THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 24, Issue of June 14, pp. 14280–14284, 1996

Printed in U.S.A.

Glycan-dependent and -independent Association of Vesicular Stomatitis Virus G Protein with Calnexin*

(Received for publication, February 26, 1996)

Kurt S. Cannon, Daniel N. Hebert, and Ari Helenius‡

From the Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06520-8002

Calnexin (CNX) is a membrane-bound molecular chaperone that associates with newly synthesized proteins in the endoplasmic reticulum. Although several studies have indicated that it interacts exclusively with glycoproteins that carry monoglucosylated *N*-linked oligosaccharides, others have reported that it can bind to proteins that have no glycans. To address this discrepancy, we translated wild-type vesicular stomatitis virus G protein and nonglycosylated mutant forms in the presence of microsomes and examined their association with CNX. Individual G protein molecules were found to efficiently associate with CNX when both glycans were present and less efficiently if there was only a single glycan. Nonglycosylated G protein also interacted with CNX, but only when misfolded and present in high molecular weight aggregates. The results indicated that CNX can interact with G protein in two ways: through an oligosaccharide-dependent mechanism that involves individual substrate proteins; and in an oligosaccharide-independent association with large aggregates.

Among the molecular chaperones present in the endoplasmic reticulum (ER),[1] calnexin (CNX) and calreticulin are unique in their ability to associate primarily with glycoproteins (1–3). Several lines of evidence indicate that binding involves lectin-like contacts between these resident ER proteins and newly synthesized substrate glycoproteins with partially trimmed *N*-linked oligosaccharides (3–10). That monoglucosylated glycans (Glc1Man9→7GlcNAc2) mediate this interaction is supported by many observations. 1) α-Glucosidase inhibitors such as castanospermine (CST) and 1-deoxynojirimycin inhibit binding of a large variety of viral and cellular ligands to these chaperones (3, 4, 11–14). 2) The same inhibitors prevent dissociation of already bound ligands, suggesting a glucosidase requirement for release (7). 3) Known monoglucosylated glycoproteins, such as tsO45 vesicular stomatitis virus (VSV) G protein, display strong binding to CNX, and the association is prevented by CST (2). 4) In mutant cells devoid of glucosidase I or II activity, complexes between substrates and CNX or calreticulin do not form (3, 8, 13). 5) Isolated monoglucosylated oligosaccharides without associated polypeptides bind specifically to CNX (5). Although these results do not exclude the possibility of protein-protein interactions between CNX and its substrates (5, 14, 15), they strongly indicate that substrate attachment to CNX and calreticulin requires a direct association with the monoglucosylated glycan moieties.

In contradiction with these findings, several reports indicate that nonglycosylated proteins can associate with CNX. When T-cell receptor ϵ, a nonglycosylated subunit of the T-cell receptor (16), is expressed together with a truncated form of CNX that lacks the ER retention motif in its cytoplasmic tail, both proteins are found to shift from the ER to what appears to be the Golgi complex, suggesting that they are associated (17). When tunicamycin treatment is used to inhibit the addition of *N*-linked oligosaccharides to thyroglobulin (18) or when mutagenesis is used to remove the consensus glycosylation sequences from P-glycoprotein and major histocompatibility complex class I and II chains (15, 19, 20) coimmunoprecipitation with CNX is observed. It has been concluded from these results that the presence of *N*-linked oligosaccharides is not necessary for substrate binding to CNX.

To address the discrepancy, we have analyzed the interactions between CNX and glycosylated and nonglycosylated forms of VSV G protein. The G protein is a transmembrane glycoprotein of M_r 67,000 with two *N*-linked glycans and several intrachain disulfide bonds (21). During its normal folding, it first interacts with an immunoglobulin heavy chain-binding protein (BiP; also called GRP78) and then with CNX (11, 22). It does not bind to calreticulin (3). A reticulocyte lysate translation system in the presence of microsomes was used, and the interaction with CNX was assessed by coimmunoprecipitation. We found that the *N*-linked glycans were necessary for the interaction of nonaggregated forms of VSV G protein with CNX. However, when the G protein was misfolded, it was present in large aggregates that associated with CNX in a glycan and glucose-trimming independent fashion.

