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Calreticulin facilitates the cell surface expression of ABCG5/G8

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Abstract

ATP-binding cassette (ABC) G5 (G5) and ABCG8 (G8) heterodimerize and function as sterol transporter that promote biliary excretion of neutral sterols. Both G5 and G8 interact with a lectin-like chaperone, calnexin (CNX), in the endoplasmic reticulum (ER) but the significance of this interaction remains unclear. Here, we show that not only CNX, but also its homologue calreticulin (CRT), is involved in the biosynthesis of G5/G8 sterol transporter. Both CNX and CRT interacted with immature forms of G5 and G8, and stimulated their productive folding by inhibiting their degradation. Interestingly, CRT predominantly enhanced the cell surface expression of mature G5/G8 whereas CNX did not have a similar effect. Inhibitors of *N*-glycan processing indicated that quality control of G5 and G8 might be differentially regulated in the ER. These findings clarify the role of CNX and CRT in the biosynthesis and quality control of G5/G8 sterol transporter.

Keywords: ABC transporter; Chaperone; Endoplasmic reticulum; Folding; Calreticulin

ATP-binding cassette (ABC) half transporters, ABCG5 (G5) and ABCG8 (G8), are highly expressed at the apical membrane in the enterocytes of the intestine and hepatocytes of the liver [1]. G5 and G8 heterodimerize and G5/G8 heterodimer functions as a sterol transporter, which limits intestinal absorption and promotes biliary excretion of neutral sterols [2–8]. Mutations and malfunction of either G5 or G8 cause sitosterolemia, an autosomal recessive disorder characterized by the accumulation of both plant-derived and animal-derived sterols in plasma and tissues [1,9,10]. In contrast, high-level expression of G5/G8 can attenuate diet-induced hypercholesterolemia and atherosclerosis [11]. Thus, it is important to understand the regulation mechanism of G5/G8 expression for maintaining cholesterol homeostasis and developing therapy for these sterol-related diseases.

Previous report showed that diets containing high cholesterol markedly increased the expression of G5 and G8 mRNA in mouse liver and intestine [12,13]. Moreover, agonists of the orphan nuclear receptor liver X receptor (LXR), such as T0901317, induce the expression of G5 and G8 at the transcriptional level [12,13]. It is known that after translation, both G5 and G8 proteins are *N*-glycosylated in the ER, and are retained in the ER until they form a G5/G8 heterodimer [3,14]. After heterodimerization, G5/G8 is exported from the ER to the Golgi where it undergoes complex glycosylation [3,7]. Ultimately, G5/G8 reaches the plasma membrane where it functions as a sterol transporter [3,7]. These reports indicate that heterodimerization in the ER seems to be a rate-limiting step for cell surface expression of G5/G8.

In this study, we investigated the role of CNX and its homologue calreticulin (CRT) on the biosynthesis and

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quality control of G5/G8. Our data demonstrate that CNX and CRT interact with immature forms of G5 and G8, and facilitate their folding. Interestingly, CRT predominantly stimulated the cell surface expression of G5/G8. Moreover, our data indicate that G5 and G8 might be regulated in distinct ER quality control mechanism. These results clarify the role of ER lectin-like chaperones in the biosynthesis and quality control of G5/G8 sterol transporter.

Materials and methods

Materials. The following antibodies were used in this study: rabbit anti-calnexin (anti-CNX; C-terminus specific; SPA-860; Stressgen Biotechnologies, Inc., San Diego, CA), rat monoclonal anti-Hsc70 (SPA-815, Stressgen), rabbit anti-calreticulin (anti-CRT; SPA-600; Stressgen), mouse monoclonal anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Myc (Upstate, Charlottesville, VA), mouse monoclonal anti-HA (HA-probe, Santa Cruz), and mouse monoclonal anti-α-tubulin (Santa Cruz) antibodies. MG-132 was purchased from Calbiochem (San Diego, CA) and digitonin, lactacystin, cycloheximide, castanospermine, and deoxymannojirimycin were from Sigma (St. Louis, MO).

DNA constructs, RNAi constructs, and recombinant adenovirus. Mouse ABCG5 and ABCG8 tagged with c-Myc or HA were constructed in pCDNA3.1 vector (Invitrogen, Carlsbad, CA) similar to a previous report [3,7]. CNX-RNAi and CRT-RNAi were constructed in pSilencer 2.1-U6 neo vector (Ambion Inc., Austin, TX) with standard methods. For CNX-RNAi, we used the 21-bases siRNA target sequence (AAUGUGGUGGUGCCUAUGUGA) of calnexin mRNA similar to a previous report [15]. For CRT-RNAi, we used the 21-bases siRNA target sequence (AAGAAGGUUCAUGU-CAUCUUC) of calreticulin mRNA. All constructs were verified by sequencing. Recombinant human calreticulin (Ad-CRT) and calnexin (Ad-CNX) were produced as described previously [16].

