Delineation of the lectin site of the molecular chaperone calreticulin

Sten P. Thomson and David B. Williams

Department of Biochemistry, Medical Sciences Building, University of Toronto, Toronto, ON M5S 1A8, Canada

Abstract Calreticulin (CRT) is a soluble molecular chaperone of the endoplasmic reticulum that functions to promote protein folding as well as to retain misfolded proteins. Similar to its membrane-bound paralog calnexin (CNX), CRT is a lectin that preferentially interacts with glycoproteins bearing Glc₁Man₅₋₉GlcNAc₂ oligosaccharides. Although the lectin site of CNX has been delineated through X-ray crystallographic and mutagenic studies, the corresponding site for CRT has not been as well characterized. To address this issue, we attempted to construct lectin-deficient CRT mutants, using the structure of CNX as a guide to identify potential oligosaccharide-binding residues. Mutation of 4 such CRT residues (Y109, K111, Y128, D317) completely abrogated oligosaccharide binding. In contrast, mutation of CRT residues M131 and D160, which correspond to important residues in the lectin site of CNX, had no effect on oligosaccharide binding. These findings suggest that the organization of the lectin site in CRT largely resembles that of CNX but is not identical. The deficiency in oligosaccharide binding by the mutants was not due to misfolding because they exhibited wild-type protease digestion patterns, were capable of binding the thiol oxidoreductase ERp57, and functioned just as efficiently as wild-type CRT in suppressing the aggregation of the nonglycosylated substrate citrate synthase. However, they were impaired in their ability to suppress the aggregation of the glycosylated substrate jack bean α -mannosidase. This provides the first direct demonstration of the importance of CRT's lectin site in suppressing the aggregation of nonnative glycoproteins.

INTRODUCTION

The endoplasmic reticulum (ER) is the site of folding for nascent polypeptides destined for various locations along the secretory pathway. Folding is facilitated by an array of molecular chaperones including BiP, Grp94, Grp170, calnexin (CNX), and calreticulin (CRT) that prevent the aggregation of nascent polypeptides under conditions of high calcium and polypeptide concentrations. Of these, CNX and CRT are unique in that they interact preferentially with Asn-linked glycoproteins by virtue of a lectin site that binds to the oligosaccharide-processing intermediates Glc₁Man₅₋₉GlcNAc₂ (Ware et al 1995; Spiro et al 1996). Substrate specificity studies have shown that the single terminal glucose residue of these oligosaccharides is particularly important for recognition by CNX and CRT (Ware et al 1995; Spiro et al 1996; Vassilakos et al 1998).

CNX is a type-I ER membrane protein that shares 39%

sequence identity with its soluble paralog CRT (Wada et al 1991). The crystal structure of the ER luminal portion of CNX has revealed 2 domains, a globular lectin domain similar to legume lectins and an extended arm domain consisting of 2 antiparallel β strands (Schrag et al 2001). The structure of the arm domain of CRT has been solved by nuclear magnetic resonance (NMR) and is similar to the arm domain of CNX (Ellgaard et al 2001). Given its identical lectin specificity (Spiro et al 1996; Vassilakos et al 1998), it is generally assumed that CRT also possesses a globular lectin domain similar to that of CNX (Kapoor et al 2003).

CNX and CRT have been shown to interact with large and overlapping arrays of substrates and to play an important role in protein folding in the ER (reviewed in Parodi 2000; Leach and Williams 2003). Indeed, CNX and CRT enhance the *in vivo* folding of various model glycoproteins including the major histocompatibility complex (MHC) class I molecules (Tector and Salter 1995; Vassilakos et al 1996; Gao et al 2002), influenza hemagglutinin (HA) (Molinari et al 2004) and the nicotinic acetylcholine receptor (Chang et al 1997). CNX and CRT also

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Correspondence to: David B. Williams, Tel: 416 978-2546; Fax: 416 978-8548; E-mail: david.williams@utoronto.ca.

function in a quality control process that retains incompletely folded or misfolded polypeptides within the ER (Jackson et al 1994; Danilczyk et al 2000; Gao et al 2002).