EXPERIMENTAL PROCEDURES

Reagents—Components of the cell-free translation system (rabbit reticulocyte lysate, an amino acid mixture lacking methionine, dithiothreitol, and RNasin) were purchased from Promega Corp. (Madison, WI). Dog pancreas microsomes were a generous gift from Dr. Reid Gilmore (Worcester, MA). Pro-mix ^{35}S metabolic labeling reagent was purchased from Amersham Corp. GSSG and Zysorbin (fixed and killed *Staphylococcus aureus*) were from Fluka Chemical Corp. (Ronkonkowa, NY) and Zymed Laboratories, Inc. (San Francisco, CA), respectively. CHAPS was obtained from Pierce. Restriction endonucleases, ribonucleotide triphosphates, and T7 RNA polymerase were from Boehringer Mannheim. All other reagents were purchased from Sigma. The polyclonal anti-VSV G protein antiserum was raised against whole viral protein purified as described (23). The polyclonal anti-CNX rabbit serum was raised against a synthetic peptide corresponding to a segment of the cytoplasmic tail of canine CNX (24).

Plasmid Construction—The cDNAs for wt and mutant forms of VSV G protein (Indiana serotype, San Juan strain) in SV40 expression vectors were a gift from C. Machamer (Baltimore, MD; Ref. 25). Con-

* This work was supported by National Institutes of Health Grant GM52972 (to A. H.) and a postdoctoral fellowship from the Donaghue Medical Research Foundation (to D. N. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence and reprint requests should be addressed: Dept. of Cell Biology, Yale University School of Medicine, P. O. Box 208002, New Haven, CT 06520-8002. Tel: 203-785-4313; Fax: 203-785-7446; E-mail: ari_helenius@qm.yale.edu.

[1] The abbreviations used are: ER, endoplasmic reticulum; CNX, calnexin; VSV, vesicular stomatitis virus; CST, castanospermine; CHAPS, 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate; HBS, Hepes-buffered saline; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; wt, wild type.

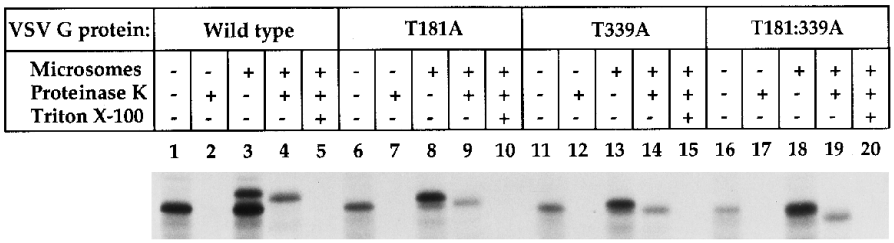

FIG. 1. **wt VSV G protein and glycosylation mutants are translocated into microsomes.** ^{35}S-Labeled wt or mutant VSV G protein was translated using a rabbit reticulocyte lysate system in the presence or absence of canine microsomes for 1 h at 27 °C. The reaction was diluted in buffer and incubated with or without proteinase K (100 μg/ml) and Triton X-100. After 30 min at 0 °C, phenylmethylsulfonyl fluoride was added to inhibit the protease. The G protein was immunoprecipitated with anti-G protein antibodies, resolved by reducing SDS-PAGE, and visualized by fluorography. The names of the mutants indicate: the original amino acid (one-letter amino acid code), the location in the wt sequence that has been changed, and the substituted amino acid.

sensus *N*-linked glycosylation sites (Asn-*X*-Ser/Thr) have been removed by changing either threonine 181, 339, or both to alanine. *Xho*I fragments carrying the coding region of wt VSV G protein or one of the glycosylation mutant forms of VSV G protein were subcloned into pBluescript (Stratagene, La Jolla, CA) to allow *in vitro* transcription. Proper orientation of the inserts was confirmed by restriction analysis.

Transcription, Translation, and Translocation—pBluescript vectors containing the cDNA for wt or mutant VSV G protein were linearized beyond the 3′-end of the inserts with *Kpn*I and used in the T7-dependent expression system to make mRNA as described previously (26). ^{35}S-labeled VSV G protein was synthesized in the presence of nuclease-treated dog pancreatic microsomes with the following mixture: 6 μl of microsomes (1 membrane eq/μl), 52 μl of reticulocyte lysate, 2 μl of amino acid mixture minus methionine (10 μM each), 8 μl of Pro-mix ^{35}S (14 mCi/ml), 1 μl of 100 mM dithiothreitol, 16 μl of nuclease-free water, 4 μl of RNasin (40 units/μl), and 3.7 μl of 100 mM GSSG. After incubation for the indicated times at either 27 or 15 °C, samples were chilled on ice, and free sulfhydryls were alkylated with 20 mM *N*-ethylmaleimide (27).

