Calreticulin is a lectin chaperone of the endoplasmic reticulum that interacts with newly synthesized glycoproteins by binding to Glc$_3$Man$_9$GlcNAc$_2$ oligosaccharides as well as to the polypeptide chain. In vitro, the latter interaction potently suppresses the aggregation of various non-glycosylated proteins. Although the lectin-oligosaccharide association is well understood, the polypeptide-based interaction is more controversial because the binding site on calreticulin has not been identified, and its significance in the biogenesis of glycoproteins in cells remains unknown. In this study, we identified the polypeptide binding site responsible for the in vitro aggregation suppression function by mutating four candidate hydrophobic surface patches. Mutations in only one patch, P19K/I21E and Y22K/F84E, impaired the ability of calreticulin to suppress the thermally induced aggregation of non-glycosylated firefly luciferase. These mutants also failed to bind several hydrophobic peptides that act as substrate mimetics and compete in the luciferase aggregation suppression assay. To assess the relative contributions of the glycan-dependent and -independent interactions in living cells, we expressed lectin-deficient, polypeptide binding-deficient, and doubly deficient calreticulin constructs in calreticulin-negative cells and monitored the effects on the biogenesis of glycoproteins in cells. In all cases, we observed a profound impairment in calreticulin function when its lectin domain and extended arm or P domain (3–5) with the tip of the arm domain comprising the binding site for ERp57 (6) and cyclophilin B (7). The specificity for glycoproteins resides within their lectin domains, which recognize a monoglucosylated oligosaccharide-processing intermediate of composition Glc$_3$Man$_9$GlcNAc$_2$ (8–10). Cycles of chaperone binding and release are regulated by the availability of the terminal glucose residue on this oligosaccharide with glucose removal catalyzed by glucosidase II and its readdition by UDP-glucose:glycoprotein glucosyltransferase I (11). UDP-glucose:glycoprotein glucosyltransferase I acts as the folding sensor in the cycle, only reglucosylating non-native glycoprotein conformers (12).

The importance of lectin-based interactions in mediating associations of Cnx and Crt with folding glycoproteins has been well established. Many studies have examined this issue using glucosidase inhibitors or glucosidase-deficient cells to prevent the formation of the monoglucosylated oligosaccharide from its triglucosylated precursor. In most cases, glycoprotein interactions with the chaperones are lost or greatly diminished, and in several instances this is accompanied by reduced folding efficiency and altered export rates from the ER (Refs. 1, 11, and 13–19 and references therein). Further detailed studies using class I histocompatibility molecules have shown that interactions with Crt are dependent on a functional lectin site and the presence of monoglucosylated oligosaccharide on the substrate (20, 21). The ability of these chaperones to suppress client protein aggregation appears to be due in part to their sequestration of glycoprotein folding intermediates between the globular lectin domain and the flexible extended arm domain. This is suggested on the basis of FRET experiments using probes within the two domains (22) as well as arm domain truncation mutants that exhibited reduced abilities to suppress aggregation in vitro (23, 24).

There is also substantial evidence supporting the existence of polypeptide-based interactions between Cnx or Crt and their client proteins, interactions that may also contribute to the suppression of aggregation. This is based on the finding that binding of these chaperones to a number of clients is unaltered or only modestly diminished in glucosidase-deficient cells, cells
treated with glucosidase inhibitors, or cells either expressing non-glycosylated clients or mutated to lack Asn-linked oligosaccharides (Refs. 2, 13, 19, and 25 and references therein). Furthermore, both Cnx and Crt bind non-glycosylated hydrophobic peptides in vitro (23, 24, 26) and are capable of suppressing the in vitro aggregation of non-glycosylated proteins under physiological conditions of the ER (24, 27, 28). This aggregation suppression function resides at a single site within the globular domain and can be blocked by the addition of hydrophobic peptides (23, 24). Efforts have been ongoing to localize more precisely the site responsible for aggregation suppression with most emphasis placed on Crt. Several protein binding sites have been identified on Crt such as those specific for GABARAP (29) and thrombospondin (30), but interacting peptides derived from these proteins fail to compete in aggregation suppression assays, indicative of distinct binding sites (24). Additional binding sites have been proposed in proximity to the lectin site (22, 31, 32), but these either do not exhibit the expected specificity for hydrophobic substrates (22) or would be expected to be blocked upon addition of monoglucosylated oligosaccharide (31), a treatment that does not affect the binding of hydrophobic peptides to Crt (24).

In this study, we tested four candidate hydrophobic sites on the globular domain of Crt for their involvement in suppressing client aggregation in vitro. One site, when mutated, disrupted the ability of Crt to bind hydrophobic peptides and resulted in an impaired ability of Crt to suppress the in vitro aggregation of both non-glycosylated and monoglucosylated protein clients. We then expressed this mutant in Crt-deficient cells and compared it with a previously described lectin-deficient mutant to assess the relative contribution of each site to chaperone function in living cells. Using three distinct assays, we show that, despite the clear involvement of the newly identified polypeptide binding site in vitro, it is lectin-based interactions that are critical for the functions of Crt with glycoprotein clients within the ER.