How do CNX and CRT promote glycoprotein folding and participate in ER quality control? In the lectin-only model, CNX and CRT interact solely with the Glc₁Man₅₋₉GlcNAc₂ oligosaccharides of glycoproteins (Hebert et al 1995). Dissociation from CNX and CRT accompanies removal of the terminal glucose residue from the oligosaccharide by the ER enzyme glucosidase II, and rebinding to the chaperones can occur by the readdition of glucose through the action of UDP-glucose:glycoprotein glucosyltransferase. The latter enzyme is the folding sensor in the cycle because it will only reglucosylate non-native glycoproteins (Sousa and Parodi 1995). Interaction with CNX and CRT serves to retain nonnative conformers in the ER, and promotion of protein folding is thought to be because of recruitment by CNX and CRT of additional folding factors. This view is supported by the finding that CRT and CNX can interact with the ER thiol oxidoreductase ERp57 through their arm domains (Oliver et al 1999; Frickel et al 2002), an interaction that enhances oxidative folding of glycoproteins in vitro (Zapun et al 1998) and possibly in vivo, given that aberrant or inefficient disulfide formation has been observed for influenza HA in the absence of CRT or CNX (Molinari et al 2004).

In the dual-binding model, CNX and CRT associate with nonnative glycoprotein conformers through polypeptide-based interactions as well as by lectin-oligosaccharide interactions (Ware et al 1995). The polypeptide-based interactions are thought to be similar to those exhibited by other chaperone systems, serving to promote more efficient folding by preventing aggregation. Because adenosine triphosphate (ATP) has been shown to bind to CRT and CNX and to enhance their ability to suppress the aggregation of nonglycosylated proteins in vitro, it is possible that cycles of polypeptide binding and release are regulated by ATP exchange or hydrolysis (Ihara et al 1999; Saito et al 1999). Dual engagement of glycoprotein polypeptide and oligosaccharide moieties by CNX and CRT would be predicted to increase substrate-binding affinity relative to nonlectin molecular chaperones. This idea is supported by the finding that CNX is 4- to 16-fold more potent in suppressing the aggregation of glycoproteins in vitro than the ER Hsp70 chaperone, BiP (Stronge et al 2001).

Given the central role that lectin-oligosaccharide interactions play in both models, it is important to characterize this interaction in molecular detail. A combination of deletion mutagenesis (Leach et al 2002) and X-ray crystallographic studies (Schrag et al 2001) has revealed that the globular domains of CNX and CRT are responsible for oligosaccharide binding. Furthermore, Schrag et al (2001) soaked CNX crystals in glucose and identified 6

residues that bind the monosaccharide largely through hydrogen bond interactions. These included 2 tyrosines, 2 aspartates, a lysine, and a methionine residue (Schrag et al 2001). In a recent study, we constructed point mutants in which each of these residues was mutated to alanine and found that Glc₁Man₆GlcNAc₂ binding was eliminated in each case, confirming that all 6 residues are critically involved in binding to the natural oligosaccharide ligand (Leach and Williams 2004). In this study, we sought to determine the organization of the lectin site of CRT. Because CNX and CRT share 39% sequence identity, we identified by sequence alignment 6 CRT residues that correspond to glucose-binding residues in CNX. Point mutations were created at each of these sites, and the mutant CRT molecules were assayed for their abilities to bind oligosaccharide and to function as chaperones for both glycoprotein and nonglycosylated substrates. The results indicate that the organization of the lectin sites of CRT and CNX are similar but not identical. Furthermore, abolition of oligosaccharide binding resulted in an impaired ability to suppress the aggregation of a denatured glycoprotein substrate, thereby demonstrating the importance of CRT's lectin function in this process. In contrast, the lectin-deficient mutants were fully capable of suppressing the aggregation of a nonglycoprotein substrate, suggesting that the lectin and polypeptide-binding sites of CRT do not overlap.

MATERIALS AND METHODS

Site-directed mutagenesis

QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA, USA) was used to mutate 6 CRT residues believed to be involved in oligosaccharide binding, using pGEX-3X-CRT as a template (Baksh and Michalak 1991). pGEX-3X-CRT (Amersham Biosciences, Baie d'Urfé, Québec, Canada) encodes glutathione S-transferase (GST) fused to the mature N-terminus of rabbit CRT. To facilitate the screening of positive clones, primers were designed to either remove or introduce a restriction endonuclease site. The mutagenic primers that were used are shown in Table 1.

Purification of GST-CRT fusion proteins

BL21-CodonPlus *Escherichia coli* cells (Stratagene) were transformed with wild-type or mutant pGEX-3X-CRT plasmids, and protein expression was induced in transformants with 0.1 mM isopropyl-D-thiogalactopyranoside for 16 hours at 25°C. GST fusion proteins were purified by glutathione-agarose chromatography, essentially as described by the manufacturer (Amersham Biosciences). Purity of the fusion proteins was assessed by sodium do-

