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Characterization of a Cytosolic Activity That Induces the Formation of Golgi Membrane Tubules in a Cell-Free Reconstitution System[†]

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ABSTRACT: Using a cell-free reconstitution system, we have characterized a cytosol- and ATP-dependent process that leads to the formation of membrane tubules from isolated Golgi complexes. These membrane tubules are uniform in diameter (50–70 nm) and morphologically identical to ones normally seen in cells and to those which are enhanced following brefeldin A treatment. Tubulation was strictly dependent on an activity present in an organelle-free extract of bovine brain cytosol and hydrolyzable ATP. Tubule formation was saturable with respect to both cytosol and ATP with half-maximal induction occurring at ~0.5 mg/mL cytosol and 10–20 μM ATP. Mild proteolytic treatment of Golgi membranes significantly reduced the extent of tubulation to subsequently added cytosol, suggesting that the tubulation activity interacts with Golgi-associated membrane proteins. The cytosolic tubulation activity was heat-labile, nondialyzable, and precipitated in ammonium sulfate. This activity could be followed through various chromatographic steps to yield fractions enriched in a major 40 kDa protein and several other minor proteins of ~80, 60, and 30 kDa. Monospecific antibodies against the 40K protein inhibited the cytosol-dependent tubulation of Golgi membranes in the cell-free system. Gel filtration chromatography suggests that the tubulation activity has a native molecular weight of ~125 000–140 000. These results establish the existence of cytosolic protein factors that regulate the formation of Golgi membrane tubules, and will provide the means for a biochemical dissection of membrane tubulation.

All eucaryotic cells contain a complex array of intracellular membrane-bounded organelles. These organelles or compartments carry out many important functions such as secretion, endocytosis, and lysosome biogenesis. These processes are dependent upon the ability to transport molecules from one compartment to another—collectively referred to as intracellular membrane trafficking. Associated with the organelles performing these tasks are numerous discrete coated vesicles and membrane tubules. Several classes of coated vesicles, COP- (known now as subclasses I and II) and clathrin-coated vesicles, have been well studied and are known to be involved in various aspects of membrane trafficking (Pearse & Robinson, 1990; Pryer et al., 1992; Rothman, 1994; Barlowe et al., 1994).

Membrane tubules of uniform diameter (50–70 nm) and varying lengths (up to several micrometers) have also been observed to emanate from a variety of organelles including the Golgi complex (Morré et al., 1970; Novikoff et al., 1971; Rambourg et al., 1979), the *trans* Golgi network (TGN) (Cooper et al., 1990; Rambourg & Clermont, 1990), and endosomes (Geuze et al., 1983; Marsh et al., 1986; Hopkins et al., 1990; Tooze & Hollingshead, 1991). In fact, tubular extensions of the Golgi complex and TGN (formerly the GERL) were observed more than 20 years ago (Morré et al., 1970; Novikoff et al., 1971). Although the existence of these discrete tubules is well documented, their functions are not well understood, except perhaps in the case of endosomes. A variety of studies have suggested that tubular membranes, emanating from early and late endosomes, are involved in the segregation and recycling of membrane and receptors to various cellular destinations (Geuze et al., 1983, 1987, 1988; Klumperman et al., 1993; Mayor et al., 1993). Thus, at least for endosomes, a probable function in intracellular trafficking has been ascribed to membrane tubules.

Interestingly, the number and length of membrane tubules emanating from the Golgi, TGN, and endosomes can be increased by the fungal metabolite brefeldin A (BFA) (Hunziker et al., 1991; Klausner et al., 1992; Lippincott-Schwartz et al., 1990, 1991; Wood et al., 1991; Wood & Brown, 1992). It is now known that at least part of BFA's effects are exerted by inhibiting the ADP-ribosylation factor (ARF)-mediated binding of both Golgi-associated COP protein complexes (Donaldson et al., 1990, 1992a,b; Helms & Rothman, 1992; Orci et al., 1991) and TGN-associated clathrin protein complexes (Robinson & Kreis, 1992; Stamnes & Rothman, 1993; Wong & Brodsky, 1992) to membranes, thereby resulting in the cessation of vesicular trafficking. For reasons that are not yet clear, Golgi and TGN membranes respond to the block in coated vesicle formation by inducing extensive tubule formation. These BFA-induced tubules are morphologically identical to those which normally emanate from Golgi and TGN membranes—there are just more of them, and they are longer in the presence of BFA. Since BFA inhibits the binding of COP-I and clathrin coat proteins which are required for vesicle formation, other factors must be responsible for the tubulation of organelles.

Although many of the proteins and factors involved in coated vesicle formation have been identified, essentially nothing is known about the specific requirements for membrane tubule formation. As one of the first steps toward a molecular dissection of membrane tubulation, we have

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recently established a cell-free system in which the tubulation of Golgi membranes has been reconstituted (Cluett et al., 1993). The tubules formed in this in vitro system are 50–70 nm in diameter, up to several micrometers in length, and morphologically identical to those seen emanating from the Golgi complex and TGN in vivo. More recently, Weidman et al. (1993) have also successfully produced membrane tubules from isolated Golgi complexes. Using this system, we found that tubulation of Golgi membranes required a factor or factors present in an organelle-free extract of cytosol (Cluett et al., 1993). We report here the further characterization of this cell-free Golgi tubulation system and the use of this assay to characterize the cytosolic tubulation activity.

MATERIALS AND METHODS

Materials. Chromatography reagents were obtained from the following suppliers: phenyl-Sepharose and Sephacryl S300 (Pharmacia, Piscataway, NJ); DE 52 (Whatman, Clifton, NJ); Affi-Gel blue (Bio-rad, Hercules, CA); Sephadex G100-125 (Sigma Chemical Co., St. Louis, MO). Most of the other reagents were from Sigma Chemical Co.