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Identification of the Polypeptide Binding Site on Calreticulin That Mediates Aggregation Suppression of Non-glycosylated Substrates—Previously, we showed that Crt suppresses the aggregation of non-glycosylated client proteins through a polypeptide binding site that resides within its globular lectin domain but at a site distinct from the site of oligosaccharide binding (24). To localize the polypeptide binding site more precisely, we turned to our crystal structure of the mouse Crt lectin domain (4) and identified four surface-exposed hydrophobic patches at locations removed from the lectin site (22, 31, 32), a treatment that does not affect the binding of hydrophobic peptides to Crt (24).

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FIGURE 1. Potential polypeptide binding sites in the lectin domain of Crt. A, locations of candidate polypeptide binding sites in the lectin domain of mouse Crt. Four hydrophobic sites (sites 1–4) were identified on the basis of surface exposure (yellow), and within each site two solvent-accessible residues were chosen for mutagenesis to polar or charged amino acids (mutated residues depicted in green). For orientation purposes, bound Glc1Man3 oligosaccharide is shown in red/blue, and residues at the beginning and end of the arm domain are in dark gray. B, aggregation suppression potency of Crt with mutations in sites 1, 3, and 4. Wild type or mutant Crt (0.5 μM) was equilibrated at 37 °C, then FFL was diluted into the reactions to a final concentration of 2 μM, and light scattering was recorded every 60 s at 370 nm.
TABLE 1

| Description | Tm<br>−G1M3 | Tm<br>+G1M3 |
|-------------|-----------|-----------|
| Wild type Crt | 42 | 47 |
| Site 2 mutant P19K/I21E | 42 | 48 |
| Site 2 mutant Y22K/F84E | 38 | 42 |
| Site 2 + lectin mutant P19K/I21E/D317A | 40 | 40 |
| Site 3 mutant I186K/V191E | 43 |
| Site 4 mutant A353E/M357E | 42 |
| Site 1 mutant Y150K/Y308Q | 43 |

mutations, although there was no change in melting temperature detected for any of these mutants (Table 1). In contrast, the P19K/I21E mutant (site 2) substantially impeded the chaperone function of Crt (Fig. 2A). Consequently, we constructed an adjacent site 2 double mutant, Y22K/F84E, and obtained similar results (Fig. 2A). Doubling the concentration of each mutant in the aggregation suppression assay resulted in potency similar to wild type Crt (Fig. 2A), suggesting that the two mutants were about 50% impaired in their aggregation suppression function. The P19K/I21E mutant was properly folded as evidenced by an unchanged circular dichroism (CD) spectrum (Fig. 2B), melting temperature (Table 1), and retention of lectin function (stabilization by Glc₃Man₃ tetrasaccharide; Table 1). The Y22K/F84E mutant exhibited some structural alterations by these criteria (Fig. 2B and Table 1). We attempted to generate several triple mutants in an effort to make variants that completely lacked aggregation suppression function, but these attempts were unsuccessful due to instability of the mutants.

To confirm that site 2 is indeed the site responsible for the aggregation suppression function of Crt, we examined site 2 mutants for their abilities to bind to the hydrophobic peptides KKAFAF and KHPYAYLAIAAEVAGTTALKLSK (herein referred to as KHP). We showed previously that these peptides competed in the FFL aggregation suppression assay by binding to the lectin site of Crt (Fig. 2A), suggesting that the correct site had been identified. Both the Lectin and Polypeptide Binding Sites of Calreticulin Participate in Suppressing the Aggregation of a Monoglycosylated Glycoprotein Substrate—To investigate the relationship between the polypeptide binding site and the lectin site of Crt in suppressing client protein aggregation, we compared the behavior of Crt constructs with mutations in either or both sites. We showed previously that the D317A mutation ablates the lectin function of Crt while maintaining native tertiary structure (33). This mutation could be combined with the polypeptide binding-deficient mutant (P19K/I21E/D317A) with no effect on its CD spectrum (Fig. 2B) and only a modest decrease in melting temperature (Table 1). The triple mutant exhibited no stabilization upon addition of Glc₃Man₃ oligosaccharide in keeping with its defect in lectin function (Table 1).

When the lectin-deficient, polypeptide binding-deficient, and combined mutants were tested for their abilities to suppress FFL aggregation, those mutants with impaired polypeptide binding exhibited reduced aggregation suppression potency (Fig. 3A). In contrast, loss of lectin function in the D317A mutant had no impact on the ability of Crt to suppress the aggregation of this non-glycosylated substrate. To determine whether both lectin and polypeptide binding sites are utilized in suppressing glycoprotein aggregation, we used jack bean α-mannosidase as substrate. This glycoprotein possesses a monoglycosylated oligosaccharide that is recognized by the lectin site of Crt and, when diluted from denaturant, aggregates very rapidly (34, 35). As shown in Fig. 3B, mutation of either the lectin site or the polypeptide binding site of Crt resulted in an impaired ability to suppress α-mannosidase aggregation. When both sites were mutated simultaneously, an even greater impairment in aggregation suppression function was observed. When the concentration of each mutant was adjusted to achieve the same degree of aggregation suppression as wild type Crt, it was observed that the polypeptide binding-deficient mutant was about 2.5-fold less potent than wild type Crt, the lectin-deficient mutant was 10-fold less potent, and the combined mutant was 20-fold less potent (Fig. 3C). These findings indicate that both lectin and polypeptide-based interactions are used by Crt to suppress the aggregation of a monoglycosylated glycoprotein in vitro.