Cell lines and transfection. CHO-K1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp.) containing 10% fetal bovine serum and antibiotics. To increase gene expression, transfected CHO-K1 cells were incubated with 1 mM sodium butyrate for 20–24 h before analysis. Transfection was performed using *Trans* IT-LT1 Transfection Reagent (Mirus, Madison, WI).

SDS–PAGE and Western blotting. Confluent (90–100%) cells grown on six-well plates were washed twice with ice-cold PBS, lysed at 4 °C in 100 μl RIPA buffer [16], and centrifuged at 15,000*g* for 10 min at 4 °C. Cell lysates were subjected to SDS–PAGE on 7% polyacrylamide gels. Protein was electroblotted from the gels to PVDF membrane. Incubation of membrane with antibodies was done as described previously [16].

Immunoprecipitation. Confluent (90–100%) cells grown on 60-mm dishes were lysed in 1 ml of 0.5% digitonin lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% digitonin, and 1% protease inhibitor cocktail) for 2 h at 4 °C and centrifuged at 15,000*g* for 10 min at 4 °C. The supernatant was incubated for 12 h at 4 °C with monoclonal anti-Myc antibody immobilized in protein G–Sepharose® 4 Fast Flow (Amersham Biosciences Corp., Piscataway, NJ). Immune complexes were precipitated and washed three times with 1 ml of 0.1% digitonin buffer. Immunoprecipitated proteins were eluted for 1 h at 37 °C with 2× concentrated loading buffer and analyzed by Western blotting.

Metabolic labeling. Subconfluent cells were incubated for 2 h in methionine- and cysteine-free DMEM, and then pulse-labeled for 15 min with 100 μCi/ml [^{35}S]methionine and [^{35}S]cysteine (>1000 Ci/mmol, Amersham Biosciences Corp.). After pulse labeling, cells were washed with ice-cold PBS three times and then lysed at 4 °C in 500 μl RIPA buffer containing 1% protease inhibitor cocktail. Cell lysates were subjected to immunoprecipitation in a similar manner as described above and then analyzed on 7% SDS–PAGE gels. The gels were dried and visualized with a BAS Imaging Plate Scanner (BAS-2000, FUJIFILM, Tokyo, Japan). The radioactivity associated with ABCG5 and ABCG8 was quantified using Image Gauge software (Ver. 3.4, FUJIFILM).

Cell surface biotinylation. Subconfluent cells were biotinylated in 1 mg/ml EZ-Link™ Sulfo-NHS-SS Biotin (PIERCE, Rockford, IL) at 4 °C for 30 min and solubilized in RIPA buffer after washing three times with PBS. Biotinylated proteins were isolated by incubation with ImmunoPure Immobilized Streptavidin (PIERCE) at 4 °C for 4 h. After washing three times with RIPA buffer, biotinylated proteins were eluted with 2× SDS sample buffer and analyzed by Western blotting.

Results

CRT overexpression enhances the cell surface expression of ABCG5/G8

To examine the role of CNX and CRT on the biosynthesis of G5/G8, we utilized overexpression system using adenovirus vector [16]. CHO-K1 cells transiently expressing both epitope (Myc or HA) -tagged G5 and G8 were infected with recombinant adenovirus expressing CNX (Ad-CNX) or CRT (Ad-CRT) at 50 MOI (multiplicity of infection). Similar to previous report [3], when G5 and G8 were co-transfected, both were expressed as two bands (Fig. 1A and B). Endoglycosidases treatment revealed that the lower bands of G5 and G8 were immature forms that were EndoH-sensitive (Fig. 1C and D). Higher bands of G5 and G8 were EndoH-resistant, but PNGase F-sensitive (Fig. 1C and D), indicating that they were mature forms that were complex-glycosylated at the post-Golgi compartments after heterodimerization (G5/G8) [3]. CNX overexpression slightly increased the immature form of G5 and significantly increased immature G8 (Fig. 1A and B; +Ad-CNX). However, CNX did not dramatically affect the steady state levels of mature G5 and G8 (Fig. 1A and B; +Ad-CNX). On the other hand, CRT overexpression increased the steady state levels of both mature and immature forms of G5 and G8 (Fig. 1A and B; +Ad-CRT).

To examine whether CRT stimulates the cell surface expression of G5/G8, cell surface biotinylation analysis was performed. Western blotting showed that CRT overexpression increased the cell surface expression of both mature G5 and G8 (Fig. 1E and F; +Ad-CRT). The immature G5, however, was also detected in the cell surface fraction (Fig. 1E; cell surface). This might be due to the fact that *N*-glycosylation of G5 is not necessary for the heterodimerization with G8 and their transport to the post-Golgi compartments [14]. CNX overexpression increased the cell surface expression of mature G5 and G8 but not to the same extent as the increase induced by CRT (Fig. 1E and F; +Ad-CNX).

