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Defining substrate interactions with calreticulin: an isothermal titration calorimetric study

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Abstract Calreticulin (CRT) is a soluble, lectin chaperone found in the endoplasmic reticulum of eukaryotes. It binds the N-glycosylated polypeptides via the glycan intermediate $Glc_1Man_{5-9}GlcNAc_2$, present on the target glycoproteins. Earlier we have studied interactions of substrate with CRT by isothermal titration calorimetry (ITC) and molecular modeling, to establish that CRT recognizes the Glcα1–3 linkage and forms contacts with each saccharide moiety of the oligosaccharide Glcα1–3Manα1–2Manα1–2Man. We also delineated the amino acid residues in the sugar binding pocket of CRT that play a crucial role in sugar–CRT binding. Here, we have used mono-deoxy analogues of the trisaccharide unit Glcα1–3Manα1–2Man to determine the role of various hydroxyl groups of the sugar substrate in sugar–CRT interactions. Using the thermodynamic data obtained by ITC with these analogues we demonstrate that the 3-OH group of Glc1 plays an important role in sugar–CRT binding, whereas the 6-OH group does not. Also, the 4-OH, 6-OH of Man2 and 3-OH, 4-OH of Man3 in the trisaccharide are involved in binding, of which 6-OH of Man2 and 4-OH of Man3 have a more significant role to play. This study sheds light further on the interactions between the substrate sugar of glycoproteins and the lectin chaperone CRT.

Keywords Calreticulin · Isothermal titration calorimetry · Sugar–CRT interaction

Abbreviations

ER	Endoplasmic Reticulum
CRT	Calreticulin
CNX	Calnexin
ERAD	Endoplasmic Reticulum Associated protein Degradation
ITC	Isothermal Titration Calorimetry
GST	Gluathione-*S*-Transferase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MOPS	3-(*N*-Morpholino)-propanesulfonic acid
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis

Introduction

The quality control of proteins in cells is monitored by many mechanisms that are generally connected to degradation pathways. The endoplasmic reticulum (ER) is the port of entry and the main folding compartment for proteins destined to the exocytic pathway. ER quality control prevents the deployment of potentially harmful molecules

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to their final destinations and maintains homeostasis within the ER [1–3]. The oligosaccharide moieties present on many ER-synthesized proteins have been shown to play a crucial role in the quality control. Protein-carbohydrate interaction and recognition play crucial roles in the folding, assembly and trafficking of these polypeptides [4, 5]. The two homologous calcium and carbohydrate binding chaperones, Calreticulin (CRT) and Calnexin (CNX), which reside in the ER, aid this process. These lectin-like chaperones bind to the core glycan structure of the glycoproteins ($Glc_1Man_{5-9}GlcNAc_2$) [6–11]. Interaction with other protein folding catalysts such as protein disulphide isomerase and ERp57 assists in the protein folding process and prevention of aggregation of incompletely folded intermediates [12, 13]. Although CRT and CNX share high sequence identity and carbohydrate specificity, the set of glycoproteins interacting with each of these lectin chaperones overlap only partially [6, 14]. The crystal structure of CNX established it as a monovalent lectin. Identification of the ligand binding site provided insight into the mechanism by which it interacts with its substrates [15]. Given the sequence similarity between CNX and CRT and the lack of crystal structure of CRT, we earlier carried out molecular modeling of CRT lectin domain based on CNX [16].