The Polypeptide Binding Site of Crt Is Dispensable in Supporting the Biogenesis of Glycoproteins in Living Cells—We used three approaches to assess the relative roles of the lectin site and newly identified polypeptide binding site of Crt in the biogenesis of glycoproteins in cells. The first approach was based on the observation that Crt plays an important role in the biogenesis of class I molecules of the major histocompatibility complex. Crt is present in a peptide loading complex (PLC) consisting of the class I heavy chain, β₂-microglobulin, Crt, ERp57, tapasin, and the transporter associated with antigen processing (TAP). The PLC ensures that class I molecules are loaded with a spectrum of high affinity peptides prior to their delivery to the cell surface. Once at the cell surface, they are examined for the presence of foreign peptide antigens by cytotoxic T cells. In Crt-deficient K42 mouse fibroblasts, class I molecules undergo inefficient peptide loading and exhibit markedly reduced expression at the cell surface (36).

We expressed wild type Crt in K42 fibroblasts as well as the lectin-deficient, polypeptide binding-deficient, and combined mutants and then examined whether the mutants were incorporated into the peptide loading complex and whether they were capable of supporting high level expression of class I molecules at the cell surface. All constructs possessed an influenza hemagglutinin (HA) epitope tag just prior to the C-terminal KDEL ER localization sequence. As shown in Fig. 4A, the constructs were expressed at similar levels when transiently transfected into K42 cells. Consistent with previous work, wild type Crt increased the cell surface levels of the class I molecules H-2Kb and H-2Db by 4–5-fold as assessed by flow cytometry (Fig. 4B). By comparison, the lectin-deficient Crt mutant (D317A; L⁻) was ineffective in supporting increased surface expression of either class I molecule, whereas the polypeptide binding-deficient mutant (P19K/I21E; P⁺) was just as effective as wild type Crt (Fig. 4B). The combined mutant (L⁻P⁺)
behaved in the same manner as the lectin-deficient mutant. Consistent with these findings, when the PLC was immunoprecipitated with anti-tapasin antibody and immunoblotted for Crt, mutants lacking a functional lectin site were not present in the complex, whereas wild type Crt and polypeptide binding-deficient Crt were readily detected (Fig. 4C). These findings indicate that a functional lectin site is critical for incorporation of Crt into the PLC and for supporting high level expression of class I molecules at the cell surface. The polypeptide binding site does not appear to participate in these processes.

Given the important contribution of the polypeptide binding site of Crt in suppressing protein aggregation in vitro, we speculated that its apparent lack of involvement in class I biogenesis might be due to Crt functioning more to stabilize the PLC rather than suppressing the aggregation of newly synthesized class I molecules at the cell surface. The polypeptide binding site does not appear to participate in these processes.

We co-expressed the lectin and polypeptide binding-deficient mutants of Crt along with the NHK or ATZ variants in K42 cells to assess the relative roles of these sites in promoting the solubility of non-native glycoprotein substrates. Cell lysates were prepared using CHAPS detergent and separated into soluble and insoluble fractions that were subsequently immunoblotted to detect NHK (Fig. 5A) or ATZ (Fig. 5B). A substantial portion of both variants was detected in the insoluble fraction in the absence of Crt (Control lanes) with a marked increase in solubility upon expression of wild type Crt (quantified in Fig. 5C). Remarkably, when wild type Crt was replaced with the lectin-deficient, polypeptide binding-deficient, or combined mutants, only those mutants with a defective lectin site were compromised in their abilities to suppress NHK or ATZ aggregation and insolubility. These findings indicate that the poly-

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**FIGURE 2. Crt mutants at site 2 exhibit attenuated chaperone function.** A. Aggregation suppression potency of Crt P19K/I21E and Crt Y22K/F84E. Crt constructs were prewarmed to 37 °C at the indicated molar ratios for 10 min. Upon dilution of luciferase to 2 μM, light scattering was recorded every 30 s at 370 nm. B, far-UV CD spectra of Crt and mutants. The CD spectra of the various constructs (5 μM) in 5 mM HEPES, pH 7.4, 150 mM NaCl, and 0.4 mM CaCl2 were monitored at 37 °C after a preincubation period of 1 h at 37 °C. C, Site 2 mutants do not bind hydrophobic peptides. The indicated Crt constructs (1 μM) were equilibrated for 10 min at 37 °C prior to the addition of KKAF or KHP peptide. Changes in intrinsic fluorescence were recorded at 346 nm with an excitation wavelength of 280 nm. Results were expressed as the fractional reduction of fluorescence intensity (quenching; 1 − F/F0, where F is the measured fluorescence intensity and F0 is the initial fluorescence). Error bars represent S.D. of three independent measurements. deg, degrees; Pep, peptide; Lec, lectin.
peptide binding site of Crt is dispensable for enhancing the solubility of the non-native NHK and ATZ variants in cells.