Protease Protection Assay—Following translation, 15-μl aliquots were diluted to 50 μl for protease digestion in a buffer containing 140 mM potassium acetate, 50 mM triethanolamine-acetic acid (pH 7.5), and 2.5 mM magnesium acetate (28). Digestion with 100 μg/ml proteinase K was performed on ice for 30 min in the presence or absence of 0.5% Triton X-100. Protease digestion was halted by the addition of phenylmethylsulfonyl fluoride to 4 mM from a 100 mM stock in ethanol, and and samples were incubated on ice for 10 min. Remaining proteins were then immunoprecipitated and analyzed as described below.

Immunoprecipitation—Equal aliquots were solubilized in 2% CHAPS/HBS (50 mM HEPES and 200 mM NaCl, pH 7.5) containing 0.5% Zysorbin and protease inhibitors (10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin). Samples were then rotated at 4 °C for 1 h, and the Zysorbin was pelleted by centrifugation at 2000 × *g* at 4 °C for 5 min. Protein A-Sepharose (1.4%) and either 2.8% anti-CNX or 1.4% anti-VSV G protein polyclonal antiserum were then added to the supernatant. The samples were rotated at 4 °C for 16 h and then centrifuged at 2000 × *g* at 4 °C for 5 min to pellet the immune complexes. Anti-VSV G protein immunoprecipitations were washed twice with a buffer containing 0.1% SDS, 0.05% Triton X-100, 10 mM Tris, and 300 mM NaCl (pH 8). Anti-CNX immunoprecipitations were washed twice with 0.5% CHAPS in HBS. Pellets were then suspended in 50 μl of sample buffer (200 mM Tris (pH 6.8), 3% SDS, 10% glycerol, 0.004% bromphenol blue, and 1 mM EDTA), heated to 95 °C for 5 min, and centrifuged at 2000 × *g* for 5 min to pellet the protein A-Sepharose. The samples were then divided; nonreducing samples were loaded directly, while reducing samples received dithiothreitol to 100 mM and were heated to 95 °C for an additional 5 min prior to analysis by 7.5% SDS-PAGE and fluorography.

Sucrose Gradient Velocity Sedimentation—Monomers of wt and nonglycosylated G protein were separated from aggregated forms by centrifugation through a 5–25% continuous sucrose gradient in 0.5% CHAPS/HBS buffer. After a 2-h translation/translocation at 27 °C, 40 μl of the reaction mixture were mixed with an equal volume of 4% CHAPS/HBS, layered on 5-ml gradients, and centrifuged for 15 h at 4 °C in a Beckman SW50.1 at 42,000 rpm. Upon completion of the ultracentrifugation, the gradients were fractionated from top to bottom by hand and immunoprecipitated as outlined above.

RESULTS

To examine the role of *N*-linked oligosaccharides in CNX binding, wt VSV G protein and three mutants with modified consensus glycosylation sequences were used. In the wt, the *N*-linked glycans are attached to asparagines 179 and 337. By changing consensus glycosylation sequence threonines 181 and 339 to alanine, Machamer *et al.* (25) generated two distinct mutant proteins with a single glycan (T181A and T339A) and a third mutant with none (T181A/T339A).

The mRNA for wt G protein and the three mutants were translated *in vitro* using a rabbit reticulocyte lysate system in the presence or absence of rough ER-derived microsomes (26). To demonstrate that translocation and glycosylation had occurred, the samples were exposed to proteinase K and analyzed by SDS-PAGE and fluorography.

