Folding of VSV G Protein: Sequential Interaction with BiP and Calnexin

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The endoplasmic reticulum (ER) contains molecular chaperones that facilitate the folding of proteins in mammalian cells. Biosynthetic labeling was used to study the interactions of two chaperones, BiP and calnexin, with vesicular stomatitis virus (VSV) glycoprotein (G protein). Coimmunoprecipitation of G protein with the chaperones showed that BiP bound maximally to early folding intermediates of G protein, whereas calnexin bound after a short lag to more folded molecules. Castanospermine, an inhibitor of ER glucosidases, blocked the binding of proteins to calnexin and inhibited G protein folding. Interaction with calnexin was necessary for efficient folding of G protein and for retention of partially folded forms.

The lumen of the ER is a specialized environment devoted to the maturation of membrane and secretory proteins (*1*). In the ER and elsewhere in the cell, many newly synthesized proteins rely on molecular chaperones to fold properly. Although these chaperones are thought to promote folding by preventing unproductive intra- and intermolecular interactions, most likely caused by exposed hydrophobic surfaces, detailed mechanisms of chaperone action remain poorly defined (*2*).

In its mature form, the glycoprotein G of VSV is a noncovalently bound, homotrimeric membrane protein composed of 67-kD monomers (495 amino acids) that contain two N-linked oligosaccharides (*3*, *4*). The ectodomain of each subunit contains 12 cysteines of which most, or all, participate in intrachain disulfide bonds (*3*, *5*). During folding, G protein monomers have been shown to associate with two chaperones, BiP (GRP78) (*5*) and calnexin (*6*). BiP is a soluble member of the heat shock protein 70 (Hsp70) family of chaperones (*7*), and calnexin is a membrane-bound chaperone with specificity for glycoproteins (*8*–*10*).

To compare the kinetics of interaction between the newly synthesized G protein and the two chaperones, we metabolically labeled CHO15B cells for 1 hour to incorporate [^{35}S]methionine into BiP and calnexin. The cells were then infected with VSV (Indiana serotype, San Juan strain), labeled with [^{35}S]methionine for 2 min, and incubated with unlabeled media for up to 20 min. The cells were rapidly cooled and lysed in the presence of 20 mM *N*-ethylmaleimide (NEM) to alkylate free sulfhydryl groups. Lysates were immunoprecipitated with antibodies to G protein, BiP, or cal- nexin (Fig. 1). On nonreducing SDS–polyacrylamide gel electrophoresis (SDS-PAGE), the incompletely oxidized folding intermediates (ITs) of G protein migrated between the positions of the fully reduced and the fully oxidized protein (Fig. 1A). They were not resolved into discrete bands. During the incubation with unlabeled media, the protein progressed to the native (NT), oxidized form, and after transport from the ER and oligosaccharide processing in the Golgi apparatus, to the fully trimmed (Tr) form.

Coprecipitation of G protein showed that BiP was maximally bound immediately after the pulse (Fig. 1B) (*5*). The pattern on nonreducing gels showed that BiP bound only to a cohort of partially oxidized, slowly migrating intermediates. The fully oxidized G protein did not coprecipitate with the chaperone. G protein dissociated from BiP with a half-time ($t_{1/2}$) of 3 to 4 min (Fig. 1B) (*5*). The maximal amount of labeled G protein coprecipitated reached only about 10%, but the precipitations were not optimal because of limited amounts of available BiP antibody (*11*). However, with the use of more of the same antibody, approximately 40% of total labeled G protein can be coprecipitated (*5*). Given that G protein folding is very efficient and degradation virtually nonexistent during the time of the experiment, it is likely that most G protein molecules associate with BiP during early stages of their folding.