Table 1 Oligonucleotide primers used to generate the CRT mutants

Oligonucleotide	Sequence (5' → 3') ^a	Restriction site
CRT Y109A forward	GAACATTGACTGCGGGGCGGCgtGTGAAGCTGTTTCCGGCCGGCCTGG	Lose <i>Bsa</i> I site
CRT K111A forward	GAACATTGACTGCGGGGCGGCTAtGTGgctCTGTTTCCGGCCGGCCTGG	Lose <i>Bsa</i> I site
CRT Y128A forward	CATGCACGGGGACTCTGAGgcCAAtATTATGTTTGGTCTGACATCTGTG	Gain <i>Sspl</i> site
CRT Y128F forward	CACGGGGACTCTGAAttCAACATCATGTTTGG	Gain <i>Sspl</i> site
CRT M131A forward	GACTCTGAGTACAAtATtgcGTTTGGTCTGAGATCTGTGGCCC	Gain <i>Sspl</i> site
CRT M131E forward	GACTCTGAGTACAAtATtgaGTTTGGTCTGACATCTGTGGCCC	Gain <i>Sspl</i> site
CRT D160G forward	GTGCTGATCAACAAGGggATCCGTGCAAGGAC	Gain <i>Bam</i> HI site
CRT D317A forward	GCTTTGCCGTGCTGGGCTTGGctCTCTGGCAGGTCAAGTCGGG	Lose <i>Eco</i> NI site

CRT, calreticulin.

^a Mutagenic bases are indicated in lowercase type.

decyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and typically found to be near 90%. In some instances, the samples were further purified by Mono Q anion-exchange chromatography (Amersham Biosciences) using a gradient of 0.05 to 1 M NaCl in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES), 5 mM CaCl₂, pH 7.2. GST-CRT was eluted from the column at 0.45 M NaCl. In some experiments, cleavage of GST from CRT-fusion constructs was carried out by digestion with Factor Xa using 20 U in a total volume of 7 mL of 3.5 mM Tris-HCl, 100 mM NaCl, 1 mM CaCl₂, pH 8.0, for 48 hours at 4°C. The reaction was stopped by the addition of 1,5-Dansyl-Glu-Gly-Arg Chloromethyl Ketone (Calbiochem, La Jolla, CA, USA) and the mixture loaded onto a glutathione-agarose column to remove free GST. An average of 15 mg of GST-CRT was initially obtained from a 2-L culture; subsequent purification procedures yielded approximately 5 mg of pure CRT.

Oligosaccharide binding assay

The dolichol-PP-oligosaccharide fraction of *Saccharomyces cerevisiae* strain *alg8* (Runge and Robbins 1986) was used to obtain radiolabeled [³H]Glc₁Man₆GlcNAc₂ (Paquet et al 2004). Wild-type or mutant GST-CRT constructs were immobilized on glutathione-agarose beads, and then 1 μM immobilized protein in 100 μL of 20 mM HEPES, 150 mM NaCl, 5 mM CaCl₂, pH 7.4 (buffer A) containing 2000 cpm of [³H]Glc₁Man₆GlcNAc₂ was incubated for 30 minutes at 4°C. The beads were centrifuged for 5 seconds at 10 000 × *g* and washed once with 100 μL buffer A. Oligosaccharide bound by GST-CRT was isolated by resuspending the beads in buffer A containing 1 mM ethylene glycol bis (2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and heating at 50°C for 5 minutes. The beads were then centrifuged for 5 seconds at 10 000 × *g*, and the radioactive oligosaccharide in the supernatant fraction was quantified by liquid scintillation counting. Wild-type CRT fused to GST and GST alone were used as positive and negative controls, respectively, in all experiments. All results are corrected for background binding to GST and are expressed as percentage of binding by

wild type–GST CRT. Approximately 15% of input oligosaccharide (300 cpm) was typically bound by GST-CRT.

Trypsin digestion of CRT mutants

Bovine heart trypsin was obtained from Sigma Chemical Co (St Louis, MO, USA). Digestions were performed using 2.8 μg trypsin and 140 μg CRT construct (previously released from GST by Factor Xa cleavage) in 100 mM Tris-HCl, 0.5 mM CaCl₂, pH 8.5, in a total volume of 300 μL. For the trypsin digest without calcium, CRT was diluted and concentrated repeatedly in 100 mM Tris-HCl, pH 8.5, using an Amicon membrane concentrator (Millipore, Bedford, MA, USA). To remove any residual calcium bound to CRT, 1 mM ethylenediaminetetraacetic acid was added before the addition of trypsin. All digestions were carried out at 37°C. Aliquots containing 14 μg of protein were removed at each time point, heated at 95°C with SDS-PAGE sample buffer, and analyzed by SDS-PAGE using 15% polyacrylamide gels. Protein bands were detected by Coomassie Blue Staining.