In the presence of microsomes, two bands of wt G protein were observed (Fig. 1, *lane 3*). Only the lower band was present after translation in the absence of microsomes. The upper band corresponded to translocated and glycosylated G protein. When the sample was treated with protease, most of the G protein in this band was protected from proteolysis. The slight increase in mobility after digestion was probably caused by removal of the short cytosolic C-terminal tail (Fig. 1, *lanes 4*, *9*, *14*, and *19*). The lower band was composed of untranslocated G protein. It co-migrated with the G proteins synthesized in the absence of microsomes (Fig. 1, *lane 1*) and was completely susceptible to protease. In the absence of microsomes, or if Triton X-100 was added to solubilize the microsomal membrane, wt and mutant VSV G proteins were all completely digested (Fig. 1, *lanes 2*, *5*, *17*, and *20*).

A large fraction of each G protein mutant, translated in the presence of microsomes, was also translocated (Fig. 1). As expected, the apparent molecular weights were lower than for the wt G protein because of the missing glycans (Fig. 1, *lanes 8*, *13*, and *18*). Taken together, the observations demonstrated that a large fraction of the wt and mutant G proteins were present as transmembrane proteins in sealed microsomal vesicles, and that oligosaccharide addition had occurred at all the available glycosylation sites.

To analyze CNX binding and folding of these proteins, translation was performed at 27 °C, and the reticulocyte lysate was complemented with GSSG to allow oxidation of the synthesized G protein (26) and to keep CNX in its active oxidized state (7). Duplicate samples contained CST, an inhibitor of α-glucosidases, to prevent trimming of glucoses and thus block glycan-specific binding to CNX (11). After 2 h, the reaction mixtures were treated with the membrane permeable alkylating agent *N*-ethylmaleimide to block remaining free sulfhydryl groups and to prevent further disulfide bond formation. The microsomes were lysed, immunoprecipitated with polyclonal anti-G protein or anti-CNX antibodies, and subjected to SDS-PAGE with or without prior reduction.

When electrophoresed under reducing conditions, the translocated and glycosylated G proteins could be easily distin-

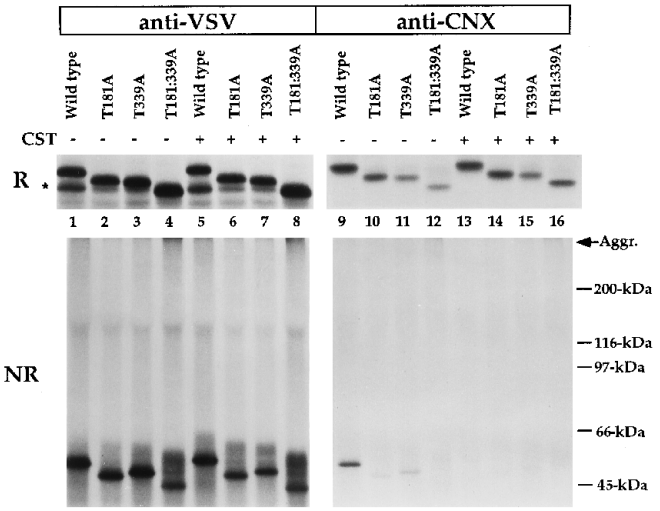

FIG. 2. **Glycosylation and glucose trimming are necessary for CNX binding to correctly folded but not to aggregated G protein.** wt, singly glycosylated (*T181A* and *T339A*), or nonglycosylated (*T181:339A*) VSV G protein was translated for 2 h at 27 °C in the absence (*lanes 1–4* and *9–12*) or presence (*lanes 5–8* and *13–16*) of the glucosidase inhibitor CST. The samples were then alkylated with *N*-ethylmaleimide and immunoprecipitated with either anti-VSV G protein (*lanes 1–8*) or anti-CNX antibodies (*lanes 9–16*). Immunoprecipitated proteins were resolved by nonreducing (*NR*) or reducing (*R*) SDS-PAGE and visualized by fluorography. Molecular weight standards shown are: myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin. *anti-CNX* lanes were exposed approximately 3-fold longer than *anti-VSV* lanes.