Fractionation of Bovine Brain Cytosol. The first steps in the characterization of cytosolic tubulation factors involve the preparation of an organelle-free cytosolic extract from bovine brain. Tubulation activity was originally discovered in cytosolic extracts from rat liver and brain (Cluett et al., 1993); however, for scaling-up purposes, we switched to bovine brain which has approximately the same amount of activity per wet weight of tissue. All fractionation steps were carried out at 4 °C. Bovine brain cytosol was prepared by a modification of the procedures described by Malhotra et al. (1989). Brains were obtained fresh from a slaughterhouse and transported in storing buffer (320 mM sucrose, 25 mM Tris, pH 7.4) on ice and washed in the same, and blood and meninges were removed. Brains were minced in a small volume of homogenization buffer [25 mM Tris-HCl, 500 mM KCl, 250 mM sucrose, 1 mM dithiothreitol (DTT), and 2 mM EGTA, pH 7.4, also containing 2 μg/mL aprotinin, 0.5 μg/mL leupeptin, 2 μM pepstatin A, 0.5 mM *o*-phenanthroline, and 1 mM PMSF as protease inhibitors]. The minced tissue was homogenized in homogenization buffer (~40% w/v) with two 30 s bursts in a Waring blender. The homogenate was centrifuged at 13000*g* for 30 min, and the postnuclear supernatant (PNS) was collected. The PNS was then clarified by centrifugation at 95500*g* for 2 h. This clarified PNS fraction will be referred to as "crude cytosol". Proteins in the crude cytosol were precipitated by the slow addition of saturated ammonium sulfate, pH 8.0, to a final concentration of 60%. After 1 h of mixing, the precipitate was pelleted at 13000*g* for 30 min in a Sorval GSA rotor. The pellets were dissolved in dialysis buffer (25 mM Tris-HCl, 50 mM KCl, and 0.5 mM DTT, pH 8.0) and dialyzed (12–14 kDa cutoff tubing) overnight against 100 volumes of dialysis buffer. In the final buffer change, DTT was not included. During dialysis, a precipitate formed which was removed by centrifugation at 290000*g* for 1 h. The supernatant was harvested and will hereafter be called "bovine brain cytosol" (BBC). Unless otherwise indicated, all experiments used this BBC preparation. Typically, 1.5 kg of brains yielded over 600 mL of BBC with a protein concentration of ~25 mg/mL.

For further fractionation of this BBC preparation, saturated ammonium sulfate was added slowly with constant stirring to a final concentration of 45%. After 1 h, the precipitate was removed by centrifugation at 245000*g* for 30 min. The supernatant, which contained the tubulation activity, was collected and loaded directly onto a column of phenyl-Sepharose (55.5 cm × 2.5 cm; flow rate of 1.8 mL/min), which had been preequilibrated with 45% ammonium sulfate in dialysis buffer (50 mM KCl, 25 mM Tris-HCl, pH 8.0). After unbound protein flowed through, the column was washed with 45% ammonium sulfate in dialysis buffer, and proteins were eluted with a decreasing ammonium sulfate concentration gradient (45–0%) in dialysis buffer. Every fifth fraction was assayed for tubulation activity (see below). Fractions containing tubulation activity were pooled and dialyzed overnight against a 200× volume of 25 mM KCl, 25 mM Tris-HCl, pH 8.5 (DE buffer). The dialyzed phenyl-Sepharose pool was loaded onto a DE52 column (37 × 1.5 cm; flow rate 1.5 mL/min) which was preequilibrated with DE buffer. The column was washed with DE buffer until the A_{280} reached background levels, and bound proteins were eluted with 1 M NaCl (in DE buffer). Both the bound and unbound fractions were tested, and the flow-through was found to contain nearly all the tubulation activity. The DE52 flow-through was loaded directly onto an Affi-Gel blue column (6.5 × 1.5 cm; flow rate of 1.5 mL/min) which was preequilibrated in 25 mM KCl, 25 mM Tris-HCl, pH 8.0, and washed with the same until the A_{280} reached background levels. Proteins bound to the column were step-eluted in succession with 0.25 M KCl and 1.0 M KCl, both in 25 mM Tris-HCl, pH 8.0. The 0.25 M KCl elution fractions (containing nearly all the tubulation activity) were pooled; protein was concentrated by Amicon filtration (YM10 membrane at 30 psi) to ~3 mg/mL and loaded onto a Sephadex G100-120 column (162 × 1.5 cm). Proteins were loaded onto and eluted from the column in 50 mM KCl, 25 mM Tris, pH 8.0, at 0.125 mL/min and assayed for tubulation activity.

Isolation of Golgi Complexes. A fraction enriched in intact Golgi complexes was prepared from rat liver as described (Cluett & Brown, 1992). Golgi membranes could be stored for several weeks at −80 °C and retained the ability to tubulate upon thawing.

Tubulation Assay. Tubulation of Golgi membranes was determined by a whole-mount, EM negative-stain procedure previously used to document the tubulation of Golgi membranes (Cluett et al., 1993). Briefly, crude cytosol, or fractions thereof, was mixed with a tubulation assay buffer (final concentrations in the complete mixture were 50 mM KCl, 1 mM $MgCl_2$, 25 mM Tris-HCl, and 10 mM Hepes, pH 7.4) and then added 1:1 to a suspension enriched in Golgi complexes. The cytosol/assay buffer was preequilibrated to 37 °C before being mixed with the organelle suspensions. Our standard assay conditions included 50 μM ATP and were performed for 30 min at 37 °C. When necessary, fractions of BBC obtained by column chromatography were microdialyzed against dialysis buffer before conducting the assays. For negative controls, bovine serum albumin (BSA) was used in place of BBC (or active fractions thereof) (all at similar concentrations). After incubation, organelles were visualized by a rapid, negative-stain electron microscopy procedure (Cluett et al., 1993).