Antibodies to calnexin coprecipitated G protein maximally after 2 to 5 min of chase (incubation in unlabeled medium) (Fig. 1C) (*6*). The pattern on nonreducing gels showed that the faster migrating, more oxidized G proteins were the predominant form coprecipitated. G protein was bound to calnexin for a longer time ($t_{1/2}$ = 10 to 12 min) than it was to BiP. The maximum amount of labeled G protein coprecipitated was more than 50% of the total, which indicated that most of the G proteins interacted with calnexin during their matura-

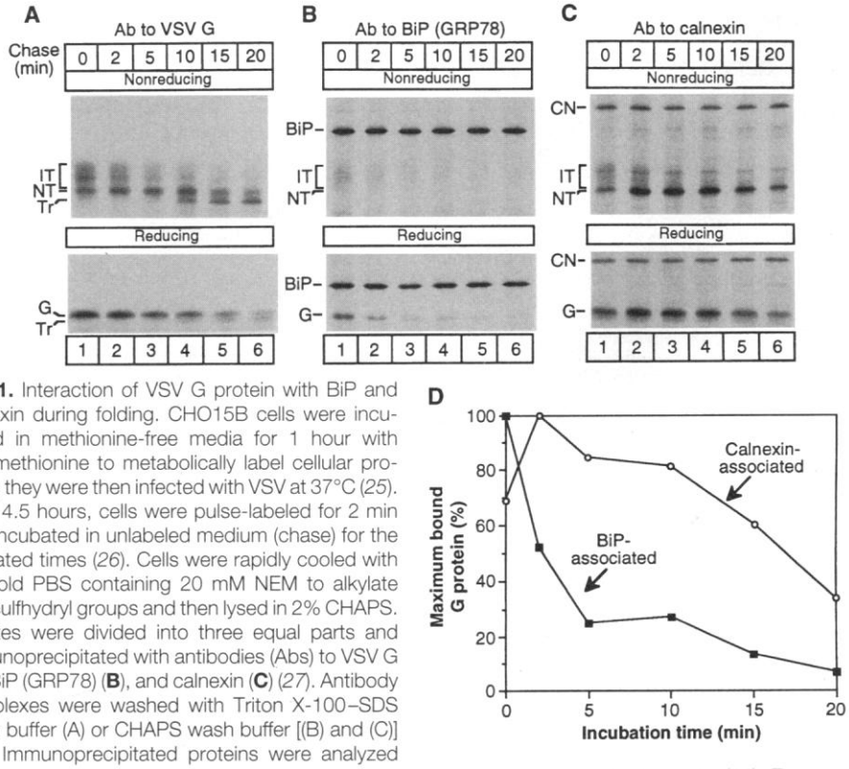

Fig. 1. Interaction of VSV G protein with BiP and calnexin during folding. CHO15B cells were incubated in methionine-free media for 1 hour with [^{35}S]methionine to metabolically label cellular proteins; they were then infected with VSV at 37°C (*25*). After 4.5 hours, cells were pulse-labeled for 2 min and incubated in unlabeled medium (chase) for the indicated times (*26*). Cells were rapidly cooled with ice-cold PBS containing 20 mM NEM to alkylate free sulfhydryl groups and then lysed in 2% CHAPS. Lysates were divided into three equal parts and immunoprecipitated with antibodies (Abs) to VSV G (**A**), BiP (GRP78) (**B**), and calnexin (**C**) (*27*). Antibody complexes were washed with Triton X-100–SDS wash buffer (A) or CHAPS wash buffer [(B) and (C)] (*28*). Immunoprecipitated proteins were analyzed on 7.5% nonreducing (upper) or reducing (lower) SDS-PAGE followed by fluorography (*29*). Exposure times for fluorography are different for (A), (B), and (C) (*11*). (**D**) The amount of G protein bound to BiP (filled squares) and calnexin (open circles) was measured by densitometry from the fluorographs shown in (B) and (C).

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tion. Thus, G protein appeared to undergo sequential interaction with the chaperones. BiP initially bound incompletely oxidized folding intermediates. Later, calnexin interacted predominantly with the more oxidized forms. Small amounts of BiP were detected coprecipitating with calnexin (Fig. 1, B and C) and vice versa. This suggested that ternary complexes of G protein, BiP, and calnexin may exist as intermediates between the BiP-bound and calnexin-bound forms (*12*).