The binding of CRT is specific for monoglucosylated N-linked oligosaccharides. As the $Glc_3Man_{5-9}GlcNAc_2$ glycan moiety is N-linked to the protein in the ER, the sequential action of glucosidase I and II removes two glucose residues leaving the monoglucosylated form of $Glc_1Man_{5-9}GlcNAc_2$, recognizable by CRT [17, 18]. Further, UDP-glucose: glycoprotein glucosyltransferase catalyzes the addition of glucose on the non-reducing end mannose of the α1–3 arm of $Man_{5-9}GlcNAc_2$, acting as a folding sensor and re-association of the unfolded glycoprotein with CRT [19–21]. Repeated cycles of de-glucosylation and re-glucosylation lead to prolonged association of the glycoprotein with CRT until proper folding occurs, failing which, the glycoprotein is targeted to the ERAD machinery [22, 23]. In our previous studies, molecular modeling of the interaction of CRT with the monoglucosylated mannose oligosaccharide identified several key residues which were involved in hydrogen bond interactions with the sugar moieties. The involvement of the identified residues was validated by carrying out site-directed mutagenesis and isothermal titration calorimetry (ITC) [16, 24, 25]. In this study we analyze the role of the atoms from sugar moieties of the trisaccharide Glcα1–3Manα1–2Man involved in hydrogen bond interactions with the protein. The mono-deoxy analogues of the trisaccharide were synthesized and used as substrate for CRT [26]. The binding affinity and thermodynamics were analyzed by carrying out ITC studies and were compared to the values for trisaccharide-CRT interaction to gain insight on the involvement of the particular atom in binding. The results corroborate the observations of molecular modeling.

Materials and methods

Materials Media components were obtained from Hi-Media (Delhi, India). GST-agarose, reduced glutathione, IPTG, MOPS and SDS-PAGE reagents were obtained from Sigma Chemicals Co. (St. Louis, MO).

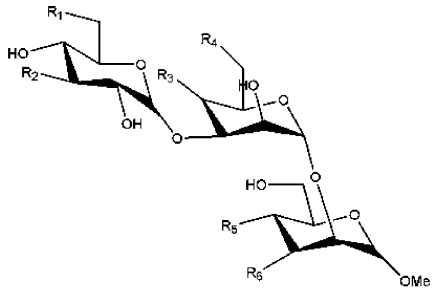
Synthesis of sugars The trisaccharide (Glcα1–3Manα1–2ManαMe) was synthesized as described in [27]. The mono-deoxy derivatives of trisaccharide (a–f) were synthesized as described in [26].

Expression and purification of recombinant calreticulin The CRT clone was a kind gift from Prof. Lars Ellgaard, University of Copenhagen. GST-CRT full-length fusion protein was purified essentially as described earlier by Helenius *et al.* [28]. Briefly, the construct was transformed into *Escherichia coli* DH5α cells. An overnight plateau phase culture was used to inoculate fresh Luria broth containing 50 mg/ml ampicillin, 2 mM $CaCl_2$ at a dilution of 1:100. Cells were grown at 37°C to A_{600} of 0.5–0.6 and induced by 0.2 mM IPTG followed by additional 6 hr incubation at 25°C. the cells were harvested and resuspended in a 1:10 culture volume of lysis buffer (500 mM Tris–HCl, 100 mM NaCl, pH 7.4) and sonicated on ice using a Branson tip sonicator. Lysate thus obtained was loaded on to the GST-agarose pre-equilibrated column. The specifically bound protein was eluted by 5 mM reduced glutathione in Tris buffer and concentrated using a 12 kDa Amicon concentrator and dialyzed against a 10 mM MOPS, 5 mM $CaCl_2$, 150 mM NaCl, pH 7.4, buffer.

Protein estimation The A_{280} for GST-CRT solution (1 mg/ml) was determined as 1.06 according to the method of Gill and von Hippel [29].

Isothermal titration calorimetry ITC was performed by using VP-ITC calorimeter from Microcal Inc. (Northampton, MA) as described previously [16]. Protein, 90–120 μM in 1.5 ml buffer (10 mM MOPS, 5 mM $CaCl_2$ and 150 mM NaCl, pH 7.4) was titrated with 8–10 μl of sugar solution (10 times the protein concentration) at an interval of 3 min using a syringe rotating at 400 rpm. The data so obtained were fitted via non-linear least squares minimization method to determine binding stoichiometry (n), binding constant K_b and change in enthalpy of binding ΔH_b°, using Origin software (Microcal). The change in free energy of binding ΔG_b° was calculated using Eq. (1):