The ERp57 binding assay

ERp57 expression was induced with 0.5 mM isopropyl-D-thiogalactopyranoside for 3 hours at 30°C in BL21-CodonPlus *E. coli* cells containing a pET vector encoding human ERp57 complementary DNA. After resuspending the bacteria in buffer A, the mixture was sonicated and centrifuged at 10 000 × *g*. ERp57 was purified by applying the supernatant to a Mono Q anion-exchange column equilibrated in 10 mM HEPES, 5 mM CaCl₂, 50 mM NaCl. The column was developed with a 0.05 M to 1 M NaCl gradient and ERp57 eluted at 0.28 M NaCl. Purified ERp57 (0.1 μM) was incubated for 30 minutes at 4°C with 1 μM GST-CRT construct immobilized on glutathione-agarose beads in 250 μL of buffer A containing 0.1% Nonidet P-40 and 100 μg/mL bovine serum albumin. After centrifuging for 5 seconds at 10 000 × *g*, the sedimented beads were washed twice with buffer A. ERp57 complexed with GST-CRT fusion protein was eluted with buffer A containing 10 mM reduced glutathione. The el-

uates were analyzed by SDS-PAGE using 10% acrylamide gels, and ERp57 was detected by immunoblotting with an anti-ERp57 antibody (gift from Dr D. Thomas, McGill University, Montréal, Québec, Canada) and observing with the enhanced chemiluminescence detection system (Amersham Biosciences).

Citrate synthase aggregation assay

Citrate synthase (CS; Sigma) was desalted using a NAP-25 gel-filtration column equilibrated in buffer A. GST-CRT fusion construct (1 μ M) was mixed with CS (1 μ M) in buffer A in a total volume of 500 μ L. To monitor aggregation, light scattering was recorded at 360 nm for 1 hour at 45°C. The data shown correspond to the average percentage of maximal aggregation relative to the control (CS alone). All assays were performed in triplicates.

α -Mannosidase aggregation assay

Jack bean α -mannosidase (MS; Sigma) was desalted by NAP-25 gel filtration in buffer A and lyophilized. MS was redissolved in 6 M guanidinium hydrochloride (Sigma) at a concentration of 24 μ M. Denatured MS was then diluted to 0.36 μ M in 500 μ L buffer A containing 0.36 μ M of GST-CRT construct at 10°C, and aggregation was measured at 360 nm for 5 minutes. The data shown correspond to the average percentage of maximal aggregation relative to the control (MS alone). All assays were performed in triplicates.

RESULTS

The goal of this study was to define the lectin site of CRT and compare it to that of CNX. Schrag et al (2001) identified 6 CNX residues as glucose contacts, Y165, K167, Y186, M189, E217, and E426, that, with the exception of M189, form hydrogen bonds with the hydroxyl groups of the glucose ring (Schrag et al 2001). Sequence alignment (Fig 1) indicates that CRT residues corresponding to the 6 CNX residues are Y109, K111, Y128, M131, D160, and D317 and they lie in well-conserved regions except for CRT residue D160. To test whether these CRT residues contribute to oligosaccharide binding, we used a site-directed mutagenesis approach, replacing each residue with the small hydrophobic amino acid alanine wherever possible. We also constructed a mutant in which CRT residue, Y128, was mutated to phenylalanine. This allowed us to determine whether either the hydroxyl group or the aromatic ring of Y128 contacts glucose. In addition, a substitution of M131 to glutamate tested the effect of a non-conservative mutation at this residue on the lectin function of CRT. Because of the inability to alter a restriction site by mutating residue D160 to alanine, this residue was

Fig 1. Sequence alignment of rabbit calreticulin (CRT) and the ER luminal domain of dog calnexin (CNX). Numbering includes the N-terminal signal sequences. The boxes indicate CNX residues known to contact glucose and their counterparts in CRT. The filled circles indicate identical residues in CNX and CRT; the open circles indicate conservative substitutions between CNX and CRT. ER, endoplasmic reticulum.

instead changed to glycine. A summary of the mutants is presented in Table 1. Wild-type CRT and the CRT mutants were purified as GST fusion proteins by glutathione-agarose affinity chromatography followed, in some instances, by Mono Q anion-exchange chromatography. Purity was typically in excess of 90%.

