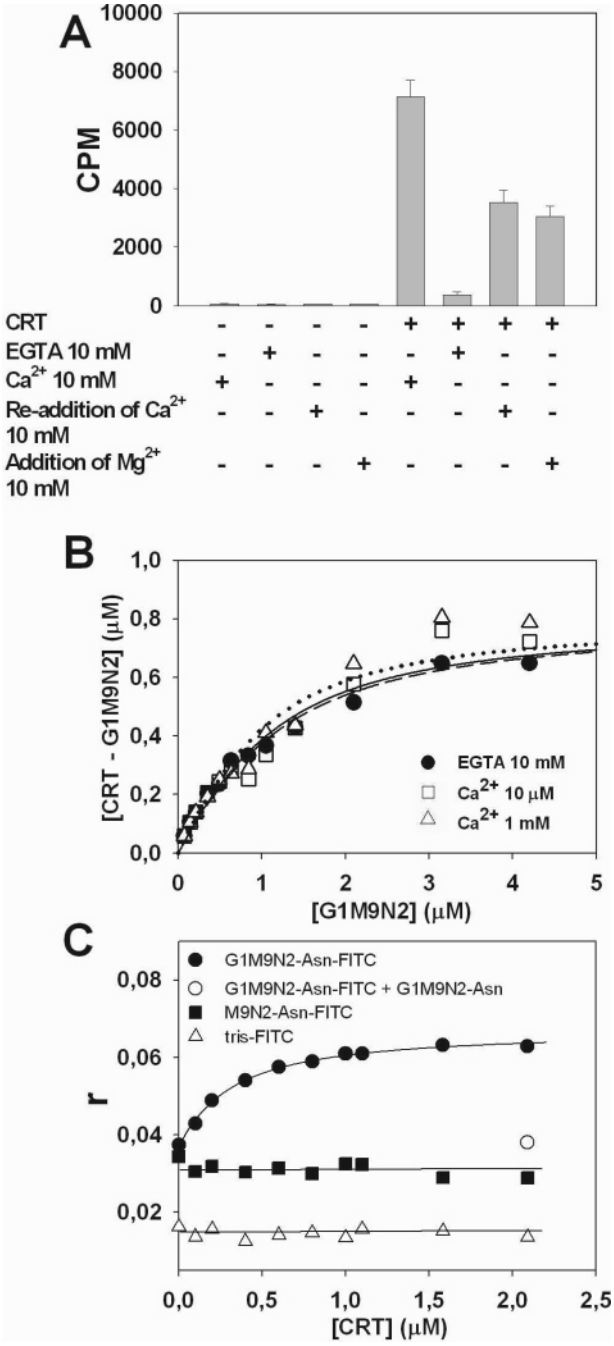

FIGURE 3: Effect of calcium on the binding of oligosaccharide to CRT. (A) Effect of EGTA, calcium, and magnesium on binding of [^{14}C]G1M9N2 to immobilized CRT on a Ni^{2+} chelating Sepharose matrix. (B) Equilibrium dialysis of G1M9N2 with 0.8 μM CRT at 25 °C using 3000 cpm of [^{14}C]G1M9N2 as a tracer. Curves are fittings of the data to a single-binding site model. (C) Fluorescence anisotropy of G1M9N2-Asn-FITC, M9N2-Asn-FITC, and Tris-FITC with increasing concentrations of CRT. The ligand concentration in all cases was fixed at 0.05 μM. The white circle indicates the addition of 30 μM G1M9N2 to the curve of G1M9N2-Asn-FITC. Binding was assessed at 25 °C in 10 mM MOPS, 150 mM NaCl, and 10 μM $CaCl_2$ (pH 7.5). Results for other conditions are listed in Table 2.

under conditions ranging from 10 mM EGTA to 1 mM calcium. To confirm this result, we developed an additional binding assay using G1M9N2-Asn modified with fluorescein isothiocyanate (FITC) at the amino group of the Asn residue. This modification allows us to detect the CRT−glycan complex by following changes in fluorescence anisotropy

Table 2: Binding of G1M9N2-Asn-FITC to CRT and CRT-ΔC

	K_d (μM)[a] for CRT		K_d (μM) for CRT-ΔC
	25 °C	37 °C	25 °C
10 mM EGTA	0.23 ± 0.06	1.6 ± 0.3	0.33 ± 0.07
10 μM Ca^{2+}	0.17 ± 0.05	1.0 ± 0.2	0.26 ± 0.05
1 mM Ca^{2+}	0.24 ± 0.06	0.9 ± 0.2	0.21 ± 0.06