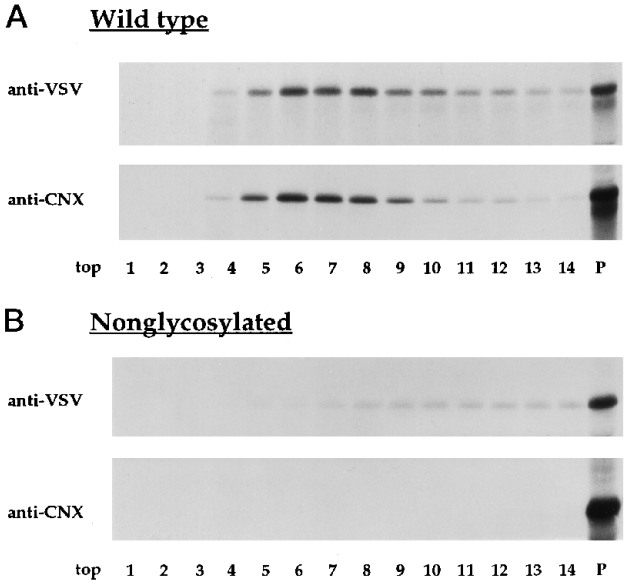

FIG. 3. **Only aggregated complexes of nonglycosylated VSV associate with CNX.** wt (*Wild type*; *A*) and nonglycosylated (*B*) VSV G protein were translated for 2 h at 27 °C. CHAPS-solubilized samples were loaded on a continuous 5–25% sucrose gradient in HBS. After centrifugation at 4 °C for 15 h at 42,000 rpm, fractions were collected from top to bottom. Each fraction was immunoprecipitated with either anti-VSV G protein or anti-CNX antibodies. The precipitated proteins were separated on reducing SDS-PAGE and visualized by fluorography.

guished from the untranslocated (Fig. 2, *R, lanes 1–3*). The latter are marked with an asterisk. The differences in gel mobility reflected the presence of two, one, or no *N*-linked glycans, respectively. That the nonglycosylated double mutant T181A/T339A migrated slightly faster than the untranslocated G protein was presumably due to cleavage of the signal peptide (Fig. 2, *R, lane 4*). CST inhibited trimming of oligosaccharides, resulting in slightly slower migration of the three glycosylated G proteins in Fig. 2, *R, lanes 5–7*, compared to controls in Fig. 2, *R, lanes 1–3*. CST had no effect on the efficiency of translation and translocation.

When all four samples were precipitated with anti-CNX antibodies, the untranslocated G protein was not precipitated (Fig. 2, *lanes 9–16*), but all the translocated forms were at least partially coprecipitated, whether glycosylated or not (Fig. 2, *lanes 9–12*). It was particularly noteworthy that T181A/T339A was brought down by the CNX antibody (Fig. 2, *lane 12*). Since this double mutant was not glycosylated, the interaction was clearly glycan independent. Moreover, coprecipitation of all four forms of G protein was unaffected by CST. Therefore, it appeared that association occurred between translocated G proteins and CNX, regardless of glycosylation and oligosaccharide trimming status.

Nonreducing gels allowed us to examine the effect of VSV G protein folding on these interactions with CNX. Fully oxidized, native G protein migrated faster than the corresponding reduced G protein due to its intrachain disulfide bonds (22). Incompletely folded proteins were seen as partially oxidized protein bands forming a smear in the gel above the band of oxidized native G protein and as large complexes at the top of the gel (Fig. 2, *NR, Aggr.*). These complexes were stabilized by interchain disulfides and thus did not enter the separation gel unless reduced prior to electrophoresis. We have previously observed disulfide cross-linked complexes in live cells when the folding of G, and other proteins that require intrachain disulfide bonds, was compromised (29–31). Incomplete folding was most prominent for mutant T181A/T339A (Fig. 2, *NR, lanes 4* and *8*), but it also occurred to some extent with the other forms including wt G protein (Fig. 2, *NR, lanes 1–3* and *5–7*).

The nonreducing gels of CNX precipitates demonstrated a glycan-specific binding of G protein. The association of nonaggregated G protein with CNX was limited to the fully oxidized fraction of wt G protein and of mutants T181A and T339A (Fig. 2, *NR, lanes 9–11*). Thus, of the forms of G protein that were not interchain disulfide cross-linked, only the glycosylated G proteins were coprecipitable with CNX. The presence of two glycans on the wt protein apparently supported more efficient binding than the single glycans of the mutants. Importantly, coprecipitation of all three forms was sensitive to CST. This confirmed the glycan-dependent nature of the attachment.