Oligosaccharide-binding assay

Initially, we tested whether the CRT point mutants were deficient in oligosaccharide binding. Both wild-type and mutant GST-CRT fusion proteins were assayed for their binding to radiolabeled [3 H]Glc₁Man₉GlcNAc₂. Because the affinity of CRT's lectin site for substrate is relatively weak ($K_d = 2 \mu$ M), we anticipated that mutagenesis of even a single residue involved in oligosaccharide binding would largely eliminate measurable oligosaccharide binding (Patil et al 2000). Indeed, 4 mutants (Y109A, K111A, Y128A, and D317A) showed no oligosaccharide binding,

Fig 2. Oligosaccharide binding by calreticulin mutants. [^3H]Glc₁Man₉GlcNAc₂ was incubated with immobilized GST-CRT or GST-CRT point mutants as indicated. After washing, bound oligosaccharide was eluted and detected by liquid scintillation counting. The data represent the level of oligosaccharide binding as percentage relative to wild-type CRT. In all cases, background binding to GST alone was subtracted. Error bars indicate the range of duplicate or triplicate measurements. GST, glutathione S-transferase; CRT, calreticulin.

whereas oligosaccharide binding by mutants M131A and D160G was comparable with that of wild-type CRT (Fig 2). In contrast to the Y128A mutant, removal of the hydroxyl group of Y128 in the Y128F mutant only partially abrogated oligosaccharide binding, suggesting that both the hydroxyl group and the aromatic ring of this residue play a role in oligosaccharide binding. The M131 did not appear to make glucose contacts, which was a surprising finding, given that a previous study had reported that this residue was important for the lectin function of CRT (Kapoor et al 2004). Consequently, we constructed a less-conservative mutation to glutamic acid at this position, but this effected only a modest reduction in oligosaccharide binding to 36% of that observed with wild-type CRT. Because mutation of 4 of the 6 residues eliminated oligosaccharide binding, it can be concluded that these residues play as crucial a role in CRT as the corresponding residues in CNX. Thus, there are both similarities and differences in the organization of the lectin site of CRT compared with that of CNX.

It was important to ensure that the point mutants did not cause substantial misfolding of CRT, thereby leading to an indirect impairment in lectin function. Unfortunately, we were unable to prepare sufficient quantities of highly purified mutants for circular dichroism (CD) analysis. Consequently, we used several approaches to assess whether the CRT point mutants had comparable tertiary structure with that of wild-type CRT. These involved test-

Fig 3. Trypsin sensitivity of oligosaccharide binding-deficient CRT mutants. Wild-type CRT and CRT point mutants Y109A, K111A, Y128A, and D317A were incubated with trypsin at a ratio of 1:50 (trypsin:protein, wt/wt) in 0.5 mM CaCl₂ (panels A, C–F) or in the absence of calcium (panel B). Aliquots were taken at the time points indicated, and the proteins were separated by reducing SDS-PAGE using 15% acrylamide gels. The arrow indicates the 27-kDa protease-resistant fragment of CRT. The mobilities of molecular weight markers are shown. CRT, calreticulin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

ing the mutants for their sensitivities to an exogenous proteolytic probe as well as assessing the integrity of the lectin and arm domains by binding to specific ligands.

Protease digests of CRT mutants

Comparative protease digests were carried out on CRT and the lectin-deficient mutants after their release from GST by Factor Xa digestion. Previously, Corbett et al (2000) showed that trypsin digestion of CRT in the presence of physiological ER calcium concentrations of 0.5–1 mM yields a protease-resistant species of 27 kDa (Corbett et al 2000). We confirmed this finding on digesting wild-type CRT with trypsin at 37°C and 0.5 mM CaCl₂ (Fig 3A). Whereas full-length CRT (60 kDa) was reduced to trace amounts after approximately 10 minutes, a prominent protease-resistant species near 27 kDa appeared after 1 minute of digestion and remained throughout the 30 minutes incubation period. In addition, a species slightly smaller than the starting material (47 kDa) appeared shortly after commencement of the digestion and disappeared after 20 minutes. The presence of a protease-resistant species (27 kDa) suggests that the tertiary struc-

ture of CRT renders portions of the protein inaccessible to trypsin.

As a positive control to demonstrate the extreme protease sensitivity of nonnative CRT, we repeated the digestion in the absence of calcium. Li et al (2001) previously established that calcium has a substantial effect on the structure of CRT. Addition of calcium at concentrations comparable with those found in the ER significantly increased the thermal stability of CRT and conferred resistance to chymotrypsin digestion (Li et al 2001). As shown in Figure 3B, the removal of calcium led to a markedly increased susceptibility to trypsin, such that only trace amounts of full-length CRT remained after 1 minute. The overall pattern of tryptic fragments was also altered. In contrast to the digest at 0.5 mM calcium, no prominent 27-kDa protease-resistant species could be observed. Rather, 2 smaller fragments of less than 19 kDa were weakly detected. Thus, trypsin is an effective probe for detecting structural perturbations in CRT.