Tubulation of Golgi membranes was quantified by determining the percentage of each stack that displayed at least one tubule. A tubule was defined as a membranous extension, 50–70 nm in diameter, and at least 3 times as

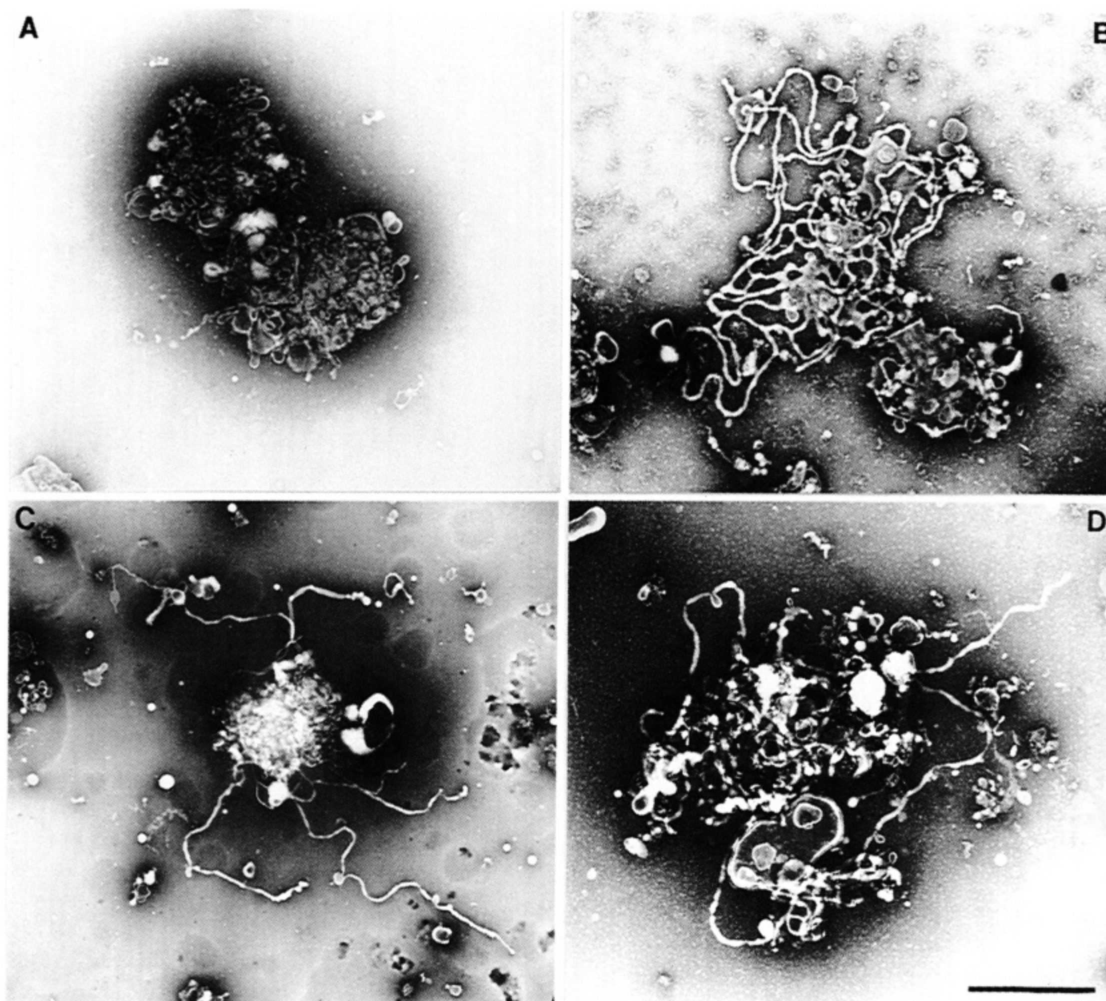

FIGURE 1: Visualization of control and tubulating Golgi membranes by negative staining. Rat liver Golgi complexes were assayed for tubulation activity by negative staining. (A) Golgi membranes treated with 1.5 mg/mL BSA as a control have associated vesicles, but few or no tubules. (B) Golgi complexes treated with unfractionated bovine brain cytosol (1.5 mg/mL) or (C and D) the peak activity fraction (91) from gel filtration (see Figure 7) induce long membrane tubules. All incubations were for 30 min at 37 °C and included 50 μM ATP, 50 μM GTP, and 1 mM $MgCl_2$. Bar = 0.5 μm.

long. For practical reasons, this assay can only provide a semiquantitative estimation of the tubulation activity. Even though the negative-stain procedure is fairly rapid, only a limited number of samples could be analyzed by electron microscopy during the fractionation procedures (e.g., usually about 30 column fractions/day). Ideally, we would like to determine the number of tubules per Golgi stack; however, this proved to be impractical during the fractionation procedure. Therefore, a Golgi stack with one tubule was equal to a stack with multiple tubules, thus making exact quantitation of tubulation per se problematic. From our qualitative observations, however, we noticed a clear positive correlation between the number of Golgi stacks with tubules and the number of tubules per stack.

Antibody Production and Characterization. Rabbit polyclonal antibodies were produced against the major 40K protein that was identified in the active fractions from the Sephadex G-100 column. Fractions highly enriched in the protein and the tubulation activity were pooled and subjected to preparative gel electrophoresis, and the single 40K band was electroeluted from gel pieces (Hunkapiller et al., 1983). Electro-eluted 40K protein was injected into rabbits using standard immunization protocols. Preimmune and immune sera were tested by Western blotting using goat anti-rabbit IgG conjugated to alkaline phosphatase as described (Park et al., 1991). Immune and preimmune IgG were prepared from sera by protein A–Sepharose affinity chromatography. Anti-40 kDa IgG was also tested for its ability to affect the cytosol-dependent tubulation of Golgi membranes in the cell-free assay system. Details of the antibody inhibition experiments are given in the figure legends.