To interact with calnexin, the G protein of the tsO45 VSV mutant and the influenza hemagglutinin (HA) glycoprotein require partially glucose-trimmed N-linked oligosaccharides (6). Castanospermine (CST) and other inhibitors of ER glucosidases I and II prevent the glucose trimming of core oligosaccharides (*13*) and thereby block the binding of the viral proteins to calnexin (6). When CST was added to uninfected CHO cells, most cellular glycoproteins that normally interact with calnexin also failed to bind (Fig. 2). Thus, the requirement for oligosaccharide trimming applied both to viral glycoproteins and to constitutive cellular glycoproteins that normally interact with calnexin.

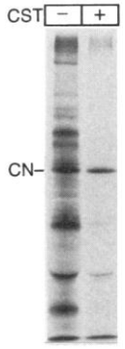


Fig. 2. Effect of CST on the association of cellular proteins with calnexin. Uninfected CHO cells were pretreated for 45 min with 200 μM CST, then pulse-labeled for 5 min in the presence of the drug (+). Control cells were mock-treated (−). The cells were then rapidly cooled, lysed with 2% CHAPS, and immunoprecipitated with cal- nexin (CN) antiserum. Immunoprecipitates were analyzed on 7.5% reducing SDS-PAGE followed by fluorography.

To determine to what extent maturation depended on calnexin binding, we analyzed G protein folding in the presence of CST. In mock-treated cells, the G protein showed the normal progression from partially oxidized forms to NT and Tr forms (Fig. 3A). Sequential interaction with BiP (Fig. 3B) and calnexin (Fig. 3C) was also seen. When G protein was synthesized in the presence of CST, its molecular weight was slightly higher and it did not bind to calnexin (Fig. 3, A and C). Folding was clearly compromised. Immediately after the labeling period, only a fraction reached a mobility on nonreduced gels equivalent to that of the fully oxidized G protein, and little additional folding took place during the subsequent 20-min incubation (Fig. 3A).

Although unable to bind to calnexin, the G protein did interact with BiP in CST-treated cells (Fig. 3B). The amount coprecipitated in the presence of CST was higher than in control cells and the binding persisted for a longer time (Fig. 3D). This indicated that glucose trimming was not needed for association with BiP and suggested that if BiP was not able to unload its ligand to calnexin the association was prolonged. The binding of the two chaperones was therefore not only sequential but also functionally connected. Clearly, calnexin provided some type of assistance needed for efficient G protein folding that could not be supplied by BiP.

Next, we analyzed the fate of G protein synthesized in the presence of CST. Cells were infected, incubated for 2 hours in the presence of CST, and prepared for immunofluorescence microscopy with antibodies to G protein. In both control and CST-treated cells, G protein was detected on the surface (Fig. 4, A and B) (*14*). About 20% of the CST-treated cells showed G protein present in a punctate pattern throughout the cytoplasm, a pattern not present in control cells (Fig. 4, C and E). Double label immunofluorescence demonstrated that the punctate structures contained p58 (Fig. 4, C and D), a marker protein for the intermediate compartment between the ER and the Golgi apparatus (*15*, *16*). The structures also contained BiP (Fig. 4, E and F). Because BiP is not a normal constituent of the intermediate compartment, it was probably relocated there as a result of its continued association with the partially folded G protein. Such a phenomenon has recently been reported for BiP bound to the misfolded tsO45 G protein (*17*, *18*).

The G protein molecules that reached

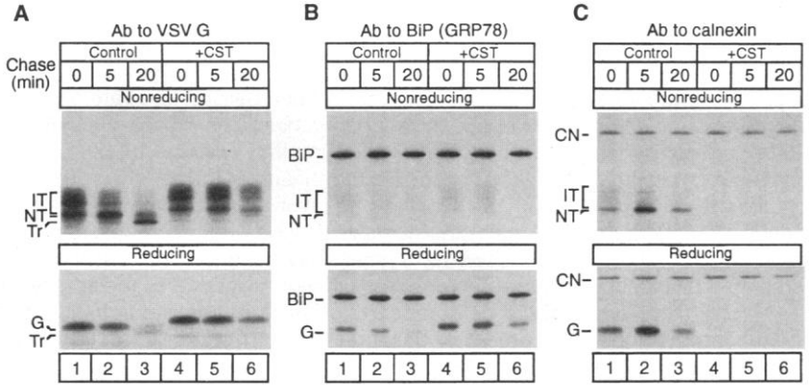
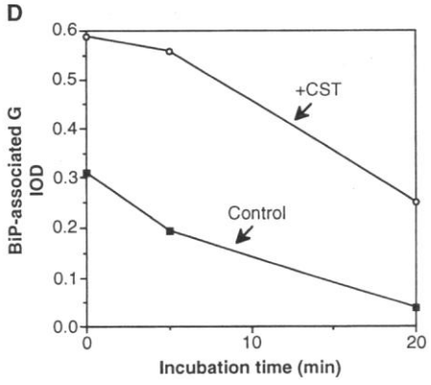