CRT and CNX increase the steady state level of immature G5 and G8

To determine whether CRT and CNX could increase the steady state levels of immature G5 and G8, we transfected CHO-K1 cells with either G5 or G8 and infected the cells with CRT or CNX. Similar to previous reports [3,14], when G5 or G8 was transfected individually, only their respective immature form was detected (Fig. 2A and B). Overexpression

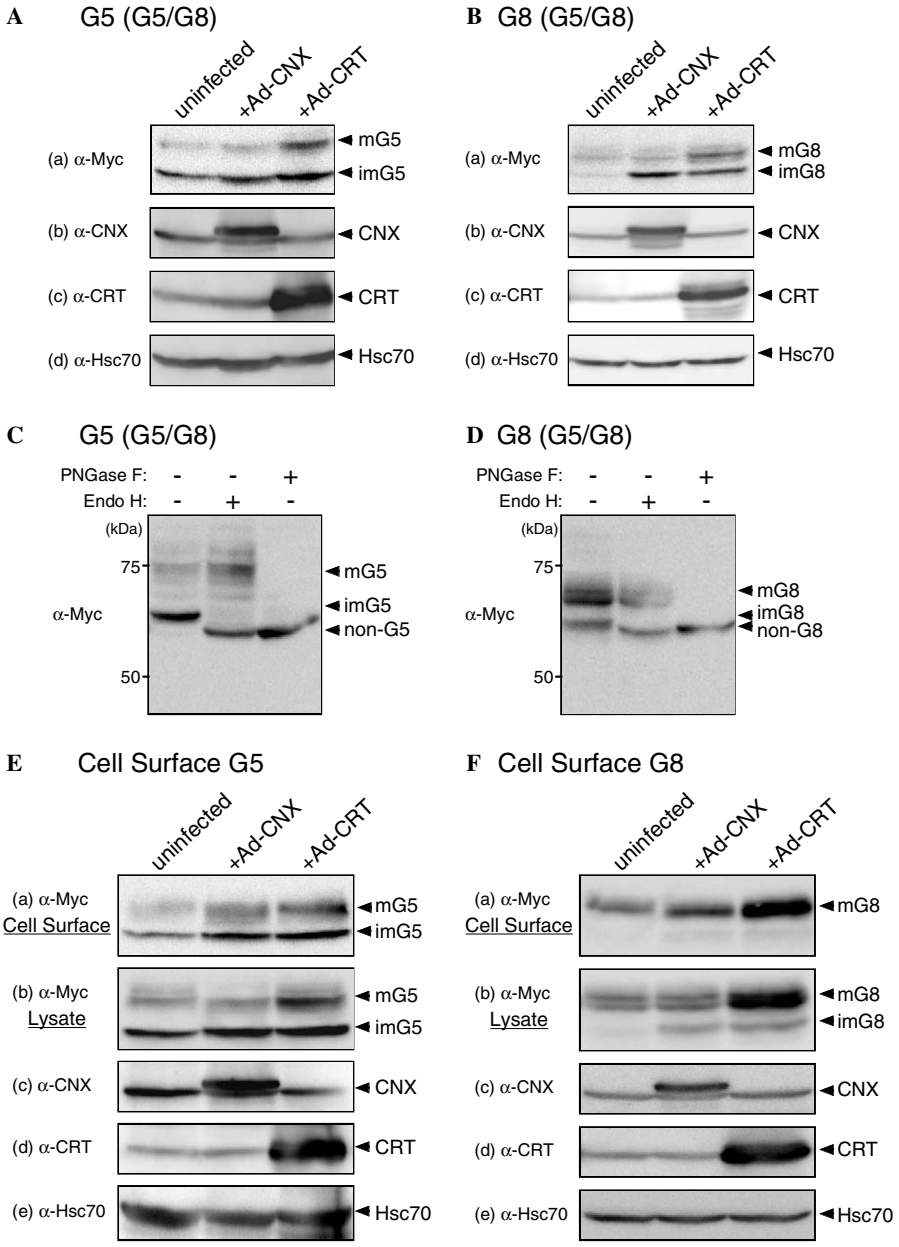

Fig. 1. CRT overexpression increases the steady state level and cell surface expression of mature G5/G8. (A–D) CHO-K1 cells were transfected with both G5-Myc and G8-HA (A,C) or both G5-HA and G8-Myc (B,D). Transfected cells were also infected with Ad-CNX (MOI 50) or Ad-CRT (MOI 50). Forty-eight hours after transfection, cell lysates were prepared for Western blotting with the indicated antibodies. For endoglycosidases digestion (C,D), cell lysates were denatured and digested with Endo H (500 U) or PNGase F (500 U) and analyzed by Western blotting. Hsc70 was used as a loading control. Note that the mobility in SDS–PAGE of exogenous human CNX (by Ad-CNX) is slightly bigger than in endogenous hamster CNX. (E,F) CHO-K1 cells transfected with both G5-Myc and G8-HA (E) or both G5-HA and G8-Myc (F) were infected with Ad-CNX (MOI 50) or Ad-CRT (MOI 50). Forty-eight hours after transfection, cell surface proteins were biotinylated for 30 min at 4 °C. Biotinylated proteins were isolated by streptavidin beads and analyzed by Western blotting. Hsc70 was used as a loading control.