RESULTS

Our previous studies established that membranes of isolated Golgi complexes are capable of forming membrane tubules of uniform diameter in a cell-free, reconstitution system (Cluett et al., 1993). Tubulation was assayed by examining the morphology of isolated Golgi complexes with electron microscopy after negative staining. As we also show here, isolated rat liver Golgi complexes incubated at 37 °C for 30 min in the absence of any cytosolic proteins (but with 50 μM ATP) exhibited a modest number of membrane buds and vesicles but few membrane tubules (Figure 1A). On the other hand, incubation of Golgi membranes with an organelle-free extract of bovine brain cytosol (BBC) and ATP at 37 °C for 30 min resulted in the striking formation of numerous, long membrane tubules which were uniformly 50−70 nm in diameter and which could reach lengths of several micrometers (Figure 1B). These tubules often extended outward from the cisternal stack (Figure 1B,C), and, therefore, could not be misinterpreted as fenestrated cisternae.

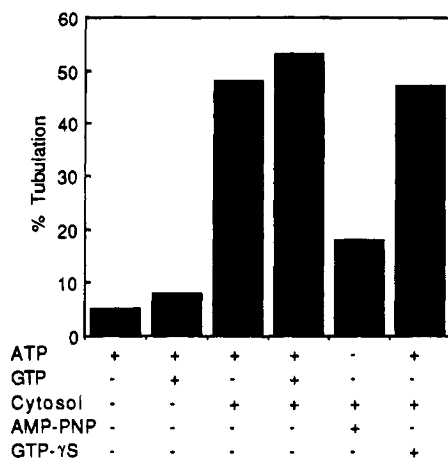

FIGURE 2: Characterization of the cytosol and energy requirements for Golgi membrane tubulation. Rat liver Golgi complexes were treated with 1.5 mg/mL bovine brain cytosol and a combination of ATP, GTP, AMP-PNP, or GTPγS as indicated on the figure. The final concentration of each nucleotide in the cytosol/assay buffer was 50 μM. The % tubulation refers to the percentage of Golgi complexes which exhibit tubules like those shown in Figure 1B and as described under Materials and Methods. Each result in the average of triplicate experiments (150 Golgi complexes counted/experiment).

Characterization of Tubulation Activity in Bovine Brain Cytosol. To characterize the tubulation activity in BBC, the extent of Golgi membrane tubulation under different conditions was measured by determining the percentage of Golgi complexes exhibiting tubular membrane extensions (as seen in Figure 1B compared to Figure 1A). In the presence of either BBC or ATP alone (but not both), a small number of Golgi complexes were found to be tubulated (Figure 2). The small number of tubules seen in samples treated with BSA alone (as a nonspecific protein control) was essentially the same as that seen when freshly isolated Golgi membranes were fixed immediately with glutaraldehyde (data not shown). However, the amount of tubulation produced by ATP alone was variably low but consistently above background levels, suggesting that some tubulation activity may be present on the isolated Golgi membranes. In contrast, the percentage of tubulated Golgi complexes increased ~10-fold following incubation with BBC and ATP (Figure 2). Because tubules did not form in the presence of just cytosol or ATP alone, tubulation cannot simply be an artifact of the negative staining procedure. Incubation with BBC and the nonhydrolyzable ATP analog AMP-PNP did not support tubulation much above control levels, suggesting that ATP hydrolysis is required (Figure 2).

An ATP dose–response in the presence of cytosol (1.5 mg/mL) revealed that half-maximal tubulation occurred at 10–20 μM ATP (Figure 3A). In addition, maximal tubule formation occurred in the presence of 0.5 mM ATP plus a regenerating system (creatine phosphate/creatine phosphokinase), showing that tubulation is not a result of low ATP levels (Figure 3A). Tubulation was neither stimulated by GTP nor inhibited by the nonhydrolyzable analog GTPγS (Figure 2).

To determine if tubulation of Golgi membranes was a saturable process, various concentrations of BBC were added to membranes. The results showed that tubulation was saturable at ~2–3 mg/mL BBC and not induced by equivalent concentrations of BSA (Figure 3B). Our qualitative impression was that the number of tubules per Golgi

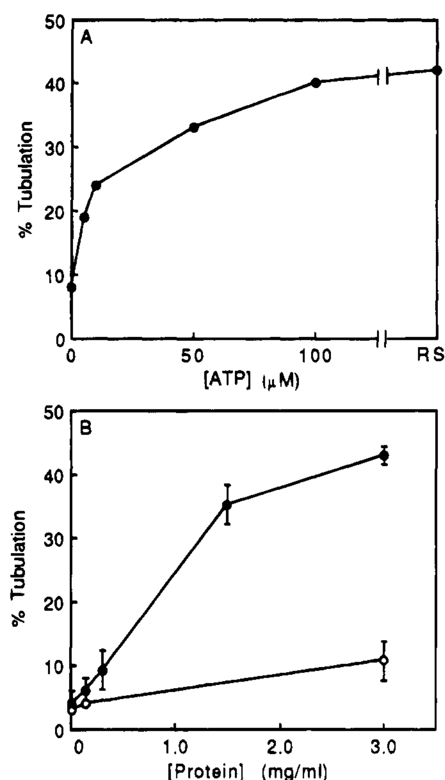

FIGURE 3: (A) ATP-dependent tubulation of Golgi membranes in vitro. Membranes were incubated with 1.5 mg/mL cytosol plus the indicated amounts of ATP. The data point as "RS" shows the extent of tubulation in 0.5 mM ATP plus an ATP regenerating system (5 mM creatine phosphate, 0.2 IU creatine phosphokinase). (B) Cytosol dose–response on Golgi membrane tubulation. Golgi complexes were incubated with increasing concentrations of bovine brain cytosol (solid circles) or BSA (open circles) in the presence of ATP and GTP (50 μM each) at 37 °C for 30 min. Each time point was determined from duplicate or triplicate samples and plotted as the mean + 1 SD.

stack did not increase significantly with higher concentrations of BBC, consistent with reaching saturation.