[a] Data are the means ± the standard deviation of three separate experiments.

when the CRT concentration was varied. In agreement with the previous assay, the CRT−glycan complex was formed with a similar dissociation constant regardless of the calcium concentration that was employed (Figure 3C and Table 2). Dissociation constants were around 0.2 μM, a value similar to that obtained by isothermal titration calorimetry (*40*). The specificity of this assay is supported by the following observations: (i) a molar excess of G1M9N2, but not of M9N2, was able to displace the complex, and (ii) the fluorescence anisotropy of M9N2-Asn-FITC and Tris-FITC remained steady with an increase in CRT concentration (Figure 3C). These observations illustrate the importance of the terminal glucose for *N*-glycan binding and show that modification with the fluorescent probe did not induce the artifactual appearance of the complex. Similar behaviors were observed at 25 and 37 °C, although lower binding constants were obtained at 37 °C (Table 2). Results show that the CRT lectin capacity is independent of calcium concentration.

We confirmed that the lectin capacity of CRT is independent of its calcium buffering activity. CRT exhibits two calcium binding sites. The first one, located in the lectin domain, binds one calcium ion with high affinity ($K_d \sim 2$ μM), while the other site, located on the C-terminal domain, binds ~20 calcium ions with low affinity ($K_d \sim 2$ mM) (*41*, *42*). This last site was deleted as it is responsible for the calcium buffering activity of the protein. The deletion did not alter significantly the secondary structure of the protein as similar far-UV spectra were observed for the full-length and truncated protein (CRT-ΔC) (Figure 4A). In addition, dissociation constants of G1M9N2-Asn-FITC for either CRT or CRT-ΔC were very similar, and furthermore, they showed a minimal variation with calcium concentration (Figure 4B and Table 2). In addition, CRT-ΔC does not bind M9N2-Asn-FITC, and that is observed with CRT. These observations agree with results reported previously (*33*) and confirm that the C-terminal domain is dispensable for CRT lectin activity.

In Vitro Binding of CRT to Glycoproteins Exhibiting either Native or Folding Intermediate Conformations Is Independent of Calcium Concentrations. Even though calcium did not affect CRT lectin activity when assayed with *N*-glycans or *N*-glycopeptides as probes, the possibility remained that the affinity of CRT for glycoproteins could respond to the ion concentrations. To explore this possibility, we measured the affinity of CRT for glycoproteins by means of a displacement assay of the CRT−G1M9N2-Asn-FITC complex by unlabeled glycoproteins (Figure 5A). As model glycoproteins, we employed chemically glycosylated proteins (neoglycoproteins) derived from chymotrypsin inhibitor 2 (CI2), a small protein whose biophysical behavior has been extensively studied (*43*). A point mutation in CI2(E7C) allowed us to covalently attach the G1M9N2-Asn glycopeptide using a chemical cross-linker (sulfoSMCC) to generate CI2(1−64)-G1M9. This protein exhibits a native conformation highly similar to that of its unglycosylated partner. We have previously used a similar approach to explore the molecular determinants recognized by UGGT (*36*, *44*). The affinity of CRT for CI2(1−64)-G1M9 measured with either 1.1 or 3 μM CRT was identical to that measured for G1M9N2-Asn-FITC at all calcium concentrations that were assayed (Table 3). In addition, binding constants measured at 37 °C were lower than those measured at 25 °C (Table 3, columns 2 and 4) and similar to those observed for the glycopeptide. It is worth mentioning that similar affinities of CRT for G1M9N2 and IgY were measured by surface plasmon resonance (*45*). This last species is a naturally monoglucosylated glycoprotein.

To gain further insight into this issue, we used a second neoglycoprotein, CI2(1−61)-G1M9, which lacks three amino acid residues at the C-terminus. This fragment exhibits a molten globule-like conformation, displaying an increased level of binding of 8-anilinonaphthalene-1-sulfonic acid (ANS), a fluorescent probe sensitive to the exposition of hydrophobic patches. It is worth mentioning that the unglucosylated derivatives of the neoglycoproteins used as probes [CI2(1−64)-M9 and CI2(1−61)-M9] exhibited strikingly different behaviors when tested as UGGT substrates. Whereas the former one was a poor glucose acceptor, the latter was