$$\Delta G_b^\circ = -RT \ln K_b \quad (1)$$



Trisaccharide (Glu1-Man2-Man3): R1=R2=R3=R4=R5=R6=OH
Glu1 6-deoxy OH (Analogue a): R1=H, R2=R3=R4=R5=R6=OH
Glu1 3-deoxy OH (Analogue b): R2=H, R1=R3=R4=R5=R6=OH
Man2 4-deoxy OH (Analogue c): R3=H, R1=R2=R4=R5=R6=OH
Man2 6-deoxy OH (Analogue d): R4=H, R1=R2=R3=R5=R6=OH
Man3 4-deoxy OH (Analogue e): R5=H, R1=R2=R3=R4=R6=OH
Man3 3-deoxy OH (Analogue f): R6=H, R1=R2=R3=R4=R5=OH

Fig. 1 A graphical representation of the six mono-deoxy derivatives of the trisaccharide studied here. The analogues *a–f* each has a single deoxy-OH at position R1–6 as shown above

where R is the gas constant and T is the temperature in kelvin. The thermodynamics quantities so obtained were used to determine the change in entropy (ΔS) using Eq. (2):

$$\Delta G_b^\circ = \Delta H_b^\circ - T\Delta S \tag{2}$$

The experimental conditions ensured that c value ranged from 2 to 130 for all the titrations, where $c=K_b M_t$ (0) and M_t (0) is the initial macromolecular concentration. The value of binding constant K_b was used to compare the relative binding of CRT with the trisaccharide. The experiments were carried out at two temperatures 15°C (288 K) and 30°C (303 K).

Results

The interactions of substrate trisaccharide Glcα1–3Manα1–2Man and its deoxy-analogues were studied by isothermal titration calorimetry (at two temperatures 288 K and 303 K) as this technique gives a direct and precise estimate of the binding constant, the enthalpy change and reaction stoichiometry. A graphical representation of the six mono-deoxy derivatives of the trisaccharide studied here is given in Fig. 1.

Isothermal titration calorimetry

ITC experiment carried out with the trisaccharide provided the binding constant $K_{b288}=(78.9\times10^4)$ M^{-1} and $\Delta G^\circ_{b288}=-7.77$kcal/mol, which is quite consistent with experiments carried out earlier [16, 24, 25]. Values of the thermodynamic parameters obtained for the trisaccharide were used as a reference for the mono-deoxy analogues. The results for analogues a–f are tabulated in Table 1 and representative ITC plots are shown in Fig. 2. The 3-deoxyGlc analogue was shown to have reduced affinity for CRT binding, while the 6-deoxyGlc analogue showed a substantially increased affinity. It can be inferred from these observations that the position 6- of Glc in the trisaccharide may not play a major role in binding of the sugar to CRT, whereas position 3- does. Similar evaluations of the other analogues show that positions 4- and 6- of Man2 and positions 3- and 4- of Man3 are involved in sugar–CRT interaction. Of all the positions evaluated, positions 3- of Glc1, 6- of Man2 and 4- of Man3, show marked decrease in affinity upon deoxygenation, underscoring their importance in substrate–lectin interaction. These results confirm the involvement of all three saccharide units in sugar–CRT interaction as was predicted by modeling and site specific mutagenic studies earlier.