We concluded that the coprecipitation of the G proteins with CNX was the sum of two distinct types of interactions. wt protein and a fraction of the mutant proteins with a single *N*-linked glycan were bound to CNX by a glycan-mediated interaction. This involved G protein chains that were not cross-linked and depended on glucose trimming. Glycan-independent association could also occur between CNX and complexes containing aggregated and partially disulfide cross-linked G protein. This accounted for most of the interaction between nonglycosylated G protein and CNX but was also seen for some of the poorly folding products of glycosylated forms.

Sucrose gradient centrifugation was used to confirm that the nonglycosylated G proteins coprecipitating with CNX were present in large aggregates. wt and T181A/T339A G protein were translated in the presence of microsomes. Detergent was added, and the lysates were loaded onto 5–25% continuous sucrose gradients. After centrifugation, fractions were immunoprecipitated with antibodies to G protein or CNX. The precipitated proteins in each fraction were reduced, resolved by SDS-PAGE, and visualized by autoradiography.

The wt VSV G protein was found to sediment in fractions 3–14, with the majority having a $s_{20,w}$ of about 6–7 S (Fig. 3*A*) as compared to standards. Some of the G protein was in the pellet, which meant that it was present in complexes with $s_{20,}$

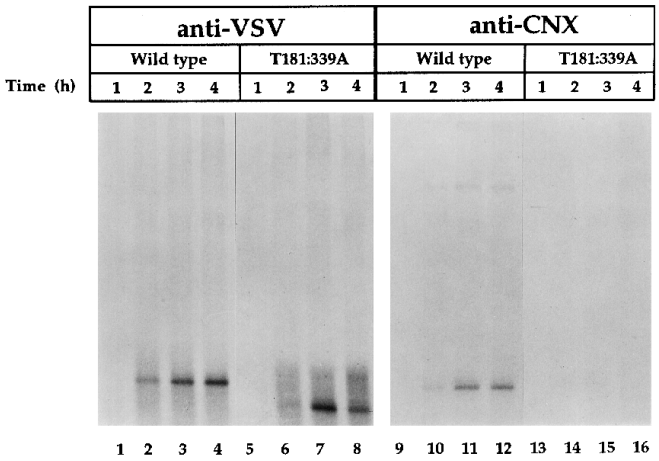

FIG. 4. **CNX does not bind to nonglycosylated VSV G protein in the absence of aggregation.** wt and nonglycosylated VSV G protein were translated for 4 h at 15 °C. Samples were removed hourly, alkylated with *N*-ethylmaleimide, and immunoprecipitated with either anti-VSV G protein (*lanes 1–8*) or anti-CNX (*lanes 9–16*) antibodies. The proteins were then separated by nonreducing SDS-PAGE and visualized by fluorography.

values higher than 19.3 S. Immunoprecipitation with anti-CNX antibodies showed that most of the slowly sedimenting G protein in fractions 4–10 was bound to CNX. When T181A/T339A was analyzed, most of it was present in the pellet fraction, with a small amount in fractions 8–14. Only the pelleted forms of the nonglycosylated G protein were precipitable with anti-CNX antibodies. This supported our conclusion that only aggregated forms of G protein associate with CNX in a glycan-independent fashion.

To further test this conclusion, we developed conditions in which we could prevent the aggregation and interchain cross-linking of nonglycosylated G protein. Since it is known that the aggregation of nonglycosylated G protein in live cells is decreased at low temperatures (32, 33), we examined folding of wt and T181A/T339A at 15 °C. Aliquots from a translation mixture were removed at various times, immunoprecipitated with anti-VSV or anti-CNX antibodies, and analyzed by nonreducing and reducing SDS-PAGE.

At 15 °C, translation was slower than at 27 °C. Therefore, full-length G protein could only be detected after 2 h (Fig. 4, *lanes 2* and *6*). At this time, fully oxidized native forms of G protein were already visible (Fig. 4, *lanes 2* and *6*). At this temperature, the nonglycosylated VSV G protein folded normally, although more slowly, than the wt. Importantly, no aggregation occurred (Fig. 4, *top*). Anti-CNX precipitation of the lysates demonstrated an interaction between wt G protein and CNX, but the nonglycosylated G protein was not coprecipitated. This confirmed our interpretation that in the absence of large aggregates, the nonglycosylated G protein does not interact with CNX. It only does so when misfolded and aggregated.