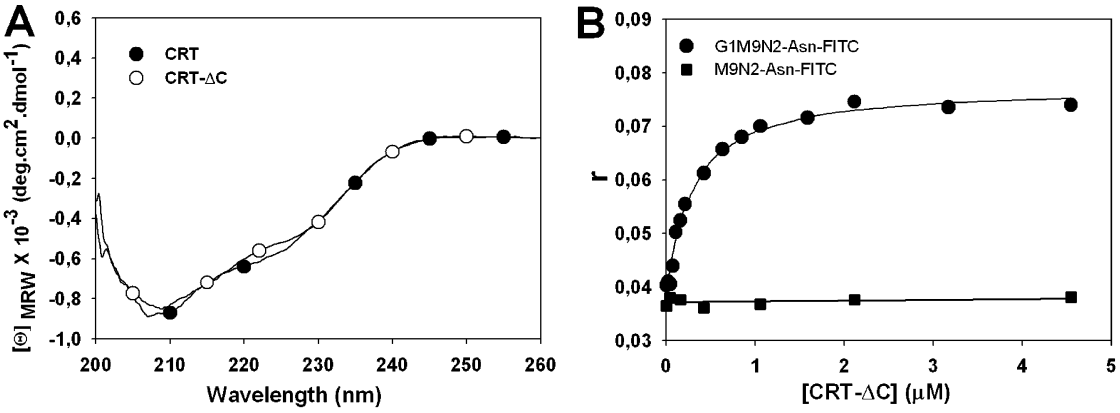

FIGURE 4: Characterization of CRT-ΔC. (A) Far-UV CD spectra of CRT and CRT-ΔC. (B) Binding of 0.05 μM G1M9N2-Asn-FITC or M9N2-Asn-FITC to CRT-ΔC followed by fluorescence anisotropy with the addition of 10 μM Ca^{2+}. Results for other conditions are listed in Table 2.