To further characterize the tubulation activity, cytosol was treated in various ways. First, heating cytosol at 65 °C for just 10 min significantly reduced the tubulation activity (Figure 4). Second, nearly all of the activity was precipitated in 60% ammonium sulfate, with little left in the supernatant; however, the tubulation activity was soluble in 45% ammonium sulfate (Figure 4). After precipitation in 60% ammonium sulfate, proteins were dissolved and dialyzed prior to testing for activity, showing that the tubulation activity is also nondialyzable. Thus, the tubulation activity in bovine brain cytosol is heat-labile, precipitated in 60% ammonium sulfate, and nondialyzable, and is therefore most likely a protein or protein complex.

To determine if the tubulation activity interacts with Golgi membranes, isolated Golgi complexes were first subjected to mild trypsinization before incubation in the tubulation assay. The results showed that mild trypsin treatment reduced the level of cytosol-dependent tubulation of Golgi membranes down to almost negative control levels (Figure 5). Treatment with similar amounts of α-chymotrypsin also reduced cytosol-dependent tubulation but only by to ~50% of positive control levels.

Identification of Tubulation Activity in Fractionated Cytosol. To further fractionate the cytosolic activity involved in Golgi membrane tubulation, proteins in cytosol were first precipitated in 60% ammonium sulfate, dissolved, dialyzed,

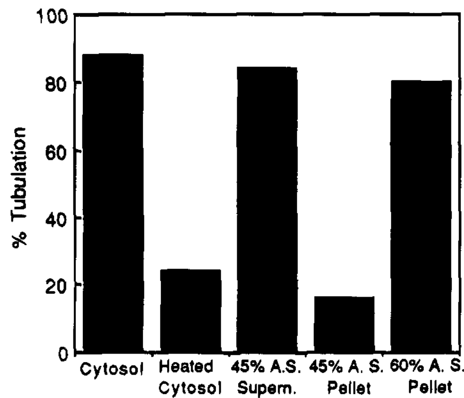

FIGURE 4: Effects of heat treatment and ammonium sulfate precipitation on Golgi membrane tubulation activity in bovine brain cytosol. Bovine brain cytosol was treated under various conditions and assayed for tubulation activity under standard conditions for 30 min at 37 °C. "Heated Cytosol" was treated for 10 min at 65 °C prior to assaying. "45% A.S. Supern." refers to material that was soluble in 45% ammonium sulfate, "45% A.S. Pellet" refers to material precipitated by 45% ammonium sulfate, and "60% A.S. Pellet" refers to material that was precipitated by 60% ammonium sulfate. Ammonium sulfate pellets and supernatants were dialyzed into the same buffer before assaying for tubulation activity.

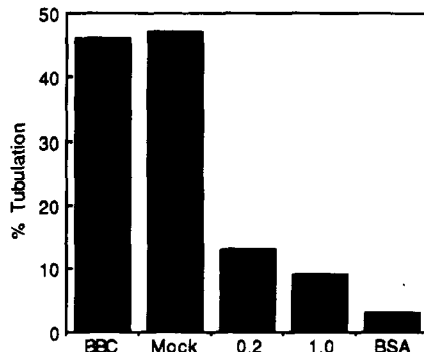

FIGURE 5: Protease treatment of Golgi membranes inhibits cytosol-dependent tubulation. Isolated Golgi complexes were treated as follows before incubation in the tubulation assay: "Control" membranes were untreated; "Mock" membranes were first incubated with 2 mg/mL (final concentration) soybean trypsin inhibitor (SBTI) for 5 min at 4 °C, and then trypsin (1.0 mg/mL final concentration) was added; "0.2" membranes were treated with 0.2 mg/mL trypsin; "1.0" membranes were treated with 1.0 mg/mL trypsin; and "BSA" indicates control Golgi membranes incubated with BSA alone during the tubulation assay to show the level of tubulation by a negative control. Mock and trypsin-treated Golgi membranes were incubated for 60 min at 4 °C, trypsin was inactivated by adding excess SBTI, and then membranes were incubated in the tubulation assay with 1.5 mg/mL cytosol (or BSA as indicated above) under standard conditions for 30 min at 37 °C.

and then subjected to precipitation with 45% ammonium sulfate. The soluble material from the 45% ammonium sulfate precipitation was then fractionated on a phenyl-Sepharose column. The peak of tubulation activity eluted at ~20% ammonium sulfate just before the major protein peak (Figure 6A). Fractions containing tubulation activity were pooled, dialyzed, and passed through a DE52 anion exchange column. The majority of the tubulation activity was found in the flow-through, although a low level of activity was observed in fractions eluted from the column with 1 M NaCl (Figure 6B). The flow-through from the DE52 column was loaded on an Affi-Gel blue column, the column was washed, and bound proteins were removed by stepwise elution with KCl. The results showed that most of the tubulation activity was retained by the column and eluted