of either CNX or CRT increased the steady state levels of both immature G5 and G8 (Fig. 2A and B). Endoglycosidases treatment confirmed that the bands were immature forms of G5 and G8 since they were EndoH-sensitive (Fig. 2C and D). These results suggest that CNX and CRT increased the expression of immature G5 and G8. However, whereas the CNX-mediated increase of immature G5 and G8 did not result in higher G5/G8 maturation, the CRT-induced up-regulation of immature G5 and G8 led to the enhanced cell surface expression of G5/G8.

Effect of CRT and CNX knockdown on the steady state expression of ABCG5/G8

We next examined the effect of CRT and CNX knockdown to further clarify their role on the biosynthesis of G5/G8. When CNX- (CNX-RNAi) and CRT-specific RNAi (CRT-RNAi) were transfected, endogenous expression of CNX and CRT was decreased, respectively (Fig. 3). We observed that CNX knockdown increased CRT expression and vice versa (Fig. 3), confirming the

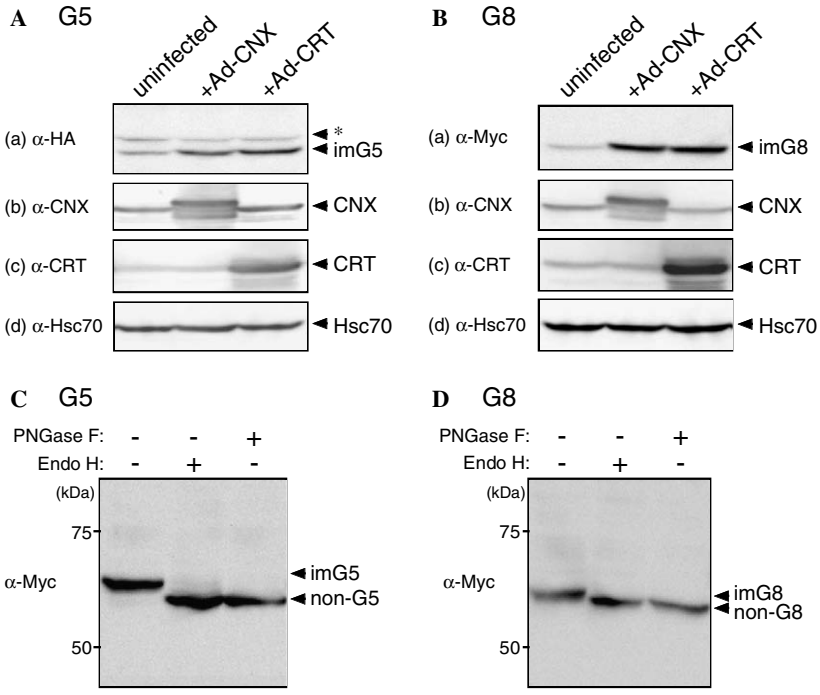

Fig. 2. CRT and CNX overexpression increase the steady state level of immature G5 and G8. (A–D) CHO-K1 cells transfected with G5-HA (A), G5-Myc (C) or G8-Myc (B,D) were infected with Ad-CNX (MOI 50) or Ad-CRT (MOI 50). Forty-eight hours post-transfection, cell lysates were prepared for Western blotting with the indicated antibodies. Hsc70 was used as a loading control. *Non-specific band.

report by Molinari et. al. [17]. Knockdown of CNX decreased both immature G5 and G8 (Fig. 3A and B; CNX-RNAi). Although CRT-RNAi had similar effects on immature G5 and G8, it was weaker than that of CNX-RNAi (Fig. 3A and B; CRT-RNAi). Knockdown of either CNX or CRT also decreased the expression of both mature G5 and G8 (Fig. 3C). These results emphasize that CNX and CRT are involved in the biogenesis of immature G5 and G8, and that both chaperones are important for maturation of G5/G8.

Interaction of CRT and CNX with G5/G8

We next investigated whether both CRT and CNX interact with G5 and G8. Similar to a previous report [14], CNX interacted with both immature G5 and G8 (Fig. 4A and C; lane 2). These interactions were increased by CNX overexpression (Fig. 4A and C; lanes 2 and 4). In contrast to CNX, we could not detect the interaction between CRT and immature G5 or G8 in control state (Fig. 4A and C; lane 2) as reported before [14]. However, upon CRT overexpression, both immature G5 and G8 weakly bound to CRT (Fig. 4A and C; lane 6). These results indicate that CNX and CRT interact with immature G5 and G8 with different affinities.