Table 1 Thermodynamic quantities for binding of sugars to calreticulin

Ligand	T (K)	N	$K_b\times10^4$ (M^{-1})	$-\Delta G_b^\circ$ (kcal/mol)	$-\Delta H_b^\circ$ (kcal/mol)	ΔS_b (cal/mol/K)
(control) Trisaccharide	288	0.96 (±0.06)	78.90 (±2.38)	7.77 (±0.04)	6.12 (±0.21)	5.73 (±0.45)
	303	0.92 (±0.04)	54.40 (±3.12)	7.95 (±0.03)	7.14 (±0.30)	2.67 (±0.56)
(a) Glu1 6-deoxy OH	288	0.79 (±0.06)	128.01 (±5.72)	8.05 (±0.05)	0.62 (±0.33)	25.79 (±0.83)
	303	0.85 (±0.02)	84.31 (±4.26)	8.21 (±0.03)	1.82 (±0.22)	21.11 (±0.97)
(b) Glu1 3-deoxy OH	288	0.87 (±0.05)	3.21 (±0.17)	5.94 (±0.04)	6.57 (±0.27)	−2.19 (±0.32)
	303	0.90 (±0.03)	1.74 (±0.15)	5.88 (±0.04)	6.93 (±0.24)	−3.47 (±0.39)
(c) Man2 4-deoxy OH	288	0.92 (±0.05)	53.22 (±3.54)	7.54 (±0.04)	6.65 (±0.35)	3.11 (±0.18)
	303	0.91 (±0.06)	38.53 (±2.76)	7.74 (±0.03)	7.12 (±0.29)	2.06 (±0.22)
(d) Man2 6-deoxy OH	288	0.87 (±0.03)	3.86 (±0.13)	6.04 (±0.03)	7.54 (±0.23)	−5.19 (±0.36)
	303	0.80 (±0.04)	2.89 (±0.13)	6.18 (±0.04)	7.98 (±0.25)	−5.92 (±0.48)
(e) Man3 4-deoxy OH	288	0.93 (±0.07)	4.30 (±0.11)	6.11 (±0.03)	8.65 (±0.24)	−8.84 (±0.56)
	303	0.90 (±0.05)	3.09 (±0.16)	6.22 (±0.04)	9.23 (±0.26)	−9.92 (±0.54)
(f) Man3 3-deoxy OH	288	0.78 (±0.02)	59.23 (±2.95)	7.61 (±0.03)	6.36 (±0.31)	4.33 (±0.25)
	303	0.83 (±0.06)	45.32 (±3.11)	7.84 (±0.05)	7.24 (±0.29)	1.98 (±0.32)