To compare the tertiary structure of the oligosaccharide binding-deficient CRT mutants with wild-type CRT, we examined the patterns generated by trypsin digestion in the presence of calcium (Fig 3C–F). The degradation rates of mutants Y109A, K111A, and Y128A were similar to that of wild-type CRT because significant amounts of full-length protein remained through 5 minutes of digestion. The mutant D317A appeared to have a slower rate of digestion because significant amounts of full-length CRT remained through 10 minutes. The overall digestion patterns generated by the mutants were also similar to that of CRT. Each mutant generated the 27-kDa protease-resistant species found in CRT, suggesting that a portion of the mutants was inaccessible to the enzyme as in wild-type CRT. Because of the reduced rate of trypsin digestion, this band was significantly stronger in D317A than in the other digests. Also, the 47-kDa band that was generated shortly after commencement of the digestion largely disappeared in wild-type CRT and in CRT mutants Y109A, K111A, and Y128A after 20 minutes but remained as a distinct species even after 30 minutes in D317A. All mutants had trypsin sensitivity that much more closely resembled wild-type CRT than CRT in the absence of calcium, suggesting that they possess tertiary structure comparable with wild-type CRT.

ERp57 binding assay

As a further test of the structural integrity of the oligosaccharide binding-deficient CRT mutants, we carried out an ERp57 binding assay. ERp57 is a thiol oxidoreductase whose catalytic activity toward monoglucosylated glycoproteins is markedly increased *in vitro* when in contact with CNX or CRT (Zapun et al 1998). ERp57 binding assays with various deletion mutants of CRT (Leach et al

Fig 4. ERp57 binding ability of lectin-deficient CRT mutants. The indicated GST-CRT fusion constructs were immobilized on glutathione-agarose beads and incubated with ERp57. Beads were washed twice, and bound ERp57 was eluted and analyzed by SDS-PAGE (10% gel) under nonreducing conditions. ERp57 was detected by immunoblotting with anti-ERp57 antibody. CRT, calreticulin; GST, glutathione S-transferase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

2002) as well as NMR studies (Frickel et al 2002) have indicated that ERp57 binds to the tip of the arm domain. Because this binding site is distant from the globular lectin domain, it would likely be affected only if the CRT point mutants caused extensive misfolding.

In the binding assay, ERp57 was incubated with GST fusions of the CRT mutants immobilized on glutathione-agarose beads. Binding was detected by immunoblotting bead eluates with anti-ERp57 antibody. The oligosaccharide binding-deficient CRT mutants Y109A, K111A, Y128A, and D317A were able to bind ERp57 at levels comparable with wild-type CRT, whereas GST was not (Fig 4). Mutants that were not deficient in oligosaccharide binding were also able to bind ERp57 (data not shown). These findings indicate that the oligosaccharide binding-deficient CRT mutants possess a functional arm domain similar to that of wild-type CRT.

Suppression of CS aggregation

As a final test of the structural integrity of the oligosaccharide binding-deficient CRT mutants, their abilities to suppress the aggregation of the nonglycosylated protein CS were assessed. Previous *in vitro* studies have shown that CNX and CRT are capable of suppressing the aggregation of not only glycoproteins, but also nonglycosylated substrates, providing evidence for a polypeptide-based interaction of CNX and CRT with their client proteins (Ihara et al 1999; Saito et al 1999). Indeed, direct binding between CNX or CRT and thermally unfolded CS or malate dehydrogenase can be demonstrated by gel filtration (Ihara et al 1999). Because this function has been mapped primarily to the globular domain of CNX and CRT (Leach et al 2002), it has the potential to be influenced by struc-

Fig 5. Suppression of citrate synthase (CS) aggregation by lectin-deficient CRT. The indicated GST-CRT constructs and CS were mixed at a 1:1 molar ratio, heated at 45°C, and the optical density at 360 nm was recorded over a period of 1 hour. The percentage aggregation shown represents the maximum aggregation after 1 hour relative to that of CS alone. CRT, calreticulin; GST, glutathione S-transferase.

tural perturbations that may be associated with the CRT point mutants.

To test whether the oligosaccharide binding-deficient mutants were still capable of a polypeptide-based chaperone function, we carried out thermal aggregation assays with CS at 45°C. In this experiment, light scattering was monitored at 360 nm as a measure of CS aggregation. As shown in Figure 5, GST-CRT suppressed the aggregation of CS to 14% of the aggregation level of CS alone. The CRT mutants were all able to suppress CS aggregation to this level or to a greater extent: Y109A suppressed aggregation to 4%, K111A suppressed aggregation to 14%, Y128A suppressed aggregation to 10%, and D317A suppressed aggregation to 7%. This indicates that the mutants can function as molecular chaperones through their polypeptide-binding site just as effectively as wild-type CRT and suggests that each has a globular domain that is deficient only in oligosaccharide binding. The results further suggest that the lectin site of CRT does not contribute to the binding site responsible for suppressing the aggregation of nonglycosylated proteins.