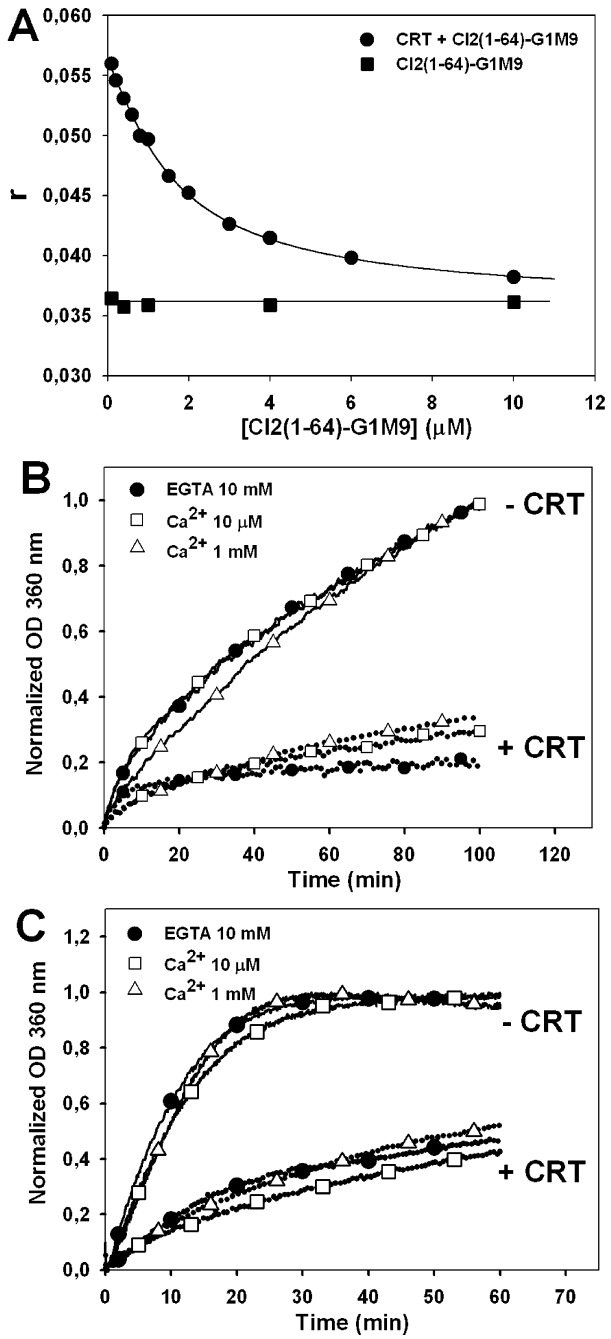

FIGURE 5: Binding of CRT to a glucosylated neoglycoprotein and chaperone activities of CRT. (A) Displacement of the CRT−G1M9N2-Asn-FITC complex by CI2(1−64)-GM9 in the presence of 1.1 μM CRT. Squares show the same experiment without CRT. (B and C) Effect of calcium on the aggregation of 0.5 μM denatured IgY (B) and PF-IgY (C) followed by the optical density at 360 nm with or without the addition of 0.5 (B) or 3 μM CRT (C). Assays were conducted in 10 mM Tris-HCl and 150 mM NaCl (pH 7.6) with the addition of calcium or EGTA as indicated, with or without CRT. The final concentration of both substrates was 0.5 μM monomeric H and L chain. The lectin concentrations were 0.5 and 3 μM for IgY and PF-IgY, respectively. Samples were constantly stirred at 37 °C for IgY or 31 °C for PF-IgY. Values at each calcium concentration were normalized to the maximal value obtained without CRT.

readily glucosylated; that is, it displayed the conformation expected for a glycoprotein folding intermediate (*36*). The affinities of CRT for CI2(1−61)-G1M9 at both 25 and 37 °C were similar to those measured for G1M9N2-Asn-FITC and CI2(1−64)-G1M9 in the presence of calcium or EGTA, indicating, first, that CRT−CI2(1−61)-G1M9 binding is independent of calcium concentration and, second, that hydrophobic protein−protein interactions do not play a major role in the binding of the molten globule-like, hydrophobic patch-exposing neoglycoprotein [CI2(1−61)-G1M9] to CRT (Table 3, columns 5−7). If hydrophobic protein−protein interactions had indeed played a role, it would have been reasonable to expect a higher affinity in the CRT−CI2(1−61)-G1M9 interaction than in that involving G1M9N2-Asn-FITC. To confirm that the observed changes in anisotropy originate from competition between ligands for CRT and not from direct binding between the neoglycoproteins and the fluorescent glycopeptides, changes in fluorescence anisotropy of G1M9N2-Asn-FITC were tested in the absence of CRT and shown to be practically undetectable (Figure 5A). It is worth noting that the absence of protein−protein interactions in the experiments described above does not absolutely preclude the occurrence of such interactions when other glycoproteins are used as model systems (see the next section).

In Vitro CRT Chaperone Activities Are Independent of Calcium Concentration. While calcium did not affect binding of CRT to glycopeptides or glycoproteins studied here, regardless of their folding status, it could have had an effect on CRT chaperone activity. To explore this issue, we measured the effect of calcium on the CRT-mediated inhibition of the aggregation of chemically denatured IgY (a glycoprotein displaying monoglucosylated glycans) and its deglycosylated form (PF-IgY). For all observed conditions, ranging from 10 mM EGTA to 1 mM calcium, CRT was able to decrease the level of IgY aggregation to a similar extent (Figure 5B). PF-IgY exhibited a stronger tendency to aggregate than IgY, probably as a consequence of the increased solubility of the latter due to the attached glycans, but CRT also decreased the level of aggregation in a calcium-independent manner (Figure 5C). It is worth remarking, however, that a 6-fold higher CRT concentration was employed with PF-IgY (Figure 5C) than with IgY (Figure 5B). Almost no effect of CRT was observed on PF-IgY aggregation when the CRT concentration employed was that used for IgY. It may be concluded, therefore, that CRT chaperone activity is not affected by calcium concentration fluctuations, for either glycosylated or nonglycosylated proteins. Since these assays were performed at 37 and 31 °C, a temperature at which CRT does not show any detectable conformational change, we can discard spurious effects due to the thermal instability of the lectin.

DISCUSSION

The ER is not only the second most active compartment in protein synthesis but also a highly dynamic intracellular calcium store. The effect of calcium on protein folding in the ER is unclear. For instance, depletion of ER calcium leads to contradictory results regarding the association of folding proteins with BiP; whereas in some cases this association is enhanced (*46*), in other cases the opposite behavior is observed (*47*). For glycoproteins, it has been proposed that retention of immature species in the ER by CRT relies on calcium.