The values in parentheses are standard deviations for each fitted parameter

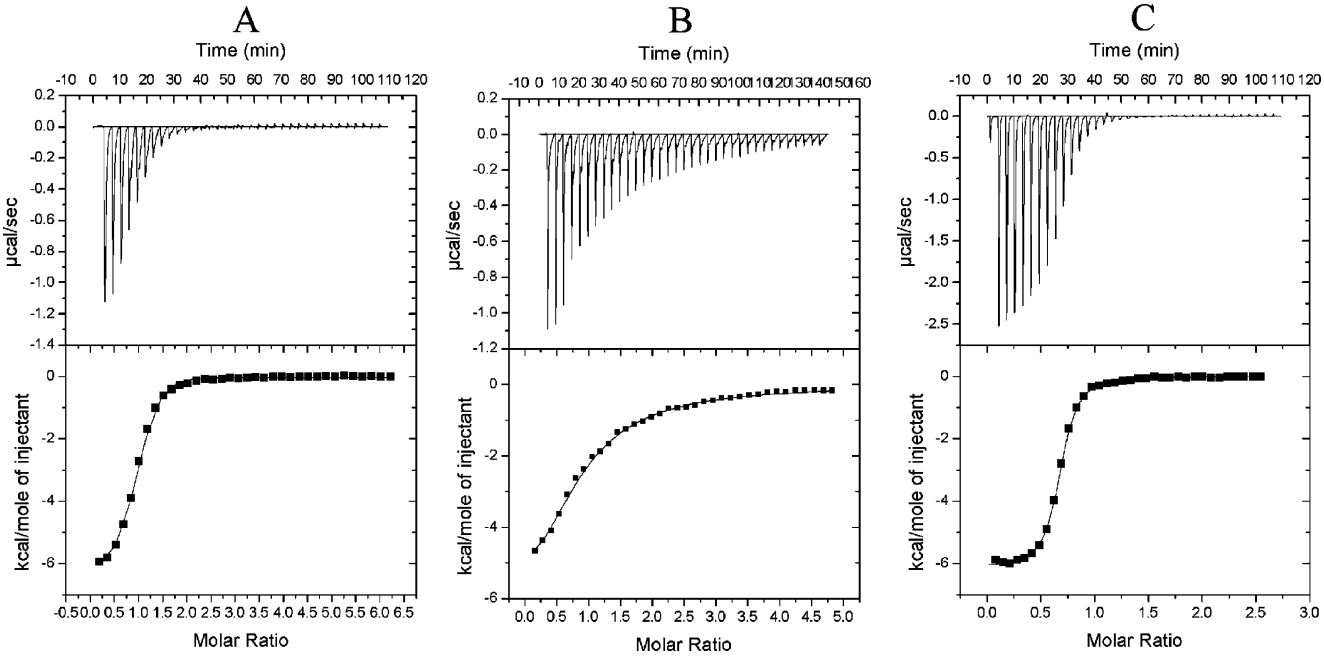


Fig. 2 Isothermal calorimetric titration of calreticulin with different sugars. The *upper panel* represents raw data obtained after injections of sugar solution into calreticulin in MOPS buffer. The *bottom panel* represents the non-linear least-squares fit of the heat released as a function of the added ligand for the titration. The data were fitted to a single-site model to obtain values of n, ΔH_b° and K_b for each sugar. The representative *curves* are **a** trisaccharide (standard), **b** Glu1 3-deoxy OH analogue, **c** Glu1 6-deoxy OH analogue

Discussion

It is known that the glycan intermediate $Glc_1Man_{5-9}GlcNAc_2$ formed in the ER mediates interaction between the glycoproteins and the lectin chaperone CRT, which leads to the proper folding of the glycoprotein substrates. The mode and thermodynamics of interaction between the sugar substrate and CRT has been the subject of study for the past many years. The unavailability of crystal or NMR structure of CRT prompted its molecular modeling based on the nearest homologue with the known structure, CNX. Molecular modeling of sugar substrates to this structure and ITC experiments were used to determine the probable mode of interaction. In our previous studies we determined that the glucoseα1–3 linkage was critical for the recognition of sugar substrate by CRT. Use of truncated glycan intermediates in ITC experiments showed that the binding constant increases by 25-fold from di- to trisaccharide unit and doubles from tri- to tetrasaccharide unit [16]. This demonstrated that all the saccharide moieties were involved in CRT–sugar interactions. Molecular modeling identified the probable amino acid residues at the sugar binding site of CRT and the atoms of sugar involved in hydrogen bonding with those amino acids. In another study, we carried out the site-directed mutagenesis of amino acid residues identified in sugar–CRT interactions and validated their involvement and role in sugar binding by ITC [24, 25]. In the present study, we determine the importance of various hydroxyl groups of the sugar in the interactions with CRT (Fig. 3).

Deoxy-analogues of sugars have frequently been used as valuable tools to determine the role of hydroxyl groups in binding [30, 31]. We chose the trisaccharide unit Glcα1–3Manα1–2Man for our study and six mono-deoxy analogues

