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Glycan specificity of a testis-specific lectin chaperone calmegin and effects of hydrophobic interactions

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ABSTRACT

Background: Testis-specific chaperone calmegin is required for the generation of normal spermatozoa. Calmegin is known to be a homologue of endoplasmic reticulum (ER) residing lectin chaperone calnexin. Although functional similarity between calnexin and calmegin has been predicted, detailed information concerned with substrate recognition by calmegin, such as glycan specificity, chaperone function and binding affinity, are obscure.
Methods: In this study, biochemical properties of calmegin and calnexin were compared using synthetic glycans and glycosylated or non-glycosylated proteins as substrates.
Results: Whereas their amino acid sequences are quite similar to each other, a certain difference in secondary structures was indicated by circular dichroism (CD) spectrum. While both of them inhibited protein heat-aggregation to a similar extent, calnexin exhibited a higher ability to facilitate protein folding. Similarly to calnexin, calmegin preferentially recognizes monoglucosylated glycans such as $Glc_1Man_9GlcNAc_2$ (G1M9). While the surface hydrophobicity of calmegin was higher than that of calnexin, calnexin showed stronger binding to substrate. We reasoned that lectin activity, in addition to hydrophobic interaction, contributes to this strong affinity between calnexin and substrate.
Conclusions: Although their similarity in carbohydrate binding specificities is high, there seems to be some differences in the mode of substrate recognition between calmegin and calnexin.
General significance: Properties of calmegin as a lectin-chaperone were revealed in comparison with calnexin.

1. Introduction

Glycan processing plays an important role in glycoprotein quality control in the endoplasmic reticulum (ER) [1,2]. As nascent glycoproteins bearing triglucosylated high-mannose-type N-glycan ($Glc_3Man_9GlcNAc_2$; G3M9) are generally unfolded, various carbohydrate active proteins and chaperones in the ER assist them in achieving mature folding. Namely, in the beginning, nascent glycoproteins are trimmed by glucosidase-I and -II to the $Glc_1Man_9GlcNAc_2$ (G1M9) glycoform and then captured by ER-residing chaperones calnexin (CNX) or calreticulin (CRT). Both CNX and CRT are characterized by their property as lectins, which are specific to monoglucosylated glycans, typically G1M9. In addition, ERp57, a member of the protein disulfide isomerase family, is in association with CNX/CRT, and facilitates disulfide bond formation and isomerization [3]. Subsequently, upon removal of the remaining glucose residue by glucosidase II, client glycoproteins are liberated from CNX/CRT. At this stage, their folding states are surveyed by a folding sensor enzyme, UDP-glucose:glycoprotein glucosyltransferase (UGGT). When the folding is incomplete, liberated glycoproteins are reglucosylated by UGGT, regenerating the monoglucosylated glycoforms (*e.g.* G1M9), which are re-captured by CNX/CRT. After repeated interaction with glucosidase II, CNX/CRT, and UGGT (CNX/CRT cycle), glycoproteins that have achieved mature folding are exported to the Golgi apparatus.

While CNX is a transmembrane protein, previous studies have shown that its lectin and chaperone regions both localize in the luminal side of the ER [4]. As shown in Fig. 1, CNX is composed of N-, P-, and C-domains. The N-domain is proposed to have a carbohydrate binding activity, while the P- and C-domains are responsible for association with ERp57 and calcium storage, respectively [5]. In addition to the C-domain, a calcium binding site was also identified in the N-domain. X-ray diffraction study has revealed that the structure of the N-domain is globular and

Abbreviations: Glc, glucose; Man, mannose; GlcNAc, N-acetylglucosamine; CNX, calnexin; CRT, calreticulin; UGGT, UDP-glucose:glycoprotein glucosyltransferase; CMG, calmegin; CRT3, calsperin or calreticulin 3; ANS, 8-anilino-1-naphtalene sulfonic acid; CAB, bovine carbonic anhydrase B; Cbz, carboxybenzyl; CS, citrate synthase

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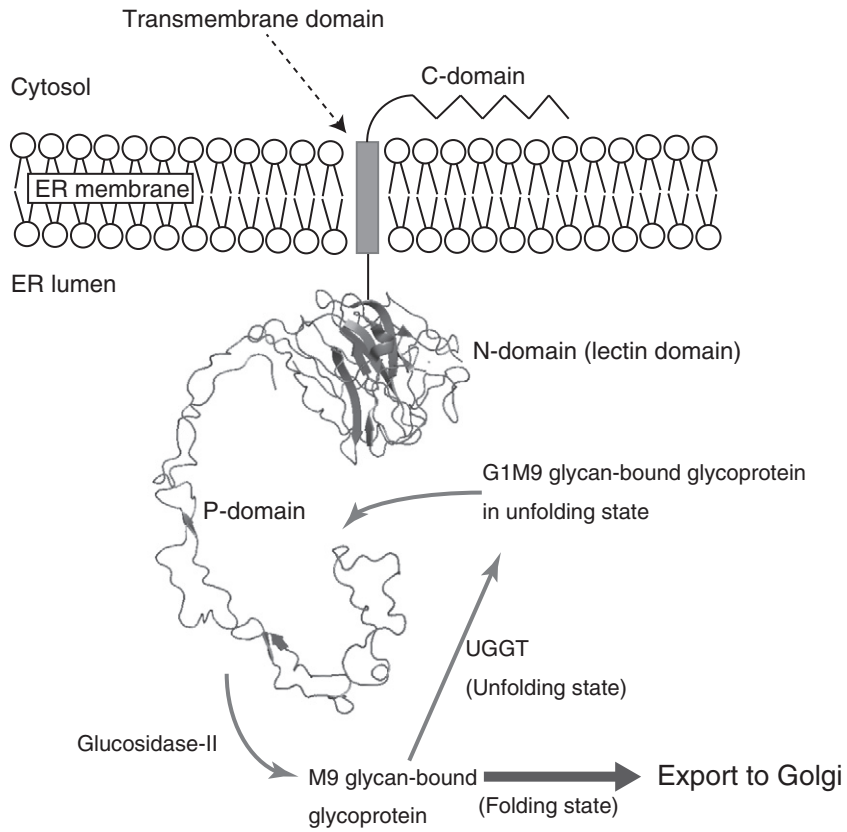

Fig. 1. Structure and schematic function of calnexin. The figure was drawn based on the structure of cCNX (PDB code 1JHN).

composed of β sandwich folds, while the P-domain holds a β strand hairpin form due to the presence of a proline-rich amino acid sequence [6].