Fig. 3. Effect of CST on the folding of VSV G protein. Cells were prelabeled, infected, and pulse-labeled as described in Fig. 1. Cells in lanes 4 to 6 were treated with 1 mM CST for 45 min before the pulse, during the pulse, and during the chase. Control cells in lanes 1 to 3 were mock-treated. Immunoprecipitates with antibodies to VSV G (**A**), BiP (GRP78) (**B**), and calnexin (**C**) were prepared as described in Fig. 1. (**D**) The amount of BiP-bound G protein in CST-treated (open circles) and control cells (filled squares) was measured by densitometry from fluorographs shown in (B) and expressed as units of integrated optical density (IOD).



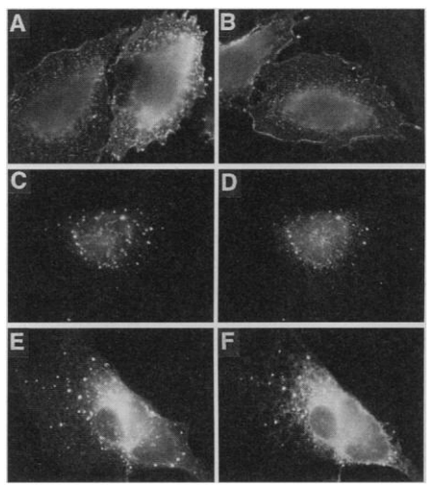


Fig. 4. Indirect immunofluorescence labeling of control and CST-treated CHO cells. Cells were infected for 1 hour at 37°C, then incubated for an additional 2 hours in the absence (**A**) or presence (**B** through **F**) of 1 mM CST. The cells were then fixed in 3.7% formaldehyde and prepared for labeling as described (*17*). Surface staining of nonpermeabilized cells with a purified polyclonal antibody VSV G is shown [(A), control, and (B), +CST]. Double labeling of Triton X-100–permeabilized cells is shown with antibodies to G protein [(C) and (E)], p58 (D), and BiP (F).

the surface of CST-treated cells may have been more extensively folded and therefore competent for transport (Fig. 3A). Alternatively, they may have been incompletely folded molecules not retained in the ER by a CST-compromised quality control system. Calnexin has been shown to be part of the retention apparatus present in the ER (*19*, *20*). The observation that incorporation of G protein from the cell surface into virus particles drops by 90% in the presence of CST suggests that much of it is, in fact, defective (*21*). The apparent transport of defective G protein to the cell surface suggests that calnexin serves not only as a folding factor for G protein but also as a retention factor.

Our results reveal that calnexin is a true chaperone in the sense that it associates transiently with G protein and promotes its folding. Because our data were obtained in living cells, they are likely to reflect a physiologically relevant activity. Our observations, moreover, provide evidence for sequential BiP and calnexin action during G protein folding in the ER. Indications of ordered chaperone binding have been found in mitochondria in vivo and for protein refolding in the presence of bacterial chaperones in vitro (*22*, *23*). In all these cases, including that of G protein, the initial interaction involves a member of the Hsp70 family of chaperones. In the mitochondrial and bacterial systems, subsequent interaction occurs with a member of the Hsp60 family. In the ER, which seems to be devoid of Hsp60 homologs, calnexin may have the role of secondary chaperone.