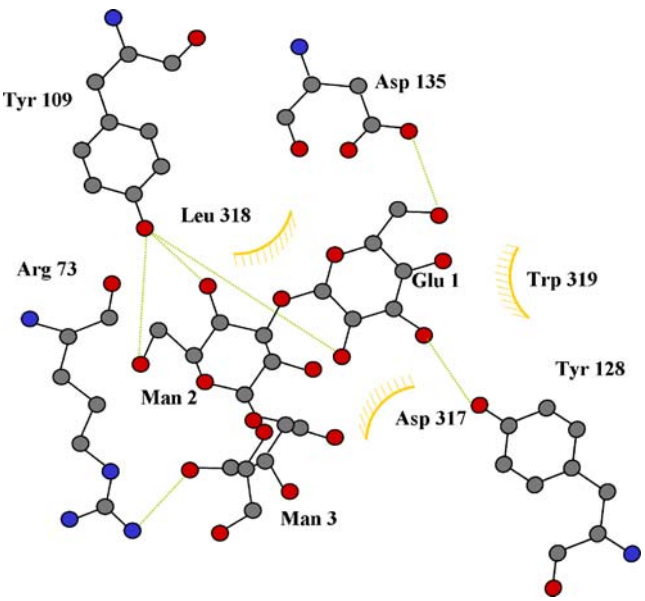


Fig. 3 A schematic representation of the interactions of trisaccharide with CRT. The *red*, *blue* and *grey* circles denote oxygen, nitrogen and carbon atoms respectively. Representative bonds between the hydroxyl groups of sugar and CRT have been denoted by *dotted green lines*. Three residues (Asp 317, Leu 318 and Trp 319) present on the other face of sugar are represented in *yellow*. They form hydrogen bonds as well as hydrophobic interactions with the sugar moieties

of this substrate. 3-deoxy and 6-deoxy Glc1 analogues of the trisaccharide were subjected to ITC. The 3-deoxy analogue showed highly reduced binding to CRT with the K_{b288} dropping to 3.21×10^4 M^{-1} from 78.9×10^4 M^{-1} of the standard trisaccharide. Also the value of ΔG_b° decreases significantly. This indicates that the 3-OH group of Glc makes important contacts with CRT during sugar–CRT interactions. It was proposed earlier that this 3-OH group makes hydrogen bonds with the Tyr128 residue of CRT. On the other hand, the 6-OH group of Glc does not seem to play a significant role in binding as was predicted earlier. Loss of this hydroxyl group greatly increases the affinity of binding of sugar to CRT. The lower enthalpy change associated with the binding can be related to the loss of –OH group from the position 6 and hence lesser extent of conformational perturbation in the binding event. This perhaps leads to decreased amount of secondary interactions, hence the enthalpy is less. Additionally the Asp side chain is liberated, due to the loss of hydrogen bonding with the –OH group resulting in more conformational freedom, thereby accounting for the more favorable entropy. In another set of experiments carried out by Y. Ito *et al.* [32] and A. Tatami *et al.* [33] it was determined that the 2-OH and 4-OH groups of Glc are also in close contact with CRT.

Both, the 4-deoxy and 6-deoxy Man2 analogues of trisaccharide unit, show decreased affinity of binding to CRT indicating that they are involved in sugar–CRT interactions. However, among the two, the 6-OH group has a more significant role to play (hydrogen bonds with Asp317, Trp319 and Tyr109) as specified by its approximately 18-fold decrease in K_{b288} and 20-fold decrease in ΔG_b° values. Of the third sugar moiety in the trisaccharide unit, 3-deoxy and 4-deoxy Man3 analogues were used. Both showed reduced binding to CRT as was predicted earlier, with the 4-OH group playing a more substantial role than the other as indicated by the binding constant. The ITC experiments were carried out at two temperatures 288 K and 303 K to determine whether change in temperature plays any major role in the binding reactions. However, no significant difference was observed in the binding of sugars to CRT at the two temperatures.

A perusal of all the results obtained for sugar–CRT interactions shows that several hydrogen bonds formed by the sugar hydroxyl groups play significant roles in binding of substrate to this lectin chaperone. Of these, some make considerably more contribution to the binding than others. Compiling results observed here with previous studies on CRT–sugar interactions, it can be summarized that the mono-glucosylated intermediate of glycan moiety is necessary for the recognition of glycoproteins by CRT. Several key residues of CRT viz. Arg73, Tyr109, Asp125, Tyr128, Asp135, Asp317, Leu318 and Trp319, are involved in sugar binding. Of these Asp 317 and Trp319 play crucial roles. They make strong hydrogen bonds with all three moieties of the trisaccharide unit; Glu1 (2-OH, 3-OH, 4-OH), Man2 (3-OH, 4-OH, 6-OH), Man3 (3-OH, 4-OH). The calorimetric studies performed on sugar–CRT interactions have significantly brought into light the mode and weightage of each predicted interaction.