As a final test of the involvement of the polypeptide binding site in the functions of Crt within cells, we examined the ability of the various Crt mutants to bind to newly synthesized glycoproteins in a pulse radiolabeling experiment. Mouse L cells transiently expressing HA-tagged wild type and mutant forms of Crt were radiolabeled with $^{35}$S]Met for 20 min and lysed in digitonin lysis buffer to preserve weak Crt-substrate interactions. WT Crt constructs (3 $\mu$m) were equilibrated at 16 °C for 10 min at which time chemically denatured $\alpha$-mannosidase was added to a final concentration of 0.3 $\mu$m. Light scattering was recorded every 30 s at 400 nm. C, comparison of the relative contributions of the lectin and polypeptide binding sites to suppression of glycoprotein aggregation. The experiment in B was repeated at the indicated molar ratios of Crt constructs to $\alpha$-mannosidase. Pep, peptide; Lec, lectin.

FIGURE 3. Contributions of the lectin and polypeptide binding sites of Crt to the suppression of aggregation of non-glycosylated and glycosylated substrates. A, aggregation suppression potency of WT Crt and its lectin-deficient (D317A), polypeptide binding-deficient (P19K/I21E), and combined deficient (P19K/I21E/D317A) mutants was assessed at 37 °C with non-glycosylated luciferase (2 $\mu$m) as substrate using the indicated molar ratios. Light scattering was recorded every 30 s at 370 nm. B, aggregation suppression potency of WT Crt and its lectin-deficient, polypeptide binding-deficient, and combined deficient mutants using glycosylated $\alpha$-mannosidase (Man) as substrate. Crt constructs (3 $\mu$m) were equilibrated at 16 °C for 10 min at which time chemically denatured $\alpha$-mannosidase was added to a final concentration of 0.3 $\mu$m. Light scattering was recorded every 30 s at 400 nm. C, comparison of the relative contributions of the lectin and polypeptide binding sites to suppression of glycoprotein aggregation. The experiment in B was repeated at the indicated molar ratios of Crt constructs to $\alpha$-mannosidase. Pep, peptide; Lec, lectin.

FIGURE 4. The lectin function of Crt is required for its participation in the biogenesis of MHC class I molecules. A, anti-Crt immunoblot showing Crt expression in wild type K41 cells or in Crt-deficient K42 cells transiently transfected for 48 h with empty pLVX IRES ZsGreen1 plasmid (C) or plasmids encoding WT Crt, lectin-deficient Crt (D317A; L), polypeptide binding-deficient Crt (P19K/I21E; P), or combined lectin and polypeptide binding-deficient Crt (P19K/I21E/D317A; L P). B, MHC class I surface expression in K42 cells expressing the Crt constructs described in A. Cells were incubated with mAb Y3 or B22–249.R1 to detect surface H-2Kb or H-2Db, respectively, followed by Alexa Fluor 647-conjugated goat anti-mouse IgG and analysis by flow cytometry. The difference in median fluorescence between GFP+ and GFP− cells was plotted as the percentage of the values obtained in cells expressing wild type Crt. Error bars indicate the standard error of the mean from six (H-2Kb) and three (H-2Db) experiments. Statistically significant mean differences ($p < 0.05$) were obtained between the WT transfectants when compared with the control, lectin-deficient Crt, and combined lectin and polypeptide binding-deficient Crt transfectants. C, detection of Crt variants within the MHC class I peptide loading complex. K42 cells transiently transfected for 48 h with the indicated Crt constructs or pLVX IRES ZsGreen1 vector (C) were lysed in digitonin lysis buffer and then subjected to immunoprecipitation with anti-tapasin antiserum. Immune complexes adsorbed on protein A beads were dissociated with 100 mM HCl at 4 °C for 30 min and then analyzed by SDS-PAGE. However, the outcome of the experiment was the same (data not shown). We conclude that the predominant interaction of Crt with newly synthesized clients occurs through lectin-based binding with little or no detectable contribution from the newly identified polypeptide binding site.
Discussion

Since the initial description of glycan-independent binding of Crt or Cnx to non-native protein conformers (10, 40) and their capacity to suppress the aggregation of non-glycosylated proteins in vitro (41, 42), there has been considerable interest in defining the binding site responsible and assessing the relative contributions of glycan-dependent and -independent interactions to chaperone function in vivo. Most effort has been directed toward Crt because it is a soluble protein, and it has been implicated in a remarkable array of protein interactions not only in the ER but also the cytosol, nucleus, cell surface, and extracellular matrix. Many of these interactions are unlikely to be lectin-mediated based on their subcellular location or the lack of monoglycosylated oligosaccharide on the Crt binding partner. Examples include binding in the cytosol to glucocorticoid receptors and the ubiquitin-like protein GABARAP as well as to the cytosolic tail of integrins. At the cell surface, there are well documented associations with thrombospondin, CD91, CD69, collectin, ficolin, the complement protein C1q, and others (for reviews, see Refs. 43 and 44). In some instances, the binding site on Crt has been identified such as residues 36–53 for thrombospondin (30) and residues 195–205 for GABARAP (29) (numbering includes signal sequence), and these sites have been considered as potential candidates responsible for the ability of Crt to suppress client protein aggregation. However, these sites could be excluded because neither GABARAP nor the thrombospondin-derived hep1 peptide could compete with the ability of Crt to suppress protein aggregation (24).