Suppression of MS aggregation.

In addition to suppressing the aggregation of nonglycosylated proteins, CNX and CRT potentially suppress the aggregation of nonnative glycoproteins bearing Glc₁Man₉GlcNAc₂ oligosaccharides (Ihara et al 1999; Saito et al 1999). Indeed, they proved to be much more effective in this regard than the Hsp70 chaperone of the ER, BiP (Stronge et al 2001). This enhanced aggregation suppression ability with glycoprotein substrates is thought to be due to the lectin-oligosaccharide interaction because it was lost on degly-

Fig 6. Suppression of jack bean α -mannosidase (MS) aggregation by lectin-deficient CRT. Chemically denatured MS was mixed with the indicated GST-CRT constructs at a 1:1 ratio at 10°C. Measurement of the optical density at 360 nm commenced immediately after mixing, and measurements were taken every 30 seconds for 5 minutes. The percentage aggregation shown represents the maximum aggregation after 5 minutes relative to the aggregation of ms alone. CRT, calreticulin; GST, glutathione S-transferase.

cosylation of the substrate (Stronge et al 2001). However, given the likelihood of altered physical properties accompanying substrate deglycosylation, it was not possible to demonstrate directly the role of CRT's lectin site in this process. The lectin-deficient CRT point mutants provided an ideal opportunity to address this issue.

In this experiment, chemically denatured MS was diluted with buffer containing GST-CRT construct at 10°C, and light scattering at 360 nm was monitored for a period of 5 minutes. At a 1:1 molar ratio, wild-type GST-CRT suppressed the aggregation of MS to 22% of the level observed with MS alone (Fig 6). In contrast, the GST-CRT mutants were severely impaired, suppressing MS aggregation to only 86% (Y109A), 88% (K111A), 77% (Y128A), and 72% (D317A) of control (Fig 6). Therefore, the lectin site of CRT plays a crucial role in suppressing the aggregation of a glycoprotein substrate possessing the Glc₁Man₉GlcNAc₂ oligosaccharide.

DISCUSSION

We previously used site-directed mutagenesis to demonstrate that the 6 residues implicated by structural studies to be involved in glucose binding by CNX (Y165, K167, Y186, M189, E217, E426) are indeed essential for this function (Leach and Williams 2004). In this study, we used a sequence alignment between CRT and CNX to identify corresponding CRT residues that may be involved in oligosaccharide binding and then mutated them to assess their functionality. Mutation to alanine of CRT residues Y109, K111, Y128, and D317, corresponding to CNX residues Y165, K167, Y186, and E426, completely

eliminated oligosaccharide binding. By contrast, mutation to alanine of M131 and to glycine of D160, corresponding to CNX residues M189 and E217, did not impair oligosaccharide binding by CRT. These findings indicate that the organization of the lectin sites of CNX and CRT are similar, although not identical.

During the course of this study, Kapoor et al published a study using isothermal titration calorimetry to measure the oligosaccharide binding of CRT mutated at residues hypothesized to interact with oligosaccharide on the basis of computer-generated models of the lectin site of CRT (Kapoor et al 2004). Consistent with our findings, they identified CRT residues Y109 and D317 as vital for oligosaccharide binding (Kapoor et al 2004). Mutations at K111 were not studied; thus, our study is the first to demonstrate the importance of this residue in oligosaccharide binding by CRT. Kapoor et al also found that mutation of Y128 to phenylalanine reduced oligosaccharide binding to 25% but did not consider any further mutations (Kapoor et al 2004). We observed a partial reduction of oligosaccharide binding to 62% when mutating this residue to phenylalanine but observed complete abrogation of oligosaccharide binding when this residue was mutated to alanine. Therefore, our results offer the additional insight that both the hydroxyl group and the aromatic ring components of Y128 are involved in oligosaccharide binding by CRT. It is noteworthy that when we mutated CRT residue D160 to glycine, it did not result in an oligosaccharide binding-deficient mutant. This residue was predicted to correspond to CNX residue E217 but because it lies in a region relatively poorly conserved between CRT and CNX, this may be a result of improper residue identification by sequence alignment. Alternatively, the negative result for D160G could be due to a difference in the organization of the lectin sites of CRT and CNX. Because Kapoor et al identified an acidic residue, D135, as a crucial oligosaccharide-binding residue (Kapoor et al 2004), it is perhaps more likely that this amino acid and not D160 performs a similar function in the lectin site of CRT as does E217 in CNX. A major discrepancy between our results and the study carried out by Kapoor et al is regarding CRT residue M131. Kapoor et al found that the M131A mutation almost completely eliminated oligosaccharide binding and concluded that M131 forms important hydrophobic contacts with glucose in a manner similar to M189 in CNX (Kapoor et al 2004). In contrast, our results indicated no reduction in oligosaccharide binding on mutation of M131 to alanine. To confirm this finding, we made a less-conservative mutation to glutamic acid and observed that it only partially affected oligosaccharide binding. These findings suggest that CRT residue M131 plays a less significant role in oligosaccharide binding than the corresponding M189 residue in CNX.