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References

1. Helenius, A., Aebi, M.: Roles of N-linked glycans in the endoplasmic reticulum. Annu. Rev. Biochem. **73**, 1019–1049 (2004)
2. Ellgaard, L., Helenius, A.: Quality control in the endoplasmic reticulum. Nat. Rev. Mol. Cell Biol. **4**, 181–191 (2003)
3. Ellgaard, L., Molinari, M., Helenius, A.: Setting the standards: quality control in the secretory pathway. Science **286**, 1882–1888 (1999)
4. Roth, J.: Protein N-glycosylation along the secretory pathway: relationship to organelle topography and function, protein quality control and cell interactions. Chem. Rev. **102**, 285–303 (2002)
5. Trombetta, E.S.: The contribution of N-glycans and their processing in the endoplasmic reticulum to glycoprotein biosynthesis. Glycobiology **13**, 77–91 (2003)
6. Caramelo, J.J., Parodi, A.J.: Getting in and out from calnexin/calreticulin cycles. J. Biol. Chem. **283**, 10221–10225 (2008)
7. Williams, D.B.: Beyond lectins: the calnexin/calreticulin chaperone system of the endoplasmic reticulum. J. Cell Sci. **119**, 615–623 (2006)
8. Patil, A.R., Thomas, C.J., Surolia, A.: Kinetics and the mechanism of interaction of the endoplasmic reticulum chaperone, calreticulin, with mono-glucosylated (Glc1Man9GlcNAc2) substrate. J. Biol. Chem. **275**, 24348–24356 (2000)
9. Michalak, M., Corbett, E.F., Mesaeli, N., Nakamura, K., Opas, M.: Calreticulin: one protein, one gene, many functions. Biochem. J. **344**, 281–292 (1999)
10. Krause, K.H., Michalak, M.: Calreticulin. Cell **88**, 439–443 (1997)
11. Ware, F., Vassilakos, A., Peterson, P.A., Jackson, M.R., Lehrman, M.A., Williams, D.B.: The molecular chaperone Calnexin binds Glc1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoproteins. J. Biol. Chem. **270**, 4697–4704 (1995)
12. Oliver, J.D., van der Wal, F.J., Bulleid, N.J., High, S.: Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. Science **275**, 86–88 (1997)
13. Baksh, S., Burns, K., Andrin, C., Michalak, M.: Interaction of calreticulin with protein disulphide isomerase. J. Biol. Chem. **270**, 31338–31344 (1995)
14. Hebert, D.N., Zhang, J.X., Chen, W., Foellmer, B., Helenius, A.: The number and location of glycans on influenza hemagglutinin determine folding and association with calnexin and calreticulin. J. Cell Biol. **139**, 613–623 (1997)
15. Schrag, J.D., Bergeron, J.J.M., Li, Y., Borisova, S., Hahn, M., Thomas, D.Y., Cygler, M.: The structure of Calnexin, an ER chaperone involved in quality control of protein folding. Mol. Cell **8**, 633–644 (2001)