Additional efforts to identify the glycan-independent binding site have involved mutagenesis of suspected regions. These have included His-170 (45) as well as a variety of residues predicted to be hydrophobic and surface-exposed based on a homology model of the Crt structure (28). However, either these mutants were destabilizing (45), or they did not impair aggregation suppression function under physiological conditions of the ER (28). More recently, interest has focused around the lectin binding site because a crystal structure of the Crt globular domain detected an N-terminal affinity tag interacting with the edge of the lectin site (31). Indeed, a mutation at Trp-319 within the lectin site has been reported to attenuate aggregation suppression in vitro (32). This region was also implicated by the finding that occupancy of the lectin site with a monoglucosylated tetrasaccharide reduced the binding of several glycosy-
lated and non-glycosylated proteins and that a fluorophore immobilized in the vicinity of the lectin site was quenched upon binding these proteins (22). Although these findings collectively suggest the presence of a protein binding site in proximity to the lectin site, this site does not exhibit the expected specificity for non-native conformers. Previous studies have established that the Crt site responsible for suppressing aggregation binds hydrophobic peptides selectively and that such peptides compete in the in vitro aggregation suppression assay (24). By contrast, the lectin-proximal binding site does not exhibit a requirement for substrate denaturation as it is capable of binding several proteins in their native states (22).

Our current efforts to identify the site responsible for aggregation suppression used two assays that report on this activity: aggregation suppression of denatured protein substrates under physiological conditions of the ER and direct binding of hydrophobic peptides. Using structure-guided mutagenesis of four candidate surface-exposed hydrophobic sites, we were able to identify a single site that, when mutated, resulted in both impaired aggregation suppression function and an inability to bind hydrophobic peptides. The mutations did not alter the overall structure of Crt as assessed by CD and melting temperature. Additional independent mutations within the same site impaired function to a similar extent, leading us to conclude that we had indeed identified the site on Crt responsible for its in vitro aggregation suppression function. Surprisingly, this site is located near the mature N terminus of Crt on the opposite face of the globular domain from the lectin site (Fig. 1A) and, as expected, is well removed from the sites of GALABARAP and thrombospondin binding. Of interest, both the lectin site and this hydrophobic polypeptide binding site participated in preventing the aggregation of the monoglucosylated glycoprotein client jack bean α-mannosidase. We showed previously that these two modes of substrate interaction resulted in Crt and a soluble form of Cnx being much more potent in suppressing glycoprotein aggregation in vitro than a non-lectin chaperone such as the ER Hsp70 BiP (33, 35). Given the lack of proximity between the two binding sites, it seems likely that they contribute independently to aggregation suppression rather than engaging a client glycoprotein simultaneously to increase binding avidity.

Three distinct assays were used to assess the relative contributions of the lectin site and hydrophobic polypeptide binding site to the chaperone functions of Crt within the ER of living cells. Disabling mutations in each site were examined for their effects on the ability of Crt to support the biogenesis and surface expression of MHC class I molecules, promote the solubility of mutant forms of α1-antitrypsin, and associate with diverse newly synthesized glycoproteins. Given the potency of the polypeptide binding site of Crt in suppressing the aggregation of both glycosylated and non-glycosylated clients in vitro, it was surprising that in all three systems there was little if any impact of mutating this site on Crt function in cells. By contrast, disabling the lectin site profoundly impacted Crt function to an extent similar to that observed in Crt-deficient cells. We considered the possibility that the mutations in the polypeptide binding site, which impaired aggregation suppression by roughly 50% in vitro, might retain greater function within the ER luminal environment. However, if this was the case, one would have expected to see partial Crt function upon mutating the lectin site or in the double site mutant. Rather, we conclude that lectin-oligosaccharide binding is the primary means whereby Crt interacts with newly synthesized glycoproteins, maintains mutant α1-antitrypsin solubility, and stabilizes the MHC class I peptide loading complex.

This conclusion is consistent with recent studies, particularly those focused on MHC class I molecules. Del Cid et al. (20) showed that when the lectin-deficient Y109A mutant of Crt (numbered Y92A without signal sequence) was expressed in Crt-deficient cells, neither it nor MHC class I molecules were stably incorporated into the class I peptide loading complex. The expression of class I molecules at the cell surface was also impaired (20). Similarly, the Y109A mutant was unable to support peptide loading onto class I molecules when the PLC was assembled from purified components in vitro (21). Lectin-deficient Crt has also been reported to be strongly impaired in its ability to bind to newly synthesized monoglucosylated glycoproteins in chemically cross-linked cell lysates (22).