Testis-specific chaperones calmegin (CMG) and calsperin (CRT3) are homologues of CNX and CRT, respectively [7–9]. Although CNX gene deletion was shown to cause early-postnatal death in mouse [10], CMG knockout mice were fully viable, while the generation of normal spermatozoa was affected [11]. In particular, expression of spermatozoal proteins such as ADAM1, ADAM2 and ADAM3, which are involved in zona pellucida binding or migration from the uterus into the oviduct, was reduced by CMG deletion, which caused male infertility [12–14]. Whereas CMG is required for the formation of a heterodimeric complex consisting of ADAM1 and ADAM2, CNX is not able to support this function [11]. Moreover, it has been reported that CMG is specifically expressed in germ cells of pachytene spermatocyte to spermatid stage, while CNX is expressed throughout all stages of spermatogenesis [15]. Therefore, the physiological role of CMG is assumed to be distinct from CNX. However, unlike CNX, the mode of substrate–CMG interaction, such as glycan specificity, chaperone activity and binding affinity, has been obscure.

Our previous studies have employed synthetic oligosaccharides modified by various aglycons as versatile substrates to study carbohydrate active proteins involved in the glycoprotein quality control system [16,17]. For instance, we have previously clarified that the reactivity of synthetic M9 derivatives as substrates of UGGT was affected by the nature of aglycons, particularly the magnitude of their hydrophobicity [18]. Synthetic N-glycan derivatives have proven valuable also as substrates of glucosidase-II and CRT [18–20]. Kinetic constants of enzyme reactions were found to be quite similar between glycoproteins and synthetic N-glycans [21], indicating that our approaches based on non-proteinic substrates are reasonable for the analyses of the glycoprotein quality control system mediated by carbohydrate–protein interactions.

In this study, to clarify its substrate recognition mechanism, we examined the biochemical properties of CMG, in comparison with CNX, using synthetic N-glycan derivatives and glycosylated or non-glycosylated proteins. Although their amino acid sequences are quite similar to each other, some differences in the secondary structure were observed by CD analysis. Both of them were able to inhibit protein aggregation to a similar extent, whereas differences were seen in their ability to assist refolding of a non-glycosylated protein. Although, similarly to CNX, CMG was able to interact specifically with monoglucosylated high-mannose-type glycans such as G1M9, subtle differences between them in terms of fine specificity of substrate recognition were observed.

2. Materials and methods

2.1. Materials

M7-BODIPY, M8-BODIPY, M9-BODIPY, G1M9-BODIPY, G2M9-BODIPY, G3M9-BODIPY, G1M8B-BODIPY, G1M8C-BODIPY, G1M7BC-BODIPY, G1M5B2C2-BODIPY, G1M6BC2-BODIPY, and G1M9-Cbz were prepared as previously reported [20,22]. ClogP values were calculated using ChemDraw Ultra version 6.0, Cambridge Soft (Cambridge, MA). Calcium chloride, calcium carbonate, guanidine hydrochloride, 10–20% Tris–glycine gels, tris–hydroxymethyl aminomethane hydrochloride, β-mercaptoethanol, dithiothreitol and acetonitrile were purchased from Wako Pure Chemical Industries (Osaka, Japan). 8-Anilino-1-naphtalene sulfonic acid (ANS) was obtained from MP Biomedicals (Illkirch, France). Recombinant firefly luciferase, citrate synthase, bovine carbonic anhydrase (CAB) and *p*-nitrophenylacetate were purchased from Sigma-Aldrich (St. Louis, MO). IgY was isolated from chicken egg yolk as previously reported [23]. VIVACON500 was obtained from Sartorius Stedim Biotech S.A. (Goettingen, Germany).

2.2. Preparation of recombinant human calnexin (hCNX) and human calmegin (hCMG)

Recombinant hCNX and hCMG were produced by an *Escherichia coli* expression system. A cDNA encoding 19–481 amino acids of hCNX was cloned into a pCold I expression plasmid (Takara Bio, Otsu, Japan), which is designed to produce N-terminally $(His)_6$-tagged proteins. Similarly, an expression plasmid encoding 18–469 amino acids of hCMG was constructed. The obtained plasmid was transformed into BL21 cells, and the recombinant proteins were expressed and purified using Ni-NTA agarose (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The obtained proteins were dialyzed in 10 mM MOPS buffer (pH 7.5) using Slide-A-Lyzer dialysis cassettes (10,000 MWCO, Pierce, IL, USA). The yields of hCNX and hCMG were 1.1 and 0.8 mg from 1 l culture, respectively.

2.3. Circular dichroism measurements of hCNX and hCMG

CD spectra were recorded from 200 to 260 nm, with 100 nm min^{-1} scanning speed on a J-720 CD spectropolarimeter (JASCO, Japan). The spectra were collected and averaged over 10 scans. All experiments were performed at 37 °C, using quartz cells of 1.0 mm optical path length. The 10 mM Tris–HCl buffer solution (pH 7.5) containing 2 μM chaperone and 10 mM $CaCO_3$ was used as samples of CD spectra assay.

2.4. Measurement of protein hydrophobicity by fluorescence probe ANS

A solution of hCNX or hCMG (10 μM) in 10 mM MOPS (pH 7.5) containing 10 mM $CaCl_2$ was incubated with 50 μM ANS at 37 °C for 10 min. The fluorescence was measured with the excitation at 360 nm and the emission recorded from 400 to 600 nm, using a FP-6500 fluorescent spectrophotometer (JASCO) at 37 °C in a 1 cm path-length cell.

2.5. Light scattering assay for protein thermal aggregation

2.5.1. CS aggregation

hCNX or hCMG was added to a final concentration of 50 nM in 10 mM MOPS buffer (pH 7.5) and 10 mM $CaCl_2$. After preincubation for 10 min at 45 °C, 50 nM CS was added into the reaction solution, and data acquisition was started while the stirring was continued throughout the measurement. Protein aggregation was monitored by light scattering at 400 nm with a FP-6500 fluorescent spectrophotometer for 5 min.

2.5.2. Luciferase aggregation

hCNX or hCMG was added to a final concentration of 10 μM in 20 mM HEPES buffer (pH 7.4) and 10 mM $CaCl_2$. After preincubation for 10 min at 37 °C, 3 μM luciferase was added into the reaction solution, and data acquisition was started while the stirring was continued throughout the measurement. Protein aggregation was monitored by light scattering at 400 nm with a FP-6500 fluorescent spectrophotometer for 5 min.

2.5.3. IgY aggregation

IgY was dialyzed overnight against denaturing buffer containing 100 mM Tris–HCl buffer (pH 7.0), 6 M guanidine hydrochloride and 40 mM dithiothreitol. The aggregation assay using IgY (0.25 μM) was performed in buffer containing 10 mM Tris–HCl (pH 7.0) and 10 mM $CaCl_2$ with or without 0.25 μM lectin chaperones [24,25]. The data acquisition was started while the stirring was continued throughout the measurement. The temperature was raised to 42 °C, and protein aggregation was monitored by light scattering at 300 nm with a FP-6500 fluorescent spectrophotometer for 5 min.