DISCUSSION

The experiments revealed two distinct types of association between newly synthesized VSV G protein and CNX: a glycan-dependent association that we have previously shown to play a role in the normal folding process of G protein in live infected cells (11); and a glycan-independent association between CNX and aggregated complexes containing newly synthesized, misfolded G protein. We think the latter type of binding corresponds to the glycan-independent association observed by others for nonglycosylated CD3ϵ, thyroglobulin, P-glycoprotein, and major histocompatibility complex class I and II polypeptides (15, 17–20).

Clearly, the two types of CNX-containing G protein complexes reflect very different modes of association. One corresponds to direct contact between individual substrate and chaperone molecules. Attachment is strictly dependent on the presence of *N*-linked glycans and requires their partial glucose trimming. This interaction is transient and important for the productive folding and maturation of the G protein (11).

The other type of interaction involves aggregates containing misfolded and disulfide cross-linked versions of the G protein. These proteins are not part of the productive maturation pathway but instead are destined for degradation (34). In addition to CNX, other resident ER proteins are likely to be present in these aggregates. For example, BiP/GRP78 is known to form stable associations with many permanently misfolded protein aggregates in the ER including the nonglycosylated G protein (22, 29).

It is well known that when the folding of proteins is compromised in cells or *in vitro*, the products tend to misfold and aggregate (32, 35). Manipulations that affect the folding process include the elimination of consensus sequences for *N*-linked glycosylation, the addition of tunicamycin or α-glucosidase inhibitors, inhibition of disulfide bond formation by the addition of dithiothreitol, elevation of the temperature, depletion of metabolic energy, and overexpression (36, 37).

Although our results may suggest that the large CNX-containing aggregates are merely experimental artifacts caused by artificially perturbed synthesis and folding conditions, it is important to note that aggregation and misfolding of newly synthesized proteins does occur both under physiological and pathological conditions in live cells (34). It has been proposed that some glycoproteins transiently pass through an aggregated state in the process of folding (18, 30, 38, 39). Aggregated forms of mutant proteins are also found to accumulate in the ER in many hereditary human diseases (40–42).

It was of interest that CNX bound to forms of the G protein with a single glycan less efficiently than to the wt, which has two glycans. Whether this reflected a lower probability of complex formation or an overall reduction in binding affinity remains unclear. The results raise, however, the possibility that CNX is multivalent, and that the affinity for a given glycoprotein substrate may in part be determined by the number of *N*-linked glycans.

Recent experiments using glycosidases have shown that CNX/substrate complexes remain stable even after the *N*-linked glycans have been severed from the polypeptide chain (5, 14, 15). This suggests that there are polypeptide-mediated contacts between the chaperone and the substrate. Thus, although a lectin-like interaction may be needed to establish the complex, we cannot rule out the presence of protein-protein interactions. Additional studies are needed to analyze the nature of these interactions and to identify the roles that they play in the conformational maturation of substrate proteins.

Acknowledgments—Special thanks to C. E. Machamer for the gift of VSV G protein glycosylation mutants, R. Gilmore for providing dog pancreas microsomes, Henry Tan for photography, and Jeff Peterson and Aylin Rodan for critical reading of the manuscript.

REFERENCES

1. Ou, W.-J., Cameron, P. H., Thomas, D. Y., and Bergeron, J. J. M. (1993) *Nature* **364,** 771–776
2. Hammond, C., Braakman, I., and Helenius, A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 913–917
3. Peterson, J. R., Ora, A., Nguyen Van, P., and Helenius, A. (1995) *Mol. Biol. Cell* **6,** 1173–1184
4. Kearse, K. P., Williams, D. B., and Singer, A. (1994) *EMBO J.* **13,** 3678–3686
5. Ware, F. E., Vassilakos, A., Peterson, P. A., Jackson, M. R., Lehrman, M. A., and Williams, D. B. (1995) *J. Biol. Chem.* **270,** 4697–4704
6. Nauseef, W. M., McCormick, S. J., and Clark, R. A. (1995) *J. Biol. Chem.* **270,** 4741–4747
7. Hebert, D. N., Foellmer, B., and Helenius, A. (1995) *Cell* **81,** 425–433
8. Ora, A., and Helenius, A. (1995) *J. Biol. Chem.* **270,** 26060–26062