Despite the predominant role of lectin–glycan interactions in the binding of Crt (and Cnx) to client glycoproteins, it is clear that lectin-independent associations do exist in cells. This has been observed in many studies using glucosidase-deficient or castanospermine-treated cells (1, 2, 13, 22, 46). Another recent example comes from the experiment of Wijeyesakere et al. (22) noted above. Although the lectin-deficient Y109A Crt was impaired in its ability to bind newly synthesized glycoproteins, significant residual interactions were readily detected (22). How are these interactions mediated given our inability to detect any involvement of the hydrophobic aggregation suppression site? The previously described glycan-independent binding site proximal to the lectin site (22) as well as contributions from the arm or P domain seem to be likely candidates (22, 24). The lectin-proximal site binds several non-glycosylated proteins with affinities similar to those observed with monoglucosylated glycoproteins albeit with different kinetics (22, 24), and mutants of Crt with arm domain truncations exhibit significantly impaired binding to purified non-glycosylated proteins in vitro (22, 24) as well as to newly synthesized proteins in lysates of castanospermine-treated cells (22). Binding contributions from both domains could occur for clients entering the cleft between the arm and lectin domains. Determining the functional relevance of these interactions in vivo, either in the biogenesis of non-glycoproteins or in providing additional binding contributions to monoglucosylated glycoproteins, must await the generation of the appropriate disabling mutations.

**Experimental Procedures**

*Materials—* The KKAFAF peptide and the KHP peptide, corresponding to the first transmembrane helix (residues 2–24) of the *Halobacterium salinarum* protein Hsmr, were synthesized on an Intavis Multipep (Intavis AG, Germany) following the manufacturer’s directions using Tentagel-S-RAM resin. Peptide purity was confirmed by reverse-phase HPLC to be greater than 90%. Peptides were dissolved in 20 mM HEPES, pH 7.4, 150 mM NaCl, and 0.4 mM CaCl2, aliquoted, and stored at −20 °C.
The Glc$_3$Man$_3$ oligosaccharide (Glc$_c$1–3Man$_a$1–2Man$_a$1–2Man-OH) was purchased from the Alberta Research Council (Edmonton, Canada). The following antibodies were used: rabbit-anti-mouse tapasin antisera raised against a C-terminal peptide of tapasin (47); Y3 and B22-249.R1 mAbs directed against the MHC class I molecules H-2Kb and H-2Dd, respectively (48, 49); rabbit-anti-mouse Crt antisera purified by immunizing rabbits with recombinant mouse calreticulin lacking arm domain residues 223–286; and rabbit anti-mouse tapasin antisera raised against a C-terminal peptide of tapasin (47). Mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH; MAB374) was purchased from Millipore (Mississauga, Canada). The following antibodies were used: rabbit antihuman tapasin antiserum (A0409) was purchased from Sigma-Aldrich. The TAP1 antiserum, a gift from Dr. Young Yang (Scripps Clinic, La Jolla, CA), was maintained in RPMI 1640 medium with 10% FBS and antibiotics (penicillin, streptomycin, and amphotericin B). Mouse embryonic fibroblasts, a gift from Dr. M. Michalak (University of Alberta), were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/liter D-glucose and supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 60 μg/ml penicillin, and 100 μg/ml streptomycin. Mouse L cells were maintained in RPMI 1640 medium with 10% FBS and antibiotics. All cells were grown at 37 °C in the presence of 5% CO$_2$.

**Mutagenesis of Calreticulin cDNA**—Amino acid numbering refers to the mouse Crt sequence with residue 1 corresponding to the first residue of the signal sequence. Constructs used for bacterial expression and purification were based on the pET15b-TEV-mCRT-CS plasmid described previously (24). It encodes an N-terminal His$_6$ tag followed by a TEV protease cleavage site fused to mouse Crt (mCrt) lacking its signal sequence and with an unpaired Cys at residue 163 mutated to Ser. pET15b-TEV-mCRT-CS was used as a template for site-directed mutagenesis of potential polypeptide binding sites using the primers listed in Table 2. Primer pairs that encoded only a single amino acid change were used in multiple rounds of site-directed mutagenesis to generate double mutants. The lectin-deficient D317A mutant and combined lectin and polypeptide binding-deficient mutant P19K/I21E/D317A were constructed by subcloning the NsiI/BamHI fragment from pQCXI1H mCRT D317A into pET15b TEV mCRT or pET15b TEV mCRT P19K/I21E, respectively. Correct introduction of all mutations was confirmed by DNA sequencing.

For expression of Crt and its lectin or polypeptide binding-deficient mutants in mammalian cells, the NsiI/BamHI DNA fragments of mouse Crt and lectin-deficient Crt D317A were synthesized with an HA tag (YPYDVPDYA; tacccgtacgatgttc) placed just upstream of the C-terminal KDEL sequence (GenScript USA). These NsiI/BamHI fragments were then subcloned into pLVX IRES ZsGreen1 mCRT or pLVX IRES ZsGreen1 mCRT D317A into pET15b TEV mCRT or pET15b TEV mCRT P19K/I21E, respectively. Correct introduction of the mutations was confirmed by DNA sequencing. Plasmids (2–3 μg) were transiently transfected into mouse K42 or L cells (1–2 × 10$^6$ cells per a p60 dish) using either Lipofectamine 2000 (Life Technologies) or PolyJet (SignaGen, Rockville, MD) according to the manufacturers’ instructions.