2.6. Refolding of denatured bovine carbonic anhydrase (CAB) in the presence of the chaperones

CAB was diluted into 350 μM as a solution in 10 mM MOPS buffer (pH 7.5) containing 5 M guanidine hydrochloride. The solution was incubated for 16 h at room temperature to denature CAB. hCNX or hCMG was dissolved in 10 mM MOPS buffer (pH 7.5) containing 10 mM $CaCl_2$. The preparation of denatured CAB was diluted with a solution of hCNX or hCMG by 500-fold. The mixtures were incubated for 24 h at 37 °C and the refolding was estimated by the esterase activity of CAB. The activity was measured by the increase of absorbance at 348 nm associated with hydrolysis of *p*-nitrophenyl acetate using with a V-650 UV–vis spectrophotometer (JASCO). The proportion of refolded CAB was determined by comparing the catalytic activities between native and refolded CAB.

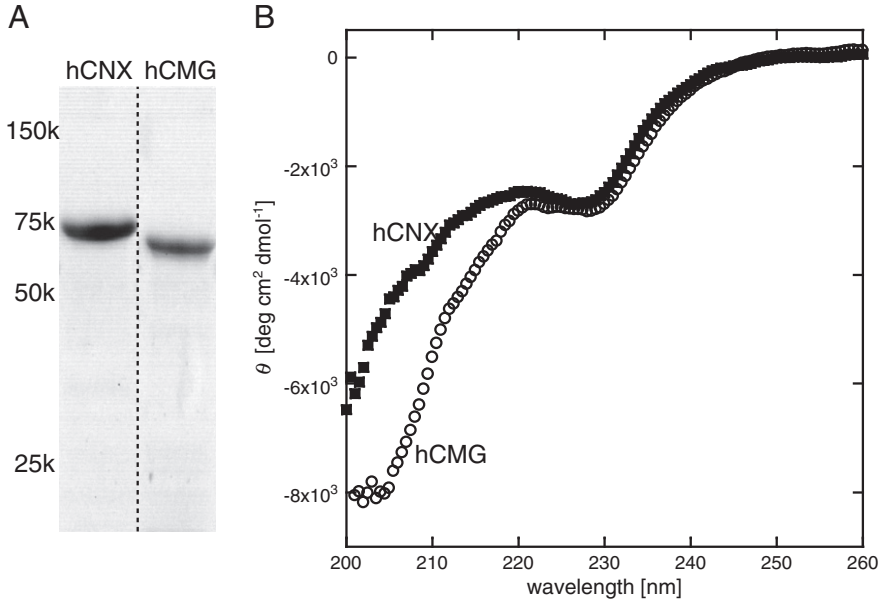

Fig. 2. Analyses of the secondary structure of hCNX and hCMG. (A) SDS-PAGE (10–20%) of recombinant hCNX and hCMG (CBB staining). (B) CD spectrum measurement of hCNX and hCMG. All CD spectra were analyzed for 2 μM chaperone solution dissolved with 10 mM Tris–HCl buffer (pH 7.5) containing 10 mM $CaCO_3$ by CD spectropolarimeter.

2.7. Multiple alignment of amino acid sequence and homology modeling

The alignment program Clustal X was used to align sequences of hCNX and hCMG, in comparison with canine CNX (cCNX). All sequences in FASTA format were obtained from the Genbank database. Their accession numbers were the following: cCNX, NP_001003232; hCNX, AAA36125; and hCMG, BAA22590. Subsequently, the PDB structure of cCNX (PDB ID: 1JHN) was used as a template to build 3D models of hCNX and hCMG in Swiss model work space (http://swissmodel.expasy.org/). All the analysis and molecular representations were rendered in PyMOL (Schrödinger).

2.8. Lectin–glycan interaction analysis by ultrafiltration method

The ultrafiltration method was employed to assess carbohydrate binding specificity of proteins as reported recently [26]. Briefly, a combined solution of hCNX or hCMG and a mixture of N-glycans were prepared in a buffer [10 mM MOPS (pH 7.5), 10 mM $CaCl_2$], which was equilibrated at room temperature. After 5 min, the solution was ultrafiltrated with VIVACON500 according to manufacturer's instructions at 5000 *g* for 2 min. The filtrate was subjected to HPLC analysis under the following conditions: Inertsil Amide column (4.6 mmϕ × 150 mm), mobile phase CH_3CN/100 mM ammonium formate (pH 4.5), linear gradient from 65:35 to 55:45 in 20 min, and flow rate 1.0 ml/min at 40 °C. BODIPY-labeled glycans were quantified by fluorescence intensities ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 520$ nm) with Waters 2475 fluorescence detector. The complexation yields were defined as the proportion of captured substrates by chaperone, which was calculated from HPLC peak areas.

The dissociation constants (K_d) between the lectin chaperone and G1M9-BODIPY were calculated by nonlinear regression curve fitting (KaleidaGraph, Synergy Software, Inc.), using the following equilibrium equation.

$$[\text{Chaperone}-\text{substrate}] = ([\text{chaperone}][\text{substrate}])/(K_d + [\text{chaperone}]).$$

3. Results

3.1. Expression and purification of hCNX and hCMG

Recombinant hCNX and hCMG used in this study were expressed as $(His)_6$-tagged soluble proteins which consisted of ER lumenal domains lacking signal sequences. In contrast to the anticipated molecular weights of hCNX and hCMG (56 and 55 kDa, respectively), SDS-PAGE analysis exhibited markedly higher molecular weights for both of them (Fig. 2A). This observation was in accordance with a previous report, in which the anomalous migration was ascribed to the high content of acidic amino acid residues [27]. As both hCNX and hCMG contain a large proportion of acidic amino acids such as glutamic acid and aspartic acid (Fig. 3), their slow migrating property is reasonable.