16. Kapoor, M., Srinivas, H., Kandiah, E., Gemma, E., Ellgaard, L., Oscarson, S., Helenius, A., Surolia, A.: Interactions of substrate with calreticulin, an endoplasmic reticulum chaperone. J. Biol. Chem. **278**, 6194–6200 (2003)
17. Totani, K., Ihara, Y., Matsuo, I., Ito, Y.: Substrate specificity analysis of endoplasmic reticulum glucosides II using synthetic high-mannose type glycans. J. Biol. Chem. **281**, 31502–31508 (2006)
18. Roth, J., Ziak, M., Zuber, C.: The role of glucosidase II and endomannosidase in glucose trimming of asparagine-linked oligosaccharides. Biochimie **85**, 287–294 (2003)
19. Ritter, C., Quirin, K., Kowarik, M., Helenius, A.: Minor folding defects trigger local modification of glycoproteins by the ER folding sensor GT. EMBO J. **24**, 1730–1738 (2005)
20. Totani, K., Ihara, Y., Matsuo, I., Koshino, H., Ito, Y.: Synthetic substrates for an endoplasmic reticulum protein-folding sensor, UDP-glucose: glycoprotein glucosyltransferase. Angew Chem. Int. Ed. Engl. **44**, 7950–7954 (2005)
21. Soldà, T., Galli, C., Kaufman, R.J., Molinari, M.: Substrate-specific requirements for UGT1-dependent release from calnexin. Mol. Cell **27**, 238–249 (2007)
22. Ruddock, L.W., Molinari, M.: N-glycan processing in ER quality control. J. Cell Sci. **119**, 4373–4380 (2006)
23. Meusser, B., Hirsch, C., Jarosch, E., Sommer, T.: ERAD: the long road to destruction. Nat. Cell Biol. **7**, 766–772 (2005)
24. Gopalakrishnapai, J., Gupta, G., Karthikeyan, T., Sinha, S., Kandiah, E., Gemma, E., Oscarson, S., Surolia, A.: Isothermal titration calorimetric study defines the substrate binding residues of calreticulin. Biochem. Biophys. Res. Comm. **351**, 14–20 (2006)
25. Kapoor, M., Ellgaard, L., Gopalakrishnapai, J., Schirra, C., Gemma, E., Oscarson, S., Helenius, A., Surolia, A.: Mutational analysis provides molecular insight into the carbohydrate-binding region of calreticulin: pivotal roles of tyrosine-109 and aspartate-135 in carbohydrate recognition. Biochemistry **43**, 97–106 (2004)
26. Gemma, E., Lahmann, M., Oscarson, S.: Synthesis of monodeoxy analogues of the trisaccharide alpha-D-Glcp-(1®3)-alpha-D-Manp-(1®2)-alpha-D-ManpOMe recognised by Calreticulin/Calnexin. Carbohydr. Res. **341**, 1533–1542 (2006)
27. Gemma, E., Lahmann, M., Oscarson, S.: Synthesis of the tetrasaccharide alpha-D-Glcp-(1®3)-alpha-D-Manp-(1®2)-alpha-D-Manp-(1®2)-alpha-D-Manp recognized by calreticulin/calnexin. Carbohydr. Res. **340**, 2558–2562 (2005)
28. Peterson, J.R., Helenius, A.: *In vitro* reconstitution of calreticulin-substrate interactions. J. Cell Sci. **112**, 2775–2784 (1999)
29. Gill, S.C., von Hippel, P.H.: Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. **182**, 319–326 (1989)
30. Oscarson, S., Svahnberg, P.: Synthesis of a tri- and tetradeoxy analogue of methyl 3,6-di-*O*-alpha-D-mannopyranosyl-alpha-D-mannopyranoside for investigation of the binding site of various plant lectins. Carbohydr Res. **309**, 207–212 (1998)
31. Oscarson, S., Tedebark, U.: Syntheses of deoxy analogues of methyl 3,6-di-*O*-alpha-D-mannopyranosyl-alpha-D-mannopyranoside for studies of the binding site of concanavalin A. Carbohydr Res. **278**, 271–287 (1995)
32. Ito, Y., Hagihara, S., Arai, M.A., Matsuo, I., Takatani, M.: Synthesis of fluorine substituted oligosaccharide analogues of monoglucosylated glycan chain, a proposed ligand of lectin-chaperone calreticulin and calnexin. Glycoconj. J. **21**, 257–266 (2004)
33. Tatami, A., Hon, Y.S., Matsuo, I., Takatani, M., Koshino, H., Ito, Y.: Analyses of carbohydrate binding property of lectin-chaperone calreticulin. Biochem. Biophys. Res. Comm. **364**, 332–337 (2007)