9. Wada, I., Imai, S., Kai, M., Sakane, F., and Kanoh, H. (1995) *J. Biol. Chem.* **270,** 20298–20304
10. Otteken, A., and Moss, B. (1996) *J. Biol. Chem.* **271,** 97–103
11. Hammond, C., and Helenius, A. (1994) *Science* **266,** 456–458
12. Chen, W., Helenius, J., Braakman, I., and Helenius, A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6229–6233
13. Balow, J. P., Weissman, J. D., and Kearse, K. P. (1995) *J. Biol. Chem.* **270,** 29025–29029
14. Zhang, Q., Tector, M., and Salter, R. D. (1995) *J. Biol. Chem.* **270,** 3944–3948
15. Arunachalam, B., and Cresswell, P. (1995) *J. Biol. Chem.* **270,** 2784–2790
16. Clevers, H., Dunlap, S., Saito, H., Georgeopoulos, K., Wileman, T., and Terhorst, C. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8623–8627
17. Rajagopalan, S., Xu, Y., and Brenner, M. B. (1994) *Science* **263,** 387–390
18. Kim, P., and Arvan, P. (1995) *J. Cell Biol.* **12,** 29–38
19. Carreno, B. M., Screiber, K. L., McKean, D. J., Stroynowski, I., and Hansen, T. H. (1995) *J. Immunol.* **154,** 5173–5180
20. Loo, T. W., and Clarke, D. M. (1994) *J. Biol. Chem.* **269,** 28683–28689
21. Rose, J. K., and Gallione, C. J. (1981) *J. Virol.* **39,** 519–528
22. Machamer, C. E., Doms, R. W., Bole, D. G., Helenius, A., and Rose, J. K. (1990) *J. Biol. Chem.* **265,** 6879–6883
23. Metsikko, K., and Simons, K. (1986) *EMBO J.* **5,** 1913–20
24. Hammond, C., and Helenius, A. (1994) *J. Cell Biol.* **126,** 41–52
25. Machamer, C. E., Florkiewicz, R. Z., and Rose, J. K. (1985) *Mol. Cell. Biol.* **5,** 3074–3083
26. Marquardt, T., Hebert, D. N., and Helenius, A. (1993) *J. Biol. Chem.* **268,** 19618–19625
27. Braakman, I., Hoover-Litty, H., Wagner, K. R., and Helenius, A. (1991) *J. Cell Biol.* **114,** 401–411
28. Nicchitta, C. V., and Blobel, G. (1993) *Cell* **73,** 989–998
29. Hurtley, S. M., Bole, D. G., Hoover-Litty, H., Helenius, A., and Copeland, C. S. (1989) *J. Cell Biol.* **108,** 2117–2126
30. de Silva, A., Braakman, I., and Helenius, A. (1993) *J. Cell Biol.* **120,** 647–655
31. Marquardt, T., and Helenius, A. (1992) *J. Cell Biol.* **117,** 505–513
32. Gibson, R., Schlesinger, S., and Kornfeld, S. (1979) *J. Biol. Chem.* **254,** 3600–3607
33. Machamer, C. E., and Rose, J. K. (1988) *J. Biol. Chem.* **263,** 5955–5960
34. Hurtley, S. M., and Helenius, A. (1989) *Annu. Rev. Cell Biol.* **5,** 277–307
35. Seckler, R., and Jaenicke, R. (1992) *FASEB J.* **6,** 2545–2552
36. Helenius, A. (1994) *Mol. Biol. Cell* **5,** 253–265
37. Gething, M.-J., and Sambrook, J. (1992) *Nature* **355,** 33–45
38. Bonifacino, J. S., and Lippincott-Schwartz, J. (1991) *Curr. Opin. Cell Biol.* **3,** 592–600
39. Marks, M. S., Germain, R. N., and Bonifacino, J. S. (1995) *J. Biol. Chem.* **270,** 10475–10481
40. Amara, J. F., Cheng, S. H., and Smith, A. E. (1992) *Trends Cell Biol.* **2,** 145–149
41. Lomas, D. A., Evans, D. L., Finch, J. T., and Carrell, R. W. (1992) *Nature* **357,** 605–607
42. Thomas, P. J., Qu, B., and Pedersen, P. L. (1995) *Trends Biochem. Sci.* **20,** 456–459