Role of CRT and CNX in the biosynthesis of G5/G8

Since CNX attenuates the proteasomal degradation of ABC transporter such as CFTR [16] and CNX and CRT increased the steady state levels of immature G5 and G8 (Fig. 2), it is possible that both chaperones may inhibit their proteasomal degradation. First, we clarified whether immature G5 and G8 undergo proteasomal degradation. Treatment with proteasome inhibitors, MG-132 and lactacystin, induced in a time-dependent manner, a high molecular weight (HMW) complex, which may be polyubiquitinated G5 and G8 (Fig. 5A and B). Additionally, low molecular weight bands, which may be non-glycosylated forms of G5 and G8, appeared after treatment with proteasome inhibitors (Fig. 5A and B; asterisk). Interestingly, proteasome inhibitors significantly induced HMW complex of G8 more than that of G5 (Fig. 5A and B). These data suggest that both immature G5 and G8 are degraded by proteasome.

We next asked whether CRT and CNX overexpression attenuate the degradation of immature G5 and G8. By quantifying the amount of the remaining proteins after cycloheximide (CHX) treatment, we determined that the half-life of immature G5 and G8 was ~4 h and ~2.5 h, respectively, confirming previous finding [3] (Fig. 5C and D; uninfected). As expected, CRT and CNX overexpression modestly increased the half-life of immature G5 and G8 (Fig. 5C and D; +Ad-CRT and +Ad-CNX), indicating that the proteasomal degradation of immature G5 and G8 might be inhibited by these chaperones.

Finally, we examined the effect of CNX and CRT overexpression on the de novo synthesis of G5 and G8 using metabolic [^{35}S]-labeling. Three bands of G5 and two bands of G8 were observed after labeling [3] (Fig. 5E and F; upper panels). CRT and CNX overexpression increased the de novo synthesis of both G5 and G8, consistent with

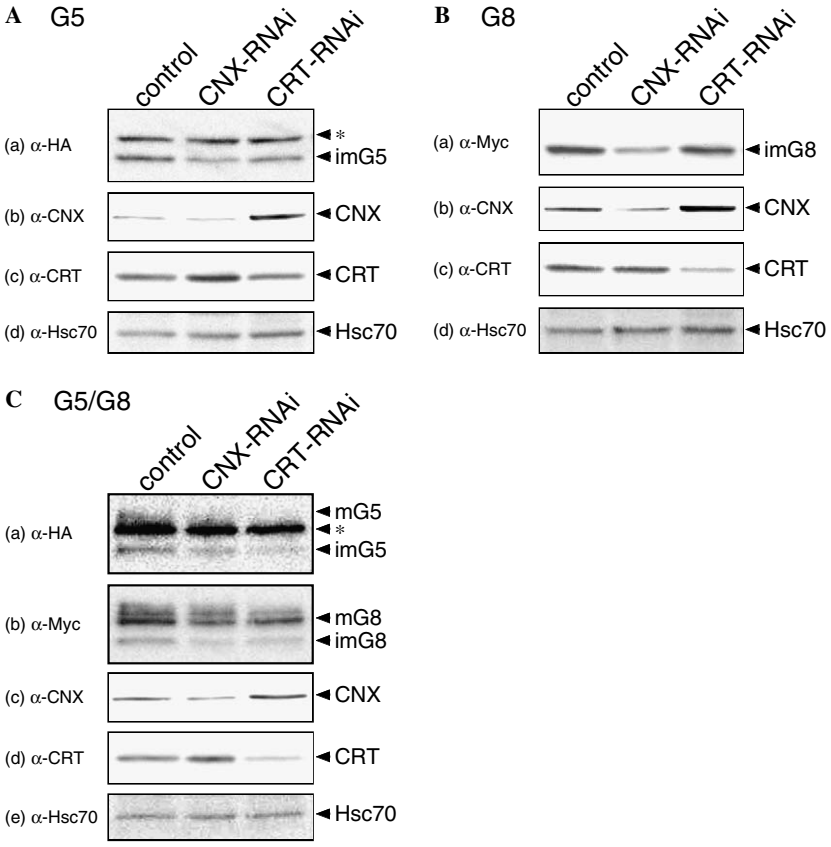

Fig. 3. Effect of CRT and CNX knockdown on the steady state expression of ABCG5/G8. (A–C) CHO-K1 cells transiently transfected with G5-HA (A), G8-Myc (B) or both (C) were transfected with control RNAi (control), CNX-RNAi or CRT-RNAi. Twenty-four hours after RNAi transfection, the culture medium was changed to medium containing 3 mg/ml G418 to select for cells expressing the RNAi constructs. Seventy-two hours after, cell lysates were prepared for Western blotting. Hsc70 was used as a loading control. *Non-specific band.