The prevailing view that CRT lectin activity depends on calcium was established using the lectin immobilized on a

Table 3: Binding of Glycoprotein to CRT

	K_d (μM)[a] for CI2(1−64)-G1M9			K_d (μM) for CI2(1−61)-G1M9		
	25 °C, 1.1 μM CRT	25 °C, 3.0 μM CRT	37 °C, 1.1 μM CRT	25 °C, 1.1 μM CRT	25 °C, 3.0 μM CRT	37 °C, 1.1 μM CRT
10 mM EGTA	0.25 ± 0.08	0.20 ± 0.07	1.2 ± 0.3	0.30 ± 0.10	0.28 ± 0.09	1.3 ± 0.3
10 μM Ca^{2+}	0.16 ± 0.07	0.18 ± 0.05	0.7 ± 0.2	0.20 ± 0.04	0.22 ± 0.06	0.9 ± 0.2
1 mM Ca^{2+}	0.17 ± 0.08	0.24 ± 0.06	0.8 ± 0.2	0.21 ± 0.06	0.26 ± 0.08	1.0 ± 0.2

[a] Data are the means ± the standard deviation of three separate experiments.

chelating matrix (*32*). This experiment was reproduced here, and consistent with previous results, we observed that immobilized CRT failed to bind G1M9N2 when incubated with a calcium-chelating agent. Unexpectedly, we found in two different assays that glycan binding did not depend on calcium concentrations when lectin activity was measured with both components in solution. This point was further supported by the observation that even in the presence of 10 mM EGTA, G1M9N2 stabilized CRT with respect to both thermally and chemically induced denaturation. If *N*-glycan binding were impeded under this condition, one would expect no stabilization effect by the ligand. It may be speculated that in the absence of calcium, immobilized CRT (a protein rich in negatively charged amino acids) would interact with the Ni^{2+}-loaded matrix through sites other than the HIS tag, thus impairing its lectin activity. Addition of a bivalent cation like calcium may conceivably disrupt this putative interaction. In this sense, addition of magnesium rendered an output similar to that observed with calcium. Since CRT does not need magnesium for its lectin activity, the recovery of binding capacity is probably due to a nonspecific effect of divalent cations on the dynamic properties of the immobilized protein.

It has been shown that ER stress may induce conformational changes in CRT that could facilitate the recognition of peptides and proteins. Thus, calcium depletion, deletion of the C-terminal domain, or heat shock triggered the appearance of oligomeric forms of CRT exhibiting peptide binding properties, suggesting that under those conditions the role of CRT could shift from that of an unconventional lectin chaperone to that of a classical one (*39*). The quantitative study we have performed rules out this speculation at least for the neoglycoprotein system used in this study. Thus, binding constants at 25 and 37 °C for two neoglycoproteins, one of them [CI2(1−64)-G1M9] displaying a native and the other [CI2(1−61)-G1M9] a molten globule-like conformation (*36*), were similar to those measured with G1M9N2-Asn-FITC under all conditions that were assayed, including the presence of EGTA, suggesting that protein−protein interactions do not play a major role in the experimental system that was used. This does not preclude the possibility that with more hydrophobic substrates protein−protein interactions may be important. In fact, CRT can suppress the aggregation of chemically denatured PF-IgY, although a higher concentration of the lectin is needed to attain an effect similar to that observed toward the glycosylated IgY (see below). In addition, although incubation at 37 °C in the presence of 10 mM EGTA promoted the appearance of heavier forms of CRT, only the monomeric form retained the ability to bind *N*-glycans. Therefore, either oligomeric forms of CRT are the result of a functional process that facilitates a new role for certain but not all protein species but obliterates lectin activity, or they are nonfunctional intermediates in the unfolding pathway triggered by calcium depletion. Nevertheless, for CRT to undergo this oligomerization process in vitro, the calcium concentration must be maintained at an extremely low concentration for a long period of time, a situation highly unlikely to occur in a living cell. Long time incubations in vitro with EGTA eventually prompt the loss of the high-affinity calcium ion bound to the lectin domain of CRT, thus triggering its oligomerization. This process is enhanced at higher temperatures and explains the slightly lower affinities measured under these conditions for G1M9N2 and both neoglycoproteins.

Finally, the ability of CRT to suppress the aggregation of chemically denatured IgY and PF-IgY was not affected by calcium absence, thus showing that not only the lectin chaperone capacity but also the classical chaperone activities of CRT do not rely on calcium. The interplay between calcium and *N*-glycans was also studied from their contributing effects on CRT stability. In this respect, both ligands affected CRT stability in an apparently additive mode, suggesting that they independently interact with the protein. In addition, the lectin activity of CRT-ΔC was identical to that observed for full-length CRT, in agreement with previous results that showed that the C-terminal domain has no effect on glycan binding (*33*).

In summary, contrary to what has been assumed, calcium concentrations do not influence either the lectin activity or the *N*-glycan-dependent and -independent chaperone activities of CRT at least in in vitro assays.

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