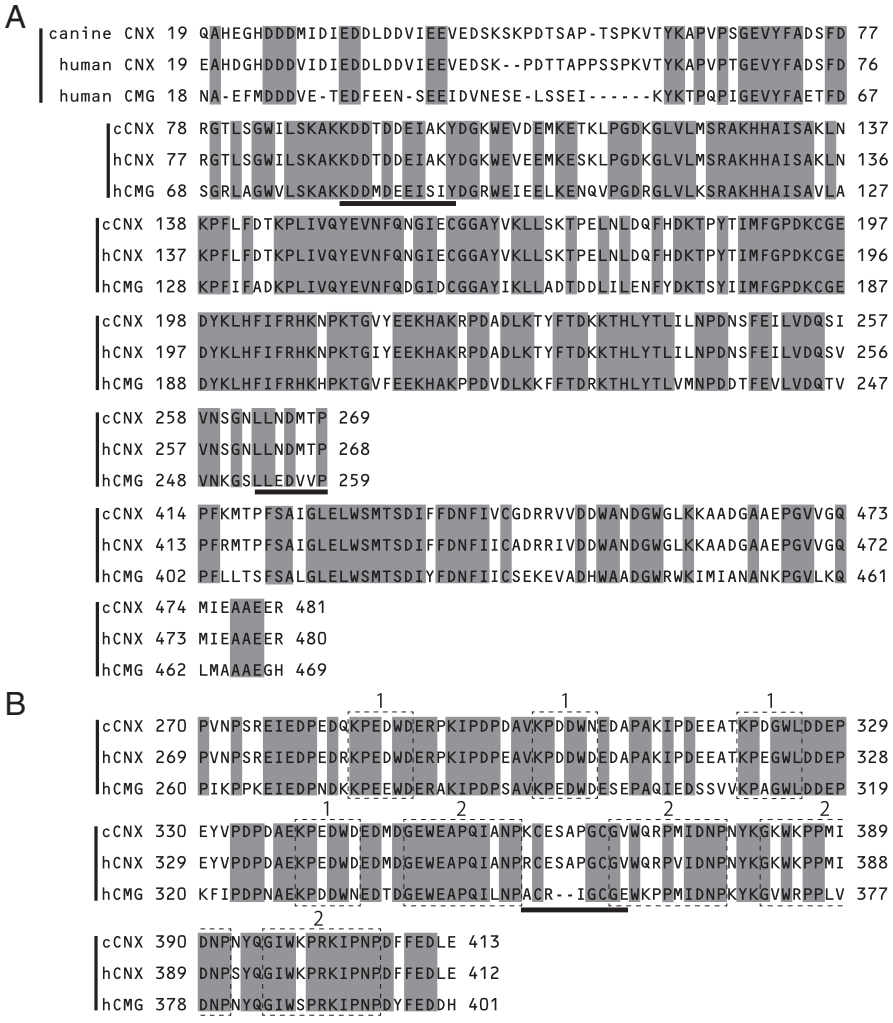

Fig. 3. Alignment of amino acid sequences. (A) Amino acid sequence of N-domain. (B) Amino acid sequence of P-domain. The enclosure shows amino acid sequences of Repeat 1 (KPEDWD) and Repeat 2 (G-W–P-I-NP).

In any event, successful expression and purification of both hCNX and hCMG were supported by the SDS-PAGE analysis, which exhibited singly migrating bands.

Subsequently, their secondary structures were analyzed by CD spectroscopy measurement (Fig. 2B). Their spectra were similar to that reported for the soluble region of canine CNX (cCNX) [28,29], with respect to the presence of a negative band at 228 nm. On the other hand, X-ray crystal study clarified that cCNX contains multiple β-sheets [6]. The seemingly abnormal CD spectrum of cCNX has been explained by the presence of a large number of tryptophan residues. The