**Purification of Crt and Mutants**—Crt or its various mutants were expressed in Escherichia coli BL21-DE3 cells and purified as described previously with some modifications (23). Briefly, cells at an A$_{600}$ nan of 0.6 were induced for 3 h at 37 °C with 1 mM isopropyl β-D-1-thiogalactopyranoside. Cells were harvested and washed once with ice-cold water. Cell pellets were resuspended in lysis buffer (50 mM Tris, pH 8, 300 mM NaCl, 3 mM CaCl$_2$, and protease inhibitors) and were lysed using a French press. The lysate was centrifuged at 16,000 × g at 4 °C for 60 min and loaded onto a 20-ml nickel-nitrilotriacetic acid column (ThermoFisher, Burlington, Canada). The column was washed extensively, and the protein was eluted in lysis buffer containing 300 mM imidazole. Peak fractions were pooled and dialyzed against 20 mM Tris, pH 8, 150 mM NaCl, 0.4 mM CaCl$_2$, and 10% glycerol. Proteins were filtered using a 0.22-μm filter, ali quoted, and stored at −70 °C.

### TABLE 2

| Primer Sequence | RE site |
|-----------------|---------|
| E. coli expression | |
| P19K/I21E FWD | cagggcccatatgacagaagccggaatattcctataaagggag |
| P19K/I21E REV | ccgctttctattgctctcgggttcgaatttggc |
| Y150K FWD | ccgatcttattgctctcgggttcgaatttggc |
| Y150K REV | ctgccttctattgctctcgggttcgaatttggc |
| Y300Q FWD | ctgccttctattgctctcgggttcgaatttggc |
| Y300Q REV | ctgccttctattgctctcgggttcgaatttggc |
| A353E/M357E FWD | ggttaccagagctcttattgctctcgggttcgaatttggc |
| A353E/M357E REV | cagggcccatatgacagaagccggaatattcctataaagggag |
| I186K/V191E FWD | ggttaccagagctcttattgctctcgggttcgaatttggc |
| I186K/V191E REV | ccgctttctattgctctcgggttcgaatttggc |
| Y22K FWD | ccgctttctattgctctcgggttcgaatttggc |
| Y22K REV | ccgctttctattgctctcgggttcgaatttggc |
| F84E FWD | ccgctttctattgctctcgggttcgaatttggc |
| F84E REV | ccgctttctattgctctcgggttcgaatttggc |
| Mammalian expression | |
| P19K/I21E FWD | gccggcccatatgacagaagccggaatattcctataaagggag |
| P19K/I21E REV | tattcctataaagggag |

* New restriction enzyme site introduced.
**Purification of Firefly Luciferase**—FFL was expressed and purified from a pPROEX-FFL vector (gift from Dr. J. Glover, University of Toronto) as described previously (50).

**Luciferase Aggregation Assay**—Purified Crt and Crt mutants were dialyzed overnight against 20 mM HEPES, pH 7.4, 150 mM NaCl, and 0.4 mM CaCl$_2$. Prior to the aggregation assay, Crt proteins at the concentrations specified in the figures were equilibrated at 37 °C for 10 min, and then FFL was added to a final concentration of 2 μM in a total volume of 200 μl. Aggregation was measured every 60 s over a period of 2.5 h at 37 °C by monitoring light scattering at 370 nm using a SpectraMax 340PC$^{384}$ microplate reader (Molecular Devices, Sunnyvale, CA).

**α-Mannosidase Aggregation Assay**—Jack bean α-mannosidase (GE Healthcare) was dialyzed against 20 mM HEPES, pH 7.4, 150 mM NaCl, and 0.4 mM CaCl$_2$. The desalted protein was lyophilized in 0.4-mg aliquots and stored at –20 °C. Freshly thawed aliquots were resuspended at a concentration of 45 μM in 6 M guanidine hydrochloride and 0.1 M Tris-HCl, pH 8, and incubated for 1 h at room temperature to denature. The various Crt constructs were dialyzed overnight against 20 mM HEPES, pH 7.4, 150 mM NaCl, and 0.4 mM CaCl$_2$, and then equilibrated at 16 °C for 10 min. Denatured α-mannosidase was rapidly diluted into the solution containing Crt to a final concentration of 0.3 μM in a total volume of 150 μl. Aggregation was measured every 30 s over a period of 10 min at 16 °C by monitoring light scattering using a Spex Fluorolog-3 (Horiba Jobin-Yvon, London, Canada) with excitation and emission wavelengths at 400 nm and 5-nm band passes.