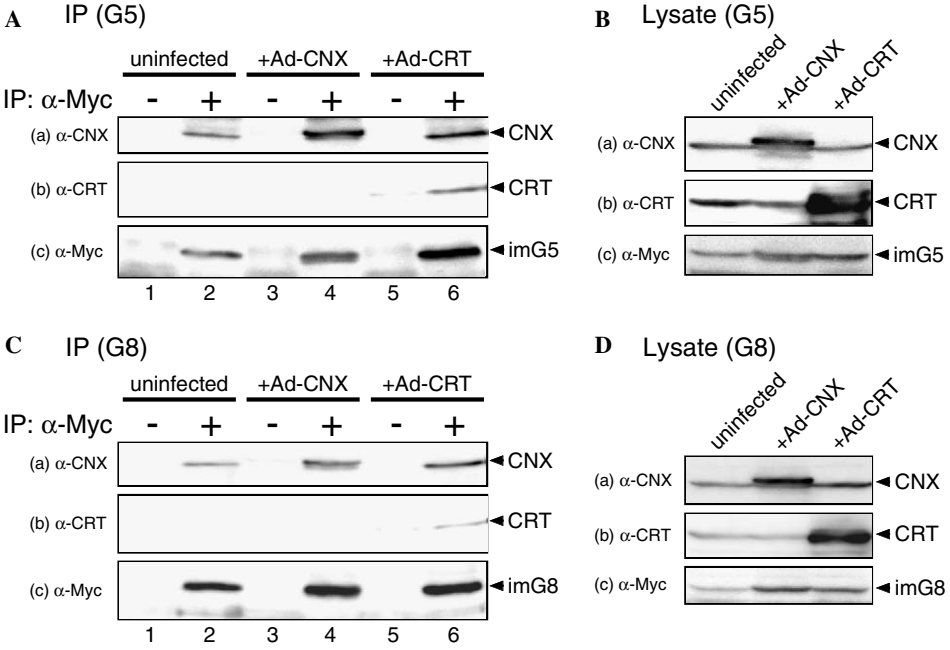

Fig. 4. Interaction of CRT and CNX with G5/G8. (A–D) CHO-K1 cells transfected with G5-Myc (A,B) or G8-Myc (C,D) were infected with Ad-CNX (MOI 50) or Ad-CRT (MOI 50). Forty-eight hours after transfection, cells were lysed in 0.5% digitonin buffer (cell lysate). G5 and G8 were immunoprecipitated using anti-Myc antibody and immunoprecipitants were analyzed by Western blotting.