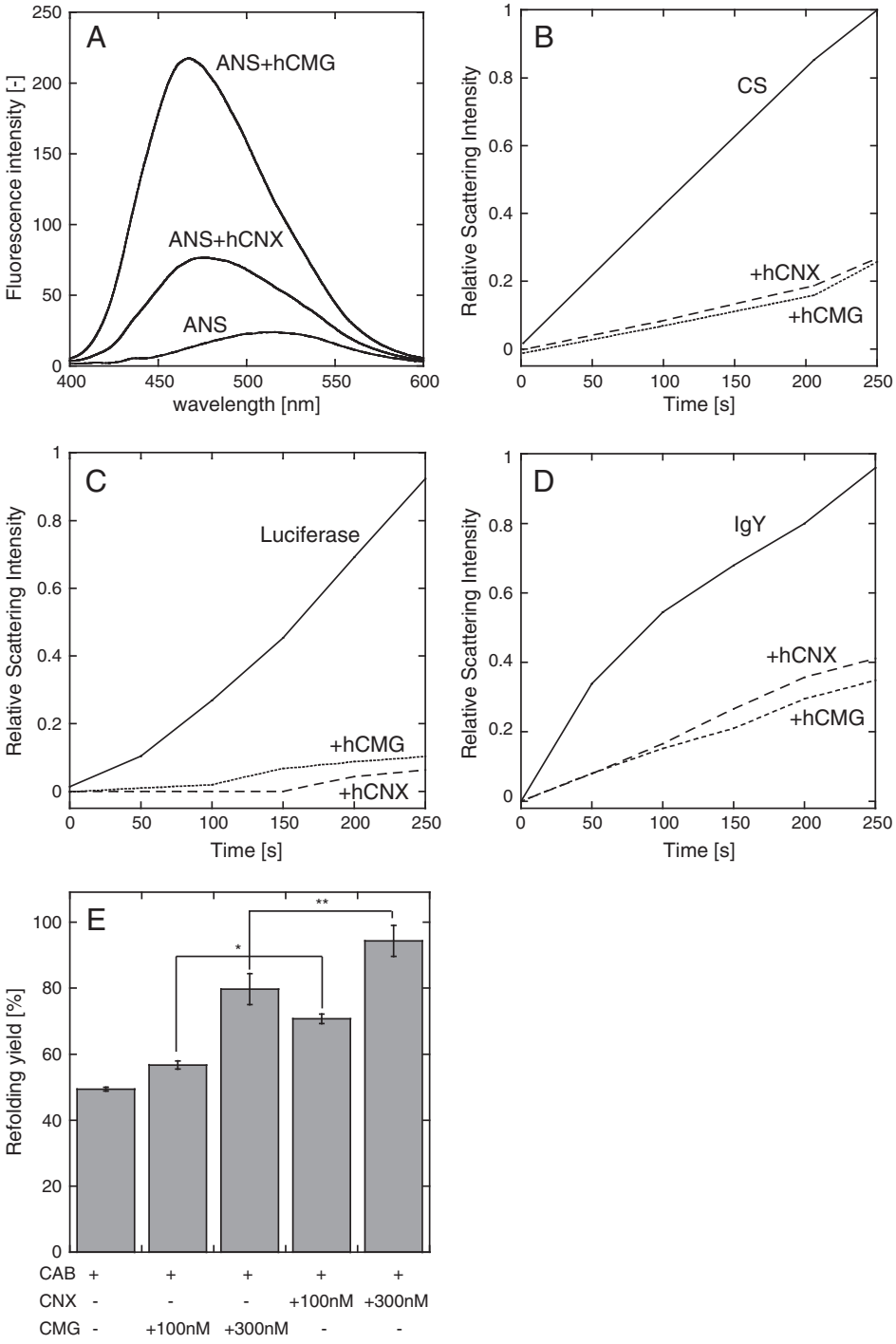

Fig. 4. Analyses of biochemical properties of hCNX and hCMG. (A) ANS binding assay for hCNX and hCMG. Both lectin chaperones (10 μM) dissolved in 10 mM MOPS (pH 7.5) and 10 mM $CaCl_2$ were incubated with 50 μM ANS at 37 °C for 10 min. The ANS-binding fluorescence was monitored with the excitation at 360 nm using a fluorescent spectrophotometer at 37 °C. (B) Effect of the chaperones on the thermal-aggregation of CS. The light scattering measurement was performed in 10 mM MOPS (pH 7.5) containing 50 nM chaperones and 50 nM CS. The reaction solution was incubated at 45 °C. (C) Effect of the chaperones on the thermal-aggregation of luciferase. The light scattering measurement was performed in 20 mM HEPES (pH 7.4) containing 10 μM chaperones and 3 μM luciferase. The reaction solution was incubated at 37 °C. (D) Effect of the chaperones on the thermal-aggregation of IgY. The light scattering measurement was performed in 10 mM Tris–HCl (pH 7.4) containing 0.25 μM chaperones and 0.25 μM IgY. The reaction solution was incubated at 42 °C. (E) Refolding of denatured CAB in the presence of the chaperones. The denatured CAB solution (350 μM) was diluted with the chaperone solution by five hundred-fold. The mixed solution was incubated for 24 h at 37 °C, and then the enzymatic activities of CAB were measured. The values shown are mean ± SE (n = 3). *$P < 0.001$; **$P < 0.05$ (Student's T-test).

side-chain of tryptophan is known to influence the CD spectrum of proteins, because aromatic rings exhibit a strong cotton effect in the far-UV region [30]. As tryptophan contents of hCNX and hCMG are both similar to cCNX (about 3%), a perturbation of CD profiles is expected for both of them (Fig. 3). Indeed, their spectra were nearly superimposable between 220 and 260 nm, whereas a significant difference was seen below 220 nm.

3.2. hCMG inhibits protein aggregation similarly to hCNX, whereas hCMG exhibits lower refolding ability than hCNX

Typically, molecular chaperones have a surface exposed hydrophobic region so as to interact with proteins in the non-native folding state [31]. Consequently, to explore its function as a chaperone, surface hydrophobicity of hCMG was measured in comparison with hCNX. To this end, ANS (8-anilino-1-naphtalene sulfonic acid) was employed as a probe, whose fluorescent intensity increases when exposed to hydrophobic environments [32].

The experiment indeed clarified that hCMG has significant hydrophobicity on its surface available to interact with ANS. Namely, as shown in Fig. 4A, both hCMG and hCNX caused an elevation of the fluorescent intensity, with a concomitant shift of the ANS's emission (515 nm) to a lower wavelength (480 nm). Notably, hCMG interacted more strongly with ANS than hCNX, as the fluorescence emission in the presence of hCMG was clearly more intense than that of hCNX, suggesting that hCMG possessed higher surface hydrophobicity.

Subsequently, in order to compare their chaperone activity, inhibitory effects against heat initiated protein aggregation were first compared by using citrate synthase (CS) as a model [28]. As shown in Fig. 4B, at 45 °C, the optical density of the solution increased over time, due to the aggregation of CS. Similarly to cCNX [33], both hCNX and hCMG inhibited the aggregation of CS, indicating their chaperone-like activity. However, under these conditions, uncertainty as for structural rigidity of these chaperones remained, because the melting temperature of cCNX was reported to be around 49 °C [28]. To remove this apprehension, a similar analysis was also performed using firefly luciferase which is known to aggregate at 37 °C, a temperature low enough to keep hCNX and hCMG in native conformation [28]. Again, both chaperones effectively inhibited luciferase aggregation (Fig. 4C). Evidently, the extent of inhibition was nearly identical between hCNX and hCMG. These results also suggest that their aggregation inhibition effects were retained both at 45 °C and 37 °C. To investigate the possible role of their lectin property in chaperone activity, we also examined a glycosylated protein, chicken IgY as a substrate. IgY is a glycoprotein abundantly available from chicken egg, whose high-mannose-type glycans are composed mainly of G1M9 [34]. The aggregation of IgY was efficiently suppressed by both chaperones to an extent similar to non-glycosylated proteins (Fig. 4D).