**Peptide Binding**—To measure peptide binding to Crt and its mutants, fluorescence was measured at 346 nm using a Spex Fluorolog-3 with an excitation wavelength of 280 nm and a 5-nm band pass. Crt (1 μM) was equilibrated in 20 mM HEPES, pH 7.4, 150 mM NaCl, and 0.4 mM CaCl$_2$. The peptide loading complexes, transiently transfected K42 cells were lysed at 4 °C for 30 min with 1% digitonin lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% digitonin, 10 mM iodoacetamide, and protease inhibitors). Lysates were centrifuged at 10,000 rpm for 10 min, and the supernatant fractions were assayed for protein content using the Pierce BCA Protein Assay kit (Thermo Scientific). Lysates (750 μg of protein) were incubated with 20 μl of anti-tapasin antibody for 1 h at 4 °C followed by the addition of 30 μl of protein A-agarose beads (Thermo Scientific) for 2 h with rocking at 4 °C. The beads were washed with 20 mM HEPES, pH 7.4, 150 mM NaCl, and 1% Nonidet P-40 followed by two washes with 20 mM HEPES, pH 7.4, 150 mM NaCl, and 0.2% digitonin. The peptide loading complex was eluted by incubating for 1 h at 4 °C with 300 μl of 100 μM tapasin C-terminal peptide (SEKATAASLTIPRNSKKSQ) used to raise the antibody. Any antibody in the eluate was cleared by incubation for 30 min at 4 °C with 30 μl of protein A-agarose beads. The beads were removed, and the supernatant was dried in a SpeedVac concentrator (Savant Instruments). Samples were dissolved in SDS-PAGE sample buffer, subjected to SDS-PAGE (10% gel) separation, and analyzed for the presence of Crt and TAP1 by immunoblotting.

**Mutant α$_1$-Antitrypsin Solubility Assay**—The effect of calreticulin mutants on the solubility of the misfolded NHK and ATZ variants of α$_1$-antitrypsin was investigated using protocols modified from Ferris et al. (39). K42 cells were transiently co-transfected with a pLVX IRES GFP plasmid containing wild type or mutant Crt and a pcDNA3.1 plasmid encoding various HA-tagged Crt constructs were washed with fresh medium after 24 h and then harvested by trypsinization after 48 h. A wash in phosphate-buffered saline (PBS) containing 3% FBS (PBS-FBS), cells were incubated on ice for 20 min in 100 μl of ice-cold PBS-FBS containing 2 μg of mAb Y3 or B22-249.R1. Cells were washed and then incubated for 30 min on ice in the dark with 2 μg of Alexa Fluor 647-conjugated goat anti-mouse IgG (Life Technologies) in 100 μl of PBS-FBS. Cells were washed twice and fixed in 300 μl of PBS containing 0.5% paraformaldehyde. Samples were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using FlowJo software (version 8.8.6). Gating was done to select for GFP-positive and -negative cells, and the difference in the median values of Alexa Fluor 647 fluorescence between GFP-positive and GFP-negative cells was compared to analyze surface expression of MHC class I molecules.
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The pellet was washed with 150 µl of lysis buffer and then solubilized in 100 µl of 1% SDS by vigorous vortexing for 30 s, sonication for 20 min, and then boiling until the pellet was completely dissolved. This was designated the insoluble fraction. The soluble and insoluble fractions were subjected to SDS-PAGE, then transferred to PVDF membrane, and stained with 0.1% Ponceau S to visualize proteins for use as loading controls. The membranes were subsequently immunoblotted to detect α1-antitrypsin variants and Crt (anti-Crt antiseraum). The NHK and ATZ band intensities, obtained from scanned films, were quantified using ImageQuant software and normalized to the Ponceau S loading control. After normalization, the soluble fraction band intensity was divided by the insoluble fraction band intensity to obtain the soluble to insoluble ratio.

Metabolic Radiolabeling—I. Cells in p60 dishes were transfected with 3 µg of plasmid encoding HA-tagged wild type or mutant Crt 24 h prior to radiolabeling. Cells were starved for 15 min in Met-deficient α-minimum Eagle’s medium and then radiolabeled for 20 min with 0.2 mCi/ml [35S]Met (>1000 Ci/mmole; PerkinElmer Life Sciences). Following lysis in digitonin lysis buffer (1% digitonin, 20 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM iodoacetamide, and protease inhibitors), Crt and associated proteins were immunosolated by incubation for 1.5 h with 21 µg of anti-HA 12CA5 antibody. Immune complexes were collected for 1 h with 30 µl of protein A-agarose, and the beads were washed using 0.25% digitonin, 20 mM HEPES, pH 7.5, and 150 mM NaCl. Proteins were separated by SDS-PAGE (10% gel), and radioactive proteins were detected by fluorography using x-ray film.

Author Contributions—R. L. designed and performed the experiments in Figs. 1–3, analyzed data, prepared figures, and wrote portions of the manuscript. S. A. designed and performed most of the experiments in Figs. 4 and 5, analyzed data, prepared figures, and wrote portions of the manuscript. S. J. H. designed and performed the experiment in Fig. 6, analyzed data, and wrote portions of the manuscript. D. C. C. performed the flow cytometry analysis in Fig. 6B, provided technical advice, and analyzed data. G. K. analyzed surface hydrophobic patches on calreticulin, provided advice, and suggested sites for mutation. D. B. W coordinated the study, helped conceive and design experiments, analyzed data, prepared figures, and wrote the final manuscript. All authors approved the final version of the manuscript.

Acknowledgments—We thank Drs. Young Yang, Tania Watts, Richard Sifers, Marek Michalak, and John Glover for generous gifts of reagents.

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