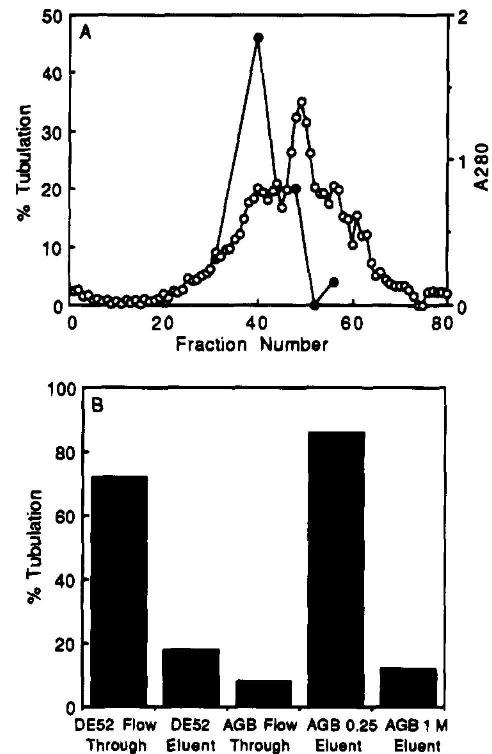

FIGURE 6: (A) Elution profile from the phenyl-Sepharose column. The % Tubulation (closed circles) and absorbance at 280 nm (open circles) were plotted versus fraction number. The percentage of Golgi complexes that were tubulated was determined by assaying various fractions under standard conditions and viewing by negative staining. The peak of Golgi membrane tubulation activity was eluted just before the major protein peak in this and numerous other preparations. (B) Golgi tubulation activity in bovine brain cytosol fractions from DE52 and Affi-Gel blue columns. The fractions containing tubulation activity from the phenyl-Sepharose column were pooled, dialyzed, and passed sequentially over DE52 and Affi-Gel blue columns. The flow-through (and wash) (DE52 Flow Through) and 1 M NaCl eluent (DE52 Eluent) from the DE52 column were collected and assayed by negative staining. Fractions from the DE52 flow-through were applied to the Affi-Gel blue column. The flow-through (AGB Flow Through) and the 0.25 M KCl (AGB 0.25 Eluent) and 1 M KCl (AGB 1 M Eluent) step elutions from the Affi-Gel blue column were collected and assayed for Golgi tubulation activity. Fractions with NaCl or KCl were dialyzed into dialysis buffer for the assay. Equal volumes of each fraction were assayed under standard conditions for 30 min at 37 °C.

with 0.25 M KCl (Figure 6B). Traces of activity were occasionally found in the 1 M KCl eluent.

Fractions containing activity from the Affi-Gel blue 0.25 M KCl elution were concentrated and then passed over a Sephadex G-100 gel filtration column (Figure 7). In this case, the peak of tubulation activity (at fraction 91) comigrated with the major protein peak (Figure 7B). SDS gel electrophoresis and silver staining of G-100 gel filtration fractions showed that a major 40 kDa protein, and several minor ones of ~80, 60, and 30 kDa (seen only on heavily overloaded gels), comigrated with the peak of tubulation activity (Figure 7A). A much more minor peak of activity was seen in this preparation (at fraction 127) but was not reproducible. When applied to isolated Golgi membranes, these peak gel filtration fractions were capable of inducing the formation of numerous membrane tubules (Figure 1C,D), similar to those seen with the bovine brain cytosol starting material (Figure 1B).

Because of the semiquantitative nature of the tubulation assay, it was not possible to obtain meaningful bookkeeping

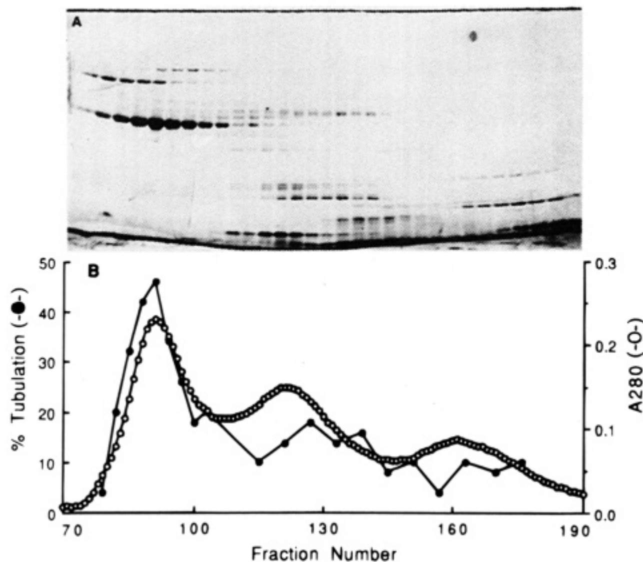

FIGURE 7: Elution profile and SDS−PAGE of fractions from the G-100 Sephadex column. (A) A silver-stained, 15% SDS−polyacrylamide gel of the assayed fractions from the G-100 column shown in (B). Equal volumes from each fraction were loaded into the lanes. Molecular weight standards ($\times 10^{-3}$) are shown on the left. (B) The % Tubulation (closed circles) and absorbance at 280 nm (open circles) were plotted versus fraction number. Percent tubulation was determined by assaying equal volumes of every fifth fraction. The peak of Golgi membrane tubulation activity comigrates with the peak of a major 40 kDa band. From this column, a major protein of ~40 kDa reproducibly comigrated with the tubulation activity. Minor bands at 80, 60, and 30 kDcan be seen on overdeveloped gels to also comigrate with the 40 kDa protein and the tubulation activity (not well-shown here).

Table 1: Enrichment of 40 kDa Protein

	total protein (mg)	amount of 40 kDa protein/ mg of protein[a]	total 40 kDa (arbitrary units $\times 10^3$)	total enrichment	yield (%)
homogenate	84937	0.32	27.56		100
high-speed supernatant	11055	0.94	10.30	2.9	37.4
BBC	8111	1.17	9.46	3.6	34.3
phenyl-Sepharose column	169.9	2.19	0.37	6.8	1.3[b]
Affi-Gel blue column	9.9	45.1	0.45	139	1.6
gel filtration	0.23	1776	0.41	5452	1.4

[a] The amount of 40 kDa protein in each fraction was determined by quantitative Western blotting followed by scanning desitometry. The numbers represent arbitrary units of immunoreactive 40 kDa/mg of protein. All scans were within the linear range of the Western blot assay. [b] Fractions from the phenyl-Sepharose column displayed a reversible loss of immunoreactivity due perhaps to the presence of ammonium sulfate.

on the enrichment of tubulation activity throughout the fractionation procedure. Thus, to estimate the enrichment of this activity, the relative amounts of the 40 kDa protein in different fractions were determined by quantitative Western blotting (see below for characterization of the anti-40 kDa antibody). Table 1 shows the results of a typical fractionation during which the 40 kDa protein was enriched >5400-fold relative to the starting homogenate. Based of the fold enrichment and yield, we conclude that the 40 kDa protein is found at a level of ~0.01−0.05% of the total protein in the homogenate and is therefore a relatively minor cytosolic protein. Starting with 1.5 kg of bovine brain (wet weight), we obtained ~0.1−0.5 mg of this 40 kDa protein, and proportionately lower amounts of the ~80, 60, and 30 kDa proteins. So far, attempts to obtain N-terminal sequence data from the 40 kDa protein that is associated with the tubulation activity have been unsuccessful presumably due to a blocked N-terminus. Figure 8 summarizes the fractionation results with a silver-stained gel of selected steps from the procedure that contain the peak of tubulation activity.