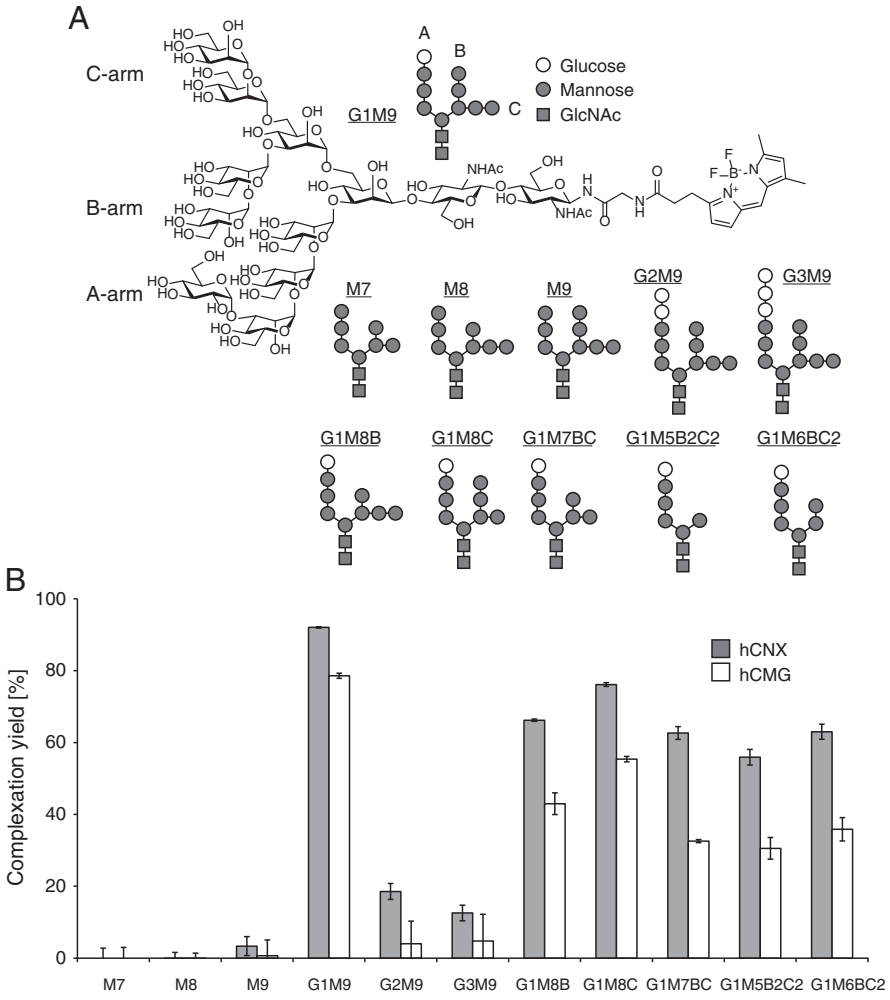

Fig. 5. Glycan-binding specificities of hCNX and hCMG. (A) Synthetic N-glycan substrates with Gly-BODIPY residues at the reducing termini. (B) Analysis of binding of 20 nM N-glycans to 10 μM lectin chaperones by the ultrafiltration method. The values shown are mean ± SE (n = 3).

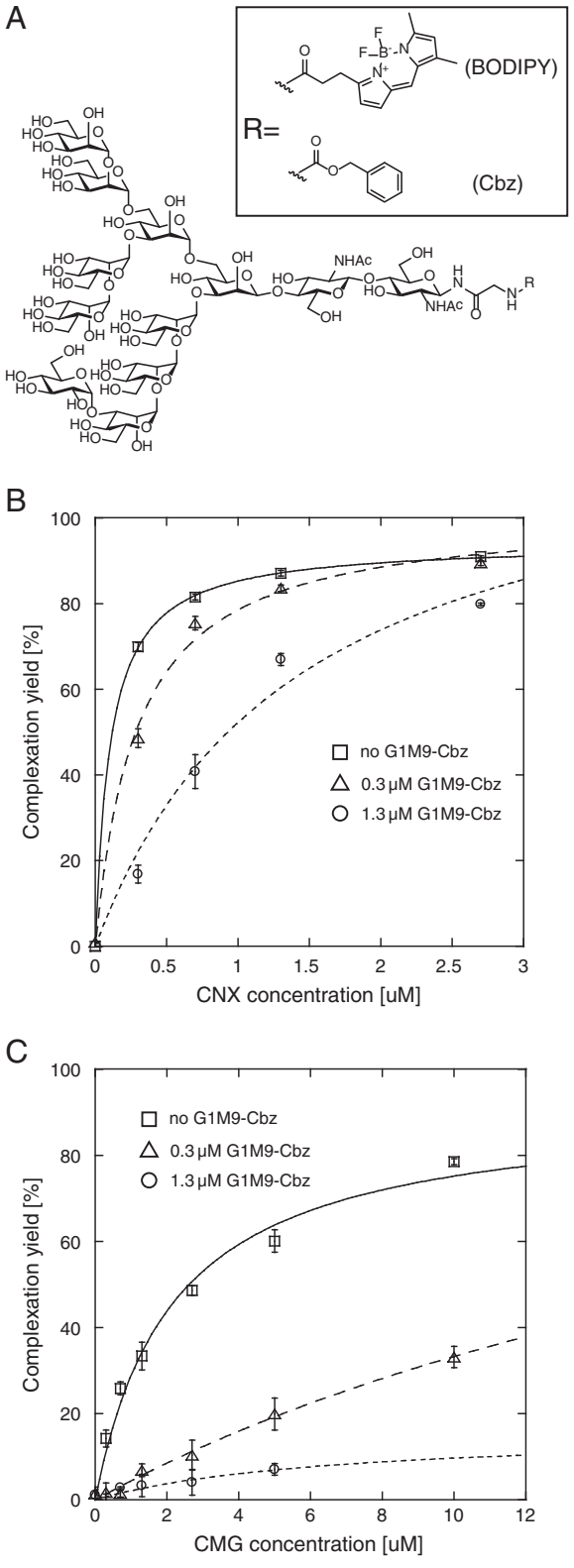

Fig. 6. Effect of aglycon on the interaction between lectin chaperones and synthetic N-glycan substrates. (A) G1M9-conjugated synthetic substrates which differed in aglycon chemicals. (B) Binding of 20 nM G1M9-BODIPY to various concentrations of hCNX in the absence or presence of G1M9-Cbz. The values shown are mean ± SE (n = 3). Coefficient determination r^2 values were over 0.98. (C) Binding of 20 nM G1M9-BODIPY to various concentrations of hCMG in the absence or presence of G1M9-Cbz. The values shown are mean ± SE (n = 3). Coefficient determination r^2 values were over 0.98.

We then examined their ability to facilitate protein refolding. For this purpose, bovine carbonic anhydrase B (CAB), a non-glycosylated enzyme with no disulfide linkage, was employed as a substrate [35]. The influences of the chaperones on the refolding of CAB are shown in Fig. 4E. In their absence, about 50% of the enzymatic activity was recovered when the 350 μM denatured CAB was diluted five hundred times without the chaperones. Inclusion of hCNX in the refolding buffer gave rise to a markedly larger recovery of the CAB activity. Effective refolding was observed even though the amount of hCNX was much lower than denatured CAB. Similarly, hCMG also exhibited the ability to assist the refolding, although its effect was clearly smaller than hCNX.

3.3. Although hCMG recognizes G1M9-N-glycans as well as hCNX, the recognition manners of each lectin chaperones to glycan are different

In light of the well-documented specificity of CNX as a lectin, CMG has been considered *a priori* to recognize monoglucosylated high-mannose-type glycans, although its specificity has not been verified experimentally. To clarify this, N-glycan substrates which were modified by a fluorophore were used for the interaction assay (Fig. 5A). Fig. 5B indicates the extent of complex formation between the chaperones and synthetic glycans. Similarly to hCNX, hCMG interacted most strongly with the G1M9 derivative. In addition, all monoglucosylated glycans exhibited interaction to hCMG, while little or no binding toward tri-, di-, and non-glucosylated substrates was observed. Since none of M7, M8 and M9 formed a complex with these lectin chaperones, complexation is glycan-dependent. Unlike denatured proteins, the hydrophobicity of a small aglycon such as BODIPY seems to be insufficient to cause an interaction strong enough to stabilize complexation with lectin chaperones. Noticeably, the strength of the interaction seems to be smaller as the number of mannose residues was reduced. Although hCMG uniformly exhibited lower carbohydrate binding than hCNX, the order of the magnitude was identical (G1M9 > G1M8C > G1M8B > G1M6BC2 > G1M7BC > G1M5G2C2 >> G2M9 ~ G3M9 > M9). All these results indicate that the glycan binding specificity of hCMG was quite similar to hCNX. However, a reduction of the complexation yield associated with the mannose truncation seems to be more apparent for hCMG. Noticeably, G1M8C-BODIPY exhibited a stronger interaction to the chaperones compared to other glycans having eight or less mannose residues, indicating the contribution of mannose residues on the B-arm toward an interaction with the chaperones.