The basis for the discrepancy between the 2 studies is

unclear. One possibility is the different species of CRT used in the 2 studies; rabbit CRT in this study and rat CRT in the experiments of Kapoor et al. However, this is an unlikely explanation because the 2 sequences are 95% identical and M131 resides within a particularly highly conserved segment. Alternatively, the M131A mutation may have altered the tertiary structure of CRT to different extents in the 2 studies. However, Kapoor et al demonstrated that M131A had a similar CD spectrum as wild-type CRT (Kapoor et al 2004). Similarly, we showed that M131A, as with all of our lectin-deficient mutants, exhibited similar proteolytic sensitivity compared with wild-type CRT. The mutants also retained full ERp57-binding ability and full capacity to suppress the thermally induced aggregation of a nonglycosylated substrate, properties associated with functional arm and globular domains, respectively. A structural determination of the globular domain of CRT complexed with oligosaccharide will be required to clarify the role of M131 and to further delineate lectin-site residues involved in binding to the natural Glc₁Man₆GlcNAc₂ ligand.

Our aggregation assays with CS demonstrated the ability of lectin-deficient CRT mutants to suppress the aggregation of nonglycosylated substrates by polypeptide-based interactions. This was not unexpected because similar experiments recently carried out in our laboratory with CNX point mutants deficient in oligosaccharide binding showed that such mutants were able to suppress CS aggregation (Leach and Williams 2004). Collectively, these findings suggest that the lectin site itself is not likely to be the site responsible for polypeptide-based aggregation suppression. This is an important finding because lectin sites frequently have hydrophobic character and thus were candidates for the polypeptide-binding sites of CNX and CRT (Drickamer 1997). Efforts to identify the polypeptide-binding sites of CNX and CRT, as well as their polypeptide-binding specificities, are in progress.

In contrast to what was observed with the nonglycosylated substrate CS, the lectin-deficient CRT mutants were substantially impaired relative to wild-type CRT in their abilities to suppress the aggregation of the monoglucosylated glycoprotein MS. This is consistent with our previous studies in which CRT and CNX were shown to be much more effective at suppressing the aggregation of a substrate bearing monoglucosylated glycans than the same substrate completely lacking glycans (Saito et al 1999; Stronge et al 2001). The latter findings were taken as evidence for the involvement of the lectin sites of CNX and CRT in their abilities to suppress the aggregation of glycoproteins. However, a firm conclusion could not be drawn because deglycosylation may have altered the physical properties of the substrate. Consequently, our current observation that lectin-deficient CRT mutants are impaired in this function provides the first direct dem-

onstration that oligosaccharide binding is indeed a vital component in suppressing the aggregation of glycoproteins.

The finding that lectin-deficient CRT mutants have an apparently unaltered tertiary structure and are otherwise completely functional provides the first step toward a detailed assessment of the importance of oligosaccharide binding in the molecular chaperone functions of CRT in vivo. For example, through the use of CRT-deficient murine fibroblasts, CRT has been shown to function in peptide loading and quality control of MHC class I molecules (Gao et al 2002). By comparing the phenotypes of such cells expressing lectin-deficient mutants or wild-type CRT, it will be possible to determine the contribution of oligosaccharide binding to CRT function in the biogenesis of MHC class I molecules. This should provide a more convincing assessment of the validity of the lectin-only vs dual-binding models for CRT function than was previously possible. Efforts in this regard with respect to CNX have revealed that oligosaccharide binding-deficient CNX retains the ability to bind MHC class I heavy chains in vivo and prevent their degradation, thus supporting the view that polypeptide-based interactions between CNX and a folding substrate do indeed occur in vivo (Leach and Williams 2004). By extension, the use of CRT and CNX mutants selectively deficient in ERp57 binding and polypeptide binding will also provide an opportunity to assess the contributions of these properties to the overall chaperone functions of CRT and CNX within the ER of living cells.

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