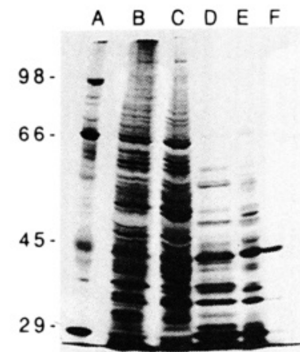

FIGURE 8: SDS−PAGE of various steps along the 40 kDa enrichment procedure. The amount of protein loaded in each lane of this silver-stained gel is shown in parentheses. Lane A: molecular weight standards ($\times 10^{-3}$). Lane B: homogenate (43 μg). Lane C: bovine brain cytosol (32 μg). Lane D: phenyl-Sepharose peak fraction (12 μg). Lane E: Affi-Gel blue pooled peak fraction (13.6 μg). Lane F: gel filtration peak fraction (0.5 μg).

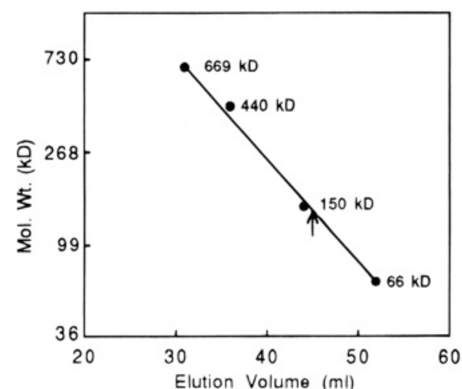

FIGURE 9: Native tubulation activity has a size consistent with a globular protein of ~125−140 kDa. The concentration tubulation activity from the 0.25 M KCl elution of the Affi-Gel blue column was run on a Sephacryl S-300 column. The peak of tubulation activity is indicated by the arrow. Molecular mass standards were thyroglobulin (669 kDa), ferritin (440 kDa), IgG (150 kDa), and BSA (66 kDa).

On the Sephadex G-100 column, the peak of tubulation activity migrated at a position corresponding to a globular protein of ~125 kDa. To obtain further evidence for this native size, material was passed through a Sephacryl S-300 column, and the tubulation activity and the 40 kDa protein again comigrated with an apparent Stoke's radius equal to that of a ~125−140 kDa globular protein (Figure 9). Likewise, the tubulation activity sedimented through a sucrose gradient at a position equal to that of a globular 125−140 kDa protein (data not shown).

Antibodies Against the 40 kDa Protein Inhibit Golgi Membrane Tubulation. Polyclonal antibodies prepared against gel-purified 40K protein specifically recognize only a single 40K band on Western blots of both crude bovine brain cytosol and gel filtration fractions highly enriched in the 40K protein (Figure 10A). Preimmune serum did not react with any proteins from either source.

To obtain further evidence that the 40K protein plays a role in the tubulation of Golgi membranes, bovine brain cytosol was immuno-depleted of the protein by preincubation with varying concentrations of anti-40 kDa IgG. Immune complexes were subsequently removed by immunoprecipitation with protein A−Separose beads. The cytosol immuno-depleted of the 40 kDa protein was then used in our standard tubulation assay. The results showed that immune IgG greatly reduced the level of Golgi membrane tubulation activity in bovine brain cytosol, whereas preimmune IgG did not (Figure 10B). Inhibition was linear with added immune IgG and saturable at 0.08 mg of anti-40 kDa IgG/mg of cytosol protein.

DISCUSSION

More than 20 years ago, and many times since, membrane tubules of uniform diameter (50−70 nm) have been observed to emanate from cisternal elements of the Golgi complex and TGN (Cooper et al., 1990; Morré et al., 1970; Novikoff et al., 1971; Rambourg et al., 1979; Rambourg & Clermont, 1990). Remarkably, however, essentially nothing is known about the nature of these tubules, including their function and the molecular requirements for their formation. Using a cell-free reconstitution system, we have characterized a cytosolic protein activity that is capable of inducing the tubulation of Golgi membranes. Fractions of bovine brain cytosol have been prepared that contain this activity and should serve as a starting point for a biochemical dissection of tubule formation from Golgi membranes.

Several lines of evidence support our contention that the Golgi membrane tubules reconstituted in this cell-free assay are physiologically relevant. First, the tubules produced in our cell-free system are morphologically identical to those seen extending from the Golgi complex in vivo under normal conditions (Cooper et al., 1990; Morré et al., 1970; Novikoff et al., 1971; Rambourg et al., 1979; Rambourg & Clermont, 1990) and following enhanced formation by BFA (Lippincott-Schwartz et al., 1990, 1991; Cluett et al., 1993). Second, tubulation of Golgi membranes both in vivo (Donaldson et al., 1990; Cluett et al., 1993) and as demonstrated here in a cell-free system is an ATP-requiring process. And third, as we have characterized here, membrane tubulation is dependent upon a very small subset of cytosolic proteins.

Tubulation appears to require ATP hydrolysis since it was not supported by the nonhydrolyzable analog AMP-PNP. Hydrolysis of ATP could be used to provide an energy source or for the phosphorylation of an unknown substrate(s). Although tubulation is not inhibited at normal ATP levels, it requires relatively low amounts of ATP (half-maximal tubulation occurring at 10−20 μM), suggesting that the ATP could be used for a phosphorylation reaction: many kinases work in this range of ATP (Kemp & Pearson, 1991). Interestingly, tubulation of Golgi membranes was neither stimulated nor inhibited by GTP or GTPγS, suggesting that GTP binding proteins are not involved in tubulation. In this regard, it is worth noting that recently Conradt et al. (1993) were able to induce the formation of tubulo-vesicular membrane structures from isolated yeast vacuoles. Although it is not clear at this point if the 50−70 nm tubules of mammalian Golgi complexes and the yeast vacuolar tubulo-vesicular membranes are produced by fundamentally similar mechanisms, tubulation of each organelle exhibited very similar cytosol, ATP, and GTP requirements.