3.4. hCMG recognizes N-glycan substrates in a different manner from hCNX

In order to examine the effect of the aglycon, carboxybenzyl (Cbz) modified G1M9 was examined as a ligand, in competition with G1M9-BODIPY (Fig. 6A). The ClogP values of BODIPY and Cbz were estimated to be 3.08 and 1.96, respectively, implicating that the hydrophobicity of BODIPY was over 10 times higher than that of Cbz. The extents of binding between G1M9-BODIPY and the chaperones were measured in the absence or presence of G1M9-Cbz using HPLC equipped with a fluorescence detector. As shown in Fig. 6B and C, responses to the addition of G1M9-Cbz were quite different between hCNX and hCMG. In the absence of G1M9-Cbz, the dissociation constants between them and G1M9-BODIPY were calculated to be 0.12 μM (hCNX) and 2.2 μM (hCMG), indicating that the affinity of hCNX toward G1M9-BODIPY is significantly higher than that of hCMG (Table 1). Interestingly, even in the presence of fifteen equivalents of G1M9-Cbz, the interaction between hCNX and G1M9-BODIPY was largely retained. This result suggests that the property of the aglycon affects the affinity of G1M9 to hCNX. In contrast, under the same conditions, the binding between G1M9-BODIPY and hCMG was more strongly suppressed, resulting in the increment of the dissociation constant, indicating that the effect of aglycon is less manifest in the hCMG–G1M9 interaction.

When a larger excess (65 equiv.) of G1M9-Cbz was added, a modest inhibition of the binding between G1M9-BODIPY and hCNX was

Table 1
Dissociation constants of the chaperones with G1M9-BODIPY in the presence of G1M9-Cbz.

Chaperone	20 nM G1M9 -BODIPY (μM)	20 nM G1M9-BODIPY with 0.3 μM G1M9-Cbz (μM)	20 nM G1M9-BODIPY with 1.3 μM G1M9-Cbz (μM)
Calnexin	0.12	0.29	1.4
Calmegin	2.2	27	–

observed, while the interaction between G1M9-BODIPY and hCMG was strongly suppressed, precluding the calculation of the dissociation constant. These results suggested that hydrophobicity of the aglycon portion of G1M9 affects more significantly in interacting with hCMG.

4. Discussion

Sequence alignment of cCNX, hCNX and hCMG revealed that a large number of amino acids are conserved among them. In particular, about 60 and 70% of amino acids in N- and P-domains, respectively, were conserved between hCNX and hCMG (Fig. 3). In the N-domain of cCNX, Tyr165, Lys167, Tyr186, Met189, Glu217 and Glu426 are known to play a crucial role in the interaction with glucose [36]. Indeed, these amino acids are fully conserved in both hCNX and hCMG. It is widely accepted that the two types of similar sequences (KPEDWD and G-W--P-I-NP) are multiply disposed in P-domains of CNX and CRT. These repeating sequences constitute a β strand which is likely to contribute in forming the structural motif of P-domain [6]. As shown in Fig. 3, the P-domain of cCNX includes the two types of four repeating sequences, while repeating sequences composed of similar amino acids are also present in hCNX and hCMG in the same region as cCNX. Therefore, the structural similarity of these chaperones is consistent with their homology at the amino acid sequence level. Nevertheless, some differences in the secondary structure between them were noticed by CD spectra (Fig. 2B), especially at 200–220 nm. The predicted tertiary structures of hCNX and hCMG were deduced by homology modeling based on the cCNX structure (Fig. 7A). Fig. 7B gives an overlay of their tertiary structures. Arrows show low-similarity regions, which are underlined in Fig. 3. Although the structural similarity of the N-domain is high, a clear difference in the tertiary structures of the two regions is predicted. In addition, the structural difference of the loop region in repeated sequences of the P-domain is also conceivable. Together, these considerations may well explain their differences in CD spectrum and strength of affinity to glycan substrates.

On the other hand, an ANS-binding assay clarified that hCMG has a higher degree of hydrophobicity than hCNX (Fig. 4A). Fig. 8 shows the distribution of hydrophobic amino acids at the protein surface based on their predicted structures. It indicates that the N-domain of hCMG seems to possess a markedly larger hydrophobic surface than the one of hCNX. Namely, the number of hydrophobic amino acids such as Phe, Val and Leu in the N-domain of hCMG is larger than that of hCNX as shown in Fig. 3A. By contrast, the hydrophobicity of their P-domain seems to be quite similar (Fig. 3B). Therefore, their difference in response to added ANS is likely to be a reflection of the level of hydrophobicity of the N-domain. Meanwhile, their inhibitory effects on protein aggregation were nearly identical (Fig. 4B, C, D), indicating that their surface hydrophobicity is high enough to interact with misfolded proteins. Nevertheless, hCNX more effectively assisted the refolding of non-native proteins to a larger extent compared to hCMG (Fig. 4E).

Given that affinities of CNX and CRT to mono-glucosylated glycoproteins are moderate [29,37], glycoprotein substrates are likely to be able to repeatedly associate with these lectin chaperones. Since glycoprotein refolding is considered to occur upon dissociation from lectin chaperones [4], an excessively strong hydrophobic interaction might cause irreversible binding between them, which would be detrimental to protein folding. This implication is reinforced by the fact that bovine serum albumin (BSA), which has high hydrophobicity, interacts with denatured proteins with no ability to assist their refolding [38].

As anticipated from its high homology to hCNX, hCMG was expected to have a glycan binding specificity similar to hCNX. Indeed, it exhibited an activity to interact with monoglucosylated glycans such as G1M9-BODIPY, while lacking binding affinity to glycans which were devoid of the glucose residue in the A-arm. In addition, truncation of a mannose residue from B- or C-arm noticeably reduced the interaction to both hCNX and hCMG. More specifically, the outermost mannose residue on the B-arm seemed to enhance the interaction, as the complexation yield of G1M8C was appreciably higher than other substrates having eight or less mannose residues. In addition, the orders of the binding specificity among various glycans are shown to be identical between hCMG and hCNX. These observations are all in accordance with their high degree of similarity of property as lectins.

