

# The Activity of Golgi Transport Vesicles Depends on the Presence of the *N*-Ethylmaleimide-sensitive Factor (NSF) and a Soluble NSF Attachment Protein ( $\alpha$ SNAP) during Vesicle Formation

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**Abstract.** An assay designed to measure the formation of functional transport vesicles was constructed by modifying a cell-free assay for protein transport between compartments of the Golgi (Balch, W. E., W. G. Dunphy, W. A. Braell, and J. E. Rothman, 1984, *Cell*, 39:405–416). A 35-kD cytosolic protein that is immunologically and functionally indistinguishable from  $\alpha$ SNAP (soluble NSF attachment protein) was found to be required during vesicle formation. SNAP, together with the *N*-ethylmaleimide-sensitive factor (NSF) have previously been implicated in the attachment and/or fusion of vesicles with their target membrane. We show that NSF is also required during

the formation of functional vesicles. Strikingly, we found that after vesicle formation, the NEM-sensitive function of NSF was no longer required for transport to proceed through the ensuing steps of vesicle attachment and fusion. In contrast to these functional tests of vesicle formation, SNAP was not required for the morphological appearance of vesicular structures on the Golgi membranes. If SNAP and NSF have a direct role in transport vesicle attachment and/or fusion, as previously suggested, these results indicate that these proteins become incorporated into the vesicle membranes during vesicle formation and are brought to the fusion site on the transport vesicles.

THE molecular mechanisms driving protein transport are being illuminated by the reconstitution of transport in cell-free and semi-intact cell systems (reviewed by Goda and Pfeffer, 1989; Balch, 1989; Wattenberg, 1990), as well as by the application of yeast molecular genetics (reviewed by Schekman, 1985; Hicke and Schekman, 1990). One of the best characterized cell-free assays for protein transport measures the transit of the membrane glycoprotein (G protein) of vesicular stomatitis virus (VSV)<sup>1</sup> from a *cis*-compartment of the Golgi apparatus to a medial-compartment (Fries and Rothman, 