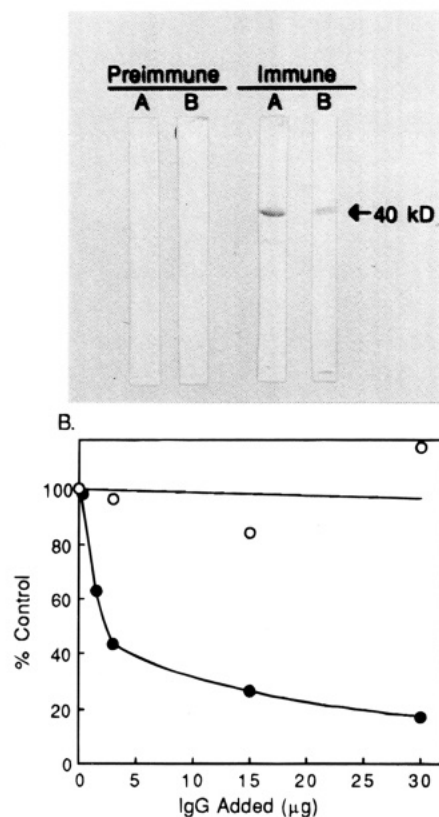

FIGURE 10: Polyclonal antibodies against the 40K protein block and cytosol-dependent tubulation of Golgi membranes. (A) Western blot using preimmune (PI) or immune (I) serum from a rabbit immunized with gel-purified 40K protein as identified in Figure 8. Lane A contains material from pooled fractions off the gel filtration column that are highly enriched in the 40K protein, and lane B contains a sample of crude bovine brain cytosol that was used as the starting material for biochemical fractionation. Immune, but not preimmune, serum specifically recognizes a 40 kDa protein in each sample. (B) Inhibition of Golgi membrane tubulation by anti-40K antibodies. Varying concentrations of preimmune (open circles) or immune anti-40 kDa IgG (solid circles) were incubated with a fixed amount of bovine brain cytosol for 15 min at room temperature followed by 90 min at 4 °C. Immune complexes were precipitated with protein A−Sepharose beads, and the resulting supernatant was used in the standard cell-free Golgi membrane tubulation assay for 15 min at 37 °C. The results are expressed as the % Control, with the control being tubulation assays performed in the absence of added IgG ("0" on the x-axis). The actual amount of tubulation in the control samples was 61.3%. Near-maximal inhibition occurred when 30 μg of immune IgG was used to immunoprecipitate tubulation activity from the bovine brain cytosol mixture (this amount corresponded to 0.088 mg of IgG/mg of cytosol protein). All data points are the average of duplicate samples.

The simplest explanation for the action of the tubulation factor(s) is that it binds to appropriate membranes and either directly or indirectly induces the formation of tubules. Support for this notion was obtained when we found that mild trypsinization of isolated Golgi membranes, under conditions that do not unstack the Golgi complex (Cluett & Brown, 1993), significantly inhibited the cytosol-dependent tubulation. This result suggests that the tubulation factor interacts with a protein "receptor" or substrate on the cytoplasmic surface of Golgi membranes.

The most highly enriched fractions of tubulation activity that we have obtained contain a major 40 kDa protein and several much more minor ones at ~80, 60, and 30 kDa. Antibodies against the 40K protein were capable of inhibiting tubulation activity in both crude cytosol and gel filtration factions highly enriched in the 40K protein consistent with

our conclusion that the 40K protein is involved in the tubulation reaction. By several methods, the native activity behaves as a large protein of ~125−140 kDa (assuming a globular shape). The 40K protein could form a homo-oligomeric complex, or it could also be complexed with one or several of the minor proteins. Additional work should reveal the subunit composition of the native protein.

Although our studies here contribute to understanding the tubulation process, they do not address the physiological role of tubules or their relationship with coated vesicles. From both in vivo and in vitro studies, some conditions which inhibit the formation of coated vesicles, e.g., BFA treatment, simultaneously induce or enhance the formation of membrane tubules (Lippincott-Schwartz et al., 1990, 1991; Orci et al., 1991; Wood et al., 1991; Wood & Brown, 1992). Klausner et al., (1992) have attempted to explain these findings by proposing that a reciprocal relationship exists between vesiculation and tubulation, although exactly what this relationship means in terms of the mechanisms by which vesicles and tubules form is not clear. For example, are the BFA-induced tubules actually membrane buds that continue to grow because coat proteins cannot bind and pinch off vesicles? Or, are tubules and vesicles produced by completely unrelated mechanisms which allow for enhanced formation of one type of membrane structure when the other is inhibited? Based on the recent stepwise reconstitution of COP-coated vesicle formation using purified components (Orci et al., 1993a,b; Ostermann et al., 1993), and our studies here on the reconstitution of tubulation in the absence of vesiculation, it would seem at first glance that tubules and vesicles are completely independent structures, each produced by different mechanisms. However, no studies to date have definitively ruled out the possibility that tubulation and vesiculation are somehow linked in order to mediate Golgi membrane trafficking [for discussions, see Klausner et al. (1992) and Weidman (1995)].

Recently, Weidman et al. (1993) were also able to induce the formation of 50−70 nm (diameter) tubules from isolated Golgi membranes. They further showed that Golgi-associated vesicles (and buds) exhibited strikingly different cytoplasmic membrane coatings than did tubules: the vesicles and buds were coated with tightly packed 10 nm particles (presumably coatomers), and the tubules were coated with a much finer material. Whether this fine coating material on tubules represents the cytosolic tubulation factor(s) characterized here, or something that is acted upon by the tubulation factor(s), remains to be determined. Our results here point directly to cytosolic protein(s) that control the process of Golgi membrane tubule formation and provide the necessary foundation for its molecular characterization.

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