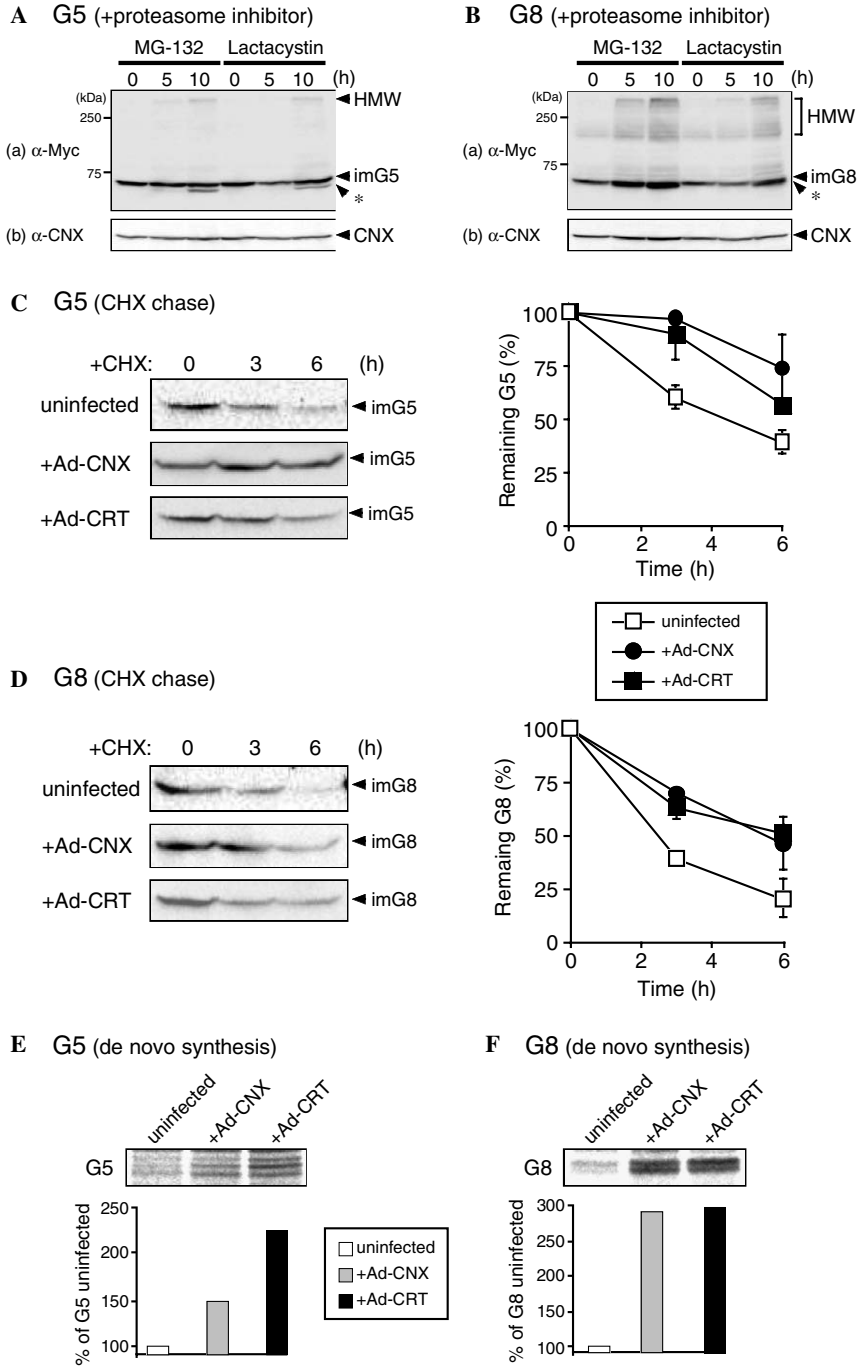

Fig. 5. Role of CRT and CNX in the biosynthesis of G5/G8. (A,B) Both immature G5 and G8 are degraded by proteasome. CHO-K1 cells transfected with G5-Myc (A) or G8-Myc (B) were treated with 10 μM MG-132 or 10 μM lactacystin for the time periods indicated. Expression of G5 and G8 was analyzed by Western blotting. CNX was used as a loading control. *Lower molecular weight band. (C,D) CRT and CNX overexpression stabilize both immature G5 (C) and G8 (D). CHO-K1 cells transfected and infected same as (A) and (B) were treated with 1 mM cycloheximide (+CHX) for the time periods indicated. After CHX treatment, cells were lysed and analyzed by Western blotting analysis (left panels). The intensity of the bands indicating immature G5 (C) and G8 (D) was quantified by Image Gauge software and expressed as a percentage of the bands at $t = 0$, respectively (right panels). Data are expressed as mean values ± SEM ($N = 3$). (E,F) CRT and CNX overexpression stimulate de novo synthesis of immature G5 and G8. CHO-K1 cells were transfected with G5-Myc (E) or G8-Myc (F) and infected with Ad-CNX (MOI 50) or Ad-CRT (MOI 50). Forty-eight hours after transfection, cells were [^{35}S]-labeled for 15 min and immediately after labeling, cells were lysed in RIPA buffer. Radio-labeled G5 and G8 were isolated by immunoprecipitation, analyzed by SDS–PAGE and autoradiography (upper panels). The intensity of the bands indicating G5 (E) and G8 (F) was quantified by Image Gauge software and expressed as a percentage of the bands in uninfected cells (lower panels).

our observations that these lectin chaperones positively affect the steady state levels of immature G5 and G8 (Fig. 2A and B). Although the effect of CRT on G5 was slightly stronger than that of CNX (Fig. 5E), the effect of CRT and CNX on G8 was comparable (Fig. 5F). Collectively, these data indicate that CRT and CNX increase de

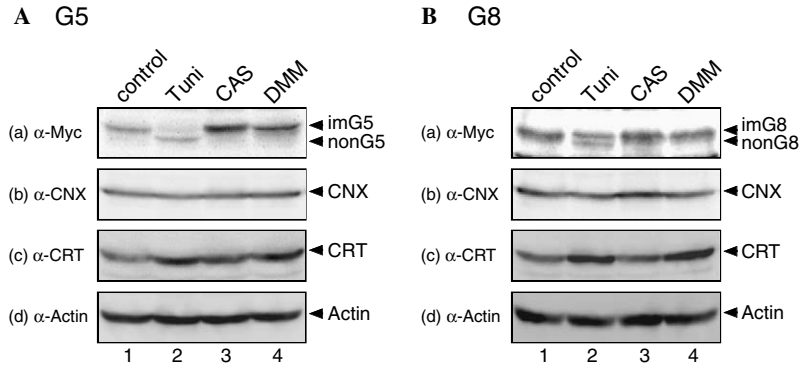

Fig. 6. Effect of inhibitors of *N*-glycan processing on the biogenesis of G5 and G8. (A,B) CHO-K1 cells transfected with G5-Myc (A) or G8-Myc (B) were treated with 5 μg/ml tunicamycin (Tun), 1 mM castanospermine (CAS) or 1 mM deoxymannojirimycin (DMM) for 24 h before lysis. Forty-eight hours after transfection, cells were lysed and analyzed by Western blotting. Actin was used as a loading control.

novo synthesis and stability of immature G5 and G8, resulting in the increase of their steady state levels and cell surface expression.