Although a large number of proteins have been shown to associate transiently with calnexin, including about 20 different proteins in CHO cells (Fig. 2) (*8*), only some are likely to be strictly dependent on it for folding. In fact, G protein belongs to a subfraction of glycoproteins whose folding is inhibited by CST and by other glucosidase inhibitors (*13*, *24*). Among the proteins that can fold independently is HA. Although it normally forms a complex with calnexin, HA folding is not inhibited by CST (*6*). Apparently calnexin constitutes a link in the folding, assembly, and retention machinery of the ER that is used by many glycoproteins but is essential for only some.

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11. Because of the limited amounts of BiP antibody available to us, immunoprecipitations could not be performed quantitatively. A longer film exposure than that shown in Fig. 1A was used to show the binding kinetics for the BiP (GRP78) coprecipitations.
12. Because VSV shuts off host protein synthesis [D. D. Dunigan and J. M. Lucas-Lenard, *J. Virol.* **45**, 618 (1983)], G protein is the most abundant itinerant protein in the ER. Thus, any BiP seen coprecipitating with calnexin antibodies (and vice versa) is likely to be bound to G protein.
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18. Control cells showed BiP in the ER and p58 in a typical punctate, predominantly perinuclear pattern.
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26. Before pulse labeling, cells were washed twice with phosphate-buffered saline (PBS) and incubated for 15 min in methionine-deficient media. Pulse media contained 0.4 mCi/ml of [^{35}S]methionine (L-[^{35}S] in vitro cell labeling mix; >1000 Ci/mM; Amersham). After labeling, we initiated incubations by adding media containing 4.5 mM methionine and 500 μM cycloheximide [I. Braakman, H. Hoover-Litty, K. Wagner, A. Helenius, *J. Cell Biol.* **114**, 401 (1991)].
27. Antibodies to calnexin and VSV G were as previously described (*17*). Cells were lysed in a buffer containing 2% CHAPS, 200 mM NaCl, 50 mM Hepes (pH 7.6), and chymostatin, leupeptin, antipain, and pepstatin (10 μg/ml each). Nuclei were pelleted by centrifugation for 5 min at 15,000*g*, and lysates were rotated at 4°C with antibody and Protein A sepharose beads (Sigma) for 3 hours.
28. Triton X-100–SDS wash buffer contained 0.1% SDS, 0.05% Triton X-100, 10 mM tris (pH 8.0), and 300 mM NaCl. CHAPS wash buffer contained 0.5% CHAPS, 50 mM Hepes (pH 7.6), and 200 mM NaCl.
29. Loss of G protein signal in Fig. 1A was seen when cells were solubilized in CHAPS, which was necessary for coprecipitation. Signal loss was not seen when cells were solubilized in 1% SDS.

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Anatomical Evidence for Cerebellar and Basal Ganglia Involvement in Higher Cognitive Function

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The possibility that neurons in the basal ganglia and cerebellum innervate areas of the cerebral cortex that are involved in cognitive function has been a controversial subject. Here, retrograde transneuronal transport of herpes simplex virus type 1 (HSV1) was used to identify subcortical neurons that project via the thalamus to area 46 of the primate prefrontal cortex. This cortical area is known to be involved in spatial working memory. Many neurons in restricted regions of the dentate nucleus of the cerebellum and in the internal segment of the globus pallidus were labeled by transneuronal transport of virus from area 46. The location of these neurons was different from those labeled after HSV1 transport from motor areas of the cerebral cortex. These observations define an anatomical substrate for the involvement of basal ganglia and cerebellar output in higher cognitive function.

The basal ganglia and cerebellum have long been regarded as contributing to the planning and execution of movement; however, there have been suggestions that these two structures are also involved in nonmotor or cognitive function. For example, Alexander, DeLong, and Strick (*1*) proposed that the basal ganglia participate in five separate loops with motor and nonmotor areas of the cerebral cortex. According to their scheme, the nonmotor output of the basal ganglia targets three cortical areas via the thalamus: dorsolateral prefrontal cortex, lateral orbitofrontal cortex, and anterior cingulate cortex. As a result of these connections, the output of the basal ganglia is

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