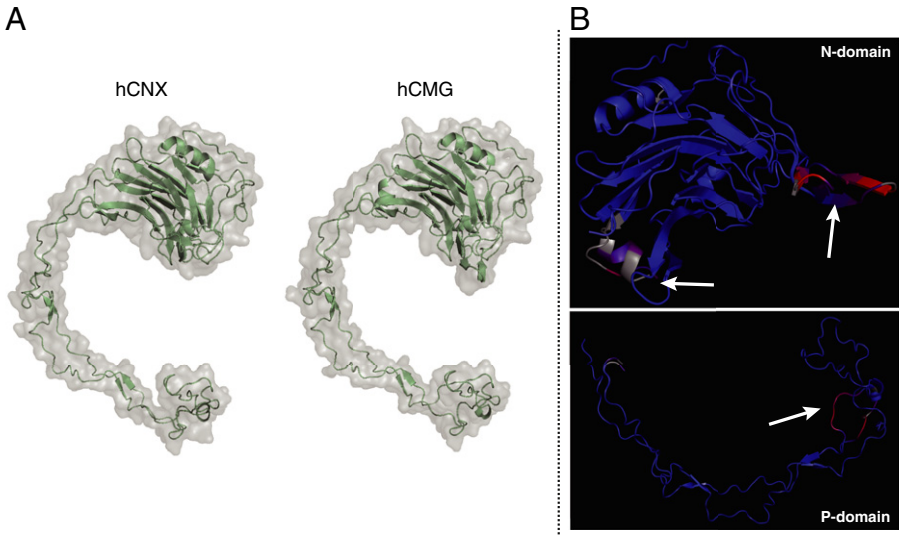

Fig. 7. (A) Predicted structures of hCNX and hCMG by homology modeling based on the cCNX structure. (B) Overlay pictures between hCNX and hCMG. Arrows indicate low-similarity regions.

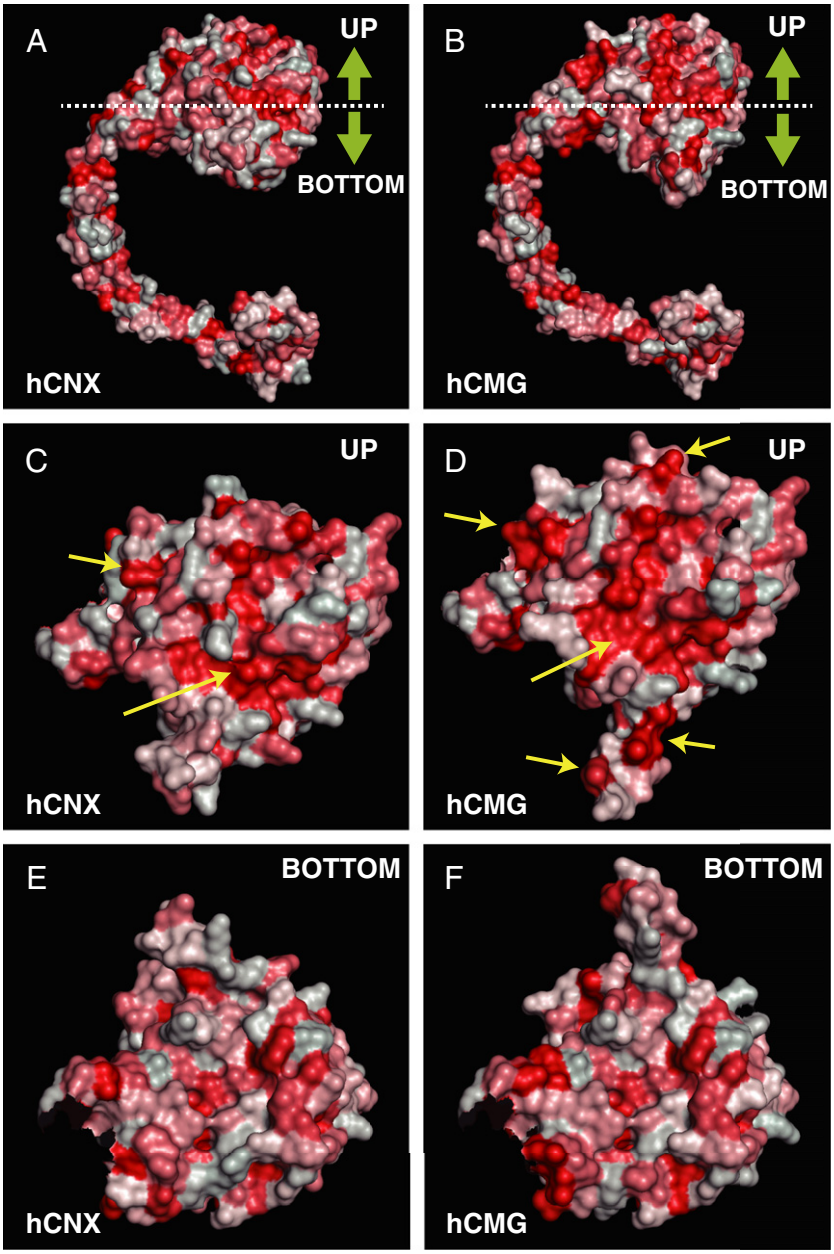

Fig. 8. Predicted hydrophobic regions at the protein surface of hCNX and hCMG according to Eisenberg's hydrophobicity scale [40]. The deep red color shows high hydrophobicity. (A, B) Whole structure of hCNX and hCMG. (C, D) Upside of N-domain. Arrows show hydrophobic regions. (E, F) Bottom side of N-domain.

While the surface hydrophobicity of hCMG was higher than that of hCNX, hCNX exhibited stronger binding to G1M9-BODIPY (Table 1). These results raise a possibility that testis-specific lectin chaperones intrinsically have lower sugar binding affinity than more widespread homologues CNX and CRT. In fact, previous analysis of CRT3, a homologue of CRT, was not able to detect its ability as a lectin [39].

Competition experiments showed a markedly reduced affinity of hCNX to G1M9-Cbz compared to G1M9-BODIPY. Since the hydrophobicity of BODIPY was estimated to be >10 times higher than Cbz, the presence of a hydrophobic interaction between hCNX and the aglycon portion of G1M9 derivatives is suggested. Somewhat to our surprise, however, the difference of the affinity to hCMG was much smaller between them. We speculate that the surface hydrophobicity of hCMG is high enough to interact strongly with both BODIPY and Cbz. Taken together, although the hydrophobicity of the aglycon affects binding to both hCMG and hCNX, the effect was more pronounced for the latter. Although their sugar binding specificities are markedly similar, there seems to be a subtle difference in the mode of substrate recognition between these lectin chaperones.

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