Effect of inhibitors of N-glycan trimming on the biogenesis of G5 and G8

To further understand the mechanism G5 and G8 biosynthesis, we treated cells with compounds that affect the trimming of N-linked oligosaccharides and hence also the interaction of substrates with CRT and CNX. Tunicamycin, an inhibitor of *N*-glycosylation [18], induced the appearance of low molecular weight band, which indicates non-glycosylated G5 and G8 (Fig. 6A and B; lane 2). Although tunicamycin induced ER stress and a resulting increase of CRT expression, it slightly decreased the expression of immature G5 and G8 (Fig. 6A and B; lane 2). Interestingly, castanospermine (CAS), which is an inhibitor of glucosidase I and II [17,19–21], increased immature G5 expression whereas it did not affect immature G8 expression (Fig. 6A and B; lane 3). It is unlikely that increased G5 expression by CAS treatment resulted from induction of CRT by ER stress since CAS did not induce CRT (Fig. 6A; lane 3). Deoxymannojirimycin (DMM), which inhibits mannosidase I [21–24], modestly increased the expression of immature G5 whereas it did not affect the expression of immature G8 (Fig. 6A and B; lane 4). Together, these data indicate that CAS- and DMM-sensitive *N*-glycan processing might be involved in the degradation of immature G5, but not of G8.

Discussion

CRT and CNX are involved in the folding and assembly of several membrane proteins [25–29]. Here we show that CRT and CNX stimulate the folding of G5 and G8. Overexpression of both chaperones increased the steady state level of immature G5 and G8 (Figs. 1 and 2). Conversely, RNAi-based knockdown of either CRT or CNX decreased the immature G5 and G8, resulting in a lower expression of mature G5/G8 (Fig. 3). CNX and CRT have distinct roles in the folding and ER quality control of glycoproteins [17]. We noted that although CRT and CNX have similar effect on immature G5 and G8 (Fig. 2), CRT stimulated the cell surface expression of mature G5/G8 more than CNX (Fig. 1). CNX is known to be involved in the ER retention of unassembled proteins [25,26,29] thus it is possible that CNX not only stimulated the folding but also enhanced the ER retention of G5 and G8. In contrast, CRT may not be involved in ER retention, rather, CRT functions to promote the cell surface expression of G5/G8 more efficiently.

Both CNX and CRT bound to immature G5 and G8 although their affinities were different (Fig. 4). Upon CRT overexpression, weak interactions between CRT and G5 or G8 were detected only when cells were lysed in 0.5% digitonin buffer (Fig. 4). Thus, CRT may transiently interact with both G5 and G8, which could induce the folding of these transporters. Such a transient interaction of CRT with substrates has been reported [30,31]. Both CNX and CRT could simultaneously interact with immature G5 and G8 since upon CRT overexpression, these interactions were detected (Fig. 4A and C; lane 6). Thus, both chaperones may be involved in the folding and assembly of G5/G8 similar to other membrane proteins such as MHC class I [27,30].

Both immature G5 and G8 underwent proteasomal degradation but G8 might be prone to ubiquitination more than G5 since proteasome inhibitors significantly induced HMW complex in G8 (Fig. 5B), suggesting a differential regulation between these two transporters. Our interpretation may be supported by the fact that the half-life of G8 (~2.5 h) was slightly shorter than that of G5 (~4 h) [3] (Fig. 5C and D). The different control mechanism between G5 and G8 is further supported by our results using inhibitors of *N*-glycan processing. CAS treatment, which prevents the interaction of CRT and CNX with their substrates [17], increased the expression of immature G5, but not of G8 (Fig. 6). Thus, interaction of these ER chaperones with immature G5, but not G8, may direct G5 to the ERAD pathway. Our interpretation could be supported by our results that DMM, which inhibits the ERAD by

preventing interaction with Man_8-lectin such as EDEM [23,24], increased the expression of immature G5, but not G8 (Fig. 6). Recently, it was reported that more than one *N*-glycan is needed for glucosidase II to allow entry of glycoproteins into CNX/CRT cycle [32]. G5 and G8 have two and one *N*-glycosylation sites, respectively [14]. Thus, *N*-glycan of G5 is trimmed by glucosidase II, enters CNX/CRT cycle, is trimmed by mannosidase I, and then G5 is directed to the ERAD pathway. In contrast, *N*-glycan of G8 is not trimmed by glucosidase II. However, CNX and CRT overexpression increased both G5 and G8 expression (Fig. 2). Thus, polypeptide-based interaction of both CNX and CRT, which is insensitive to CAS treatment [33], may be involved in G8 folding. This interpretation may be supported by a previous report that CNX interacts with non-glycosylated form of G8 [14]. Given our results, we suggest a plausible model in which the early productive folding of G5 and G8 involves polypeptide-based interaction of CNX/CRT whereas their ER quality control is mediated by oligosaccharide-based interaction. In summary, we demonstrate that CRT stimulates the cell surface expression of G5/G8. Moreover, our study proposes a possibility that G5 and G8 are regulated in distinct ER quality control mechanism. Our findings could contribute to the understanding of the biogenesis pathway of G5/G8 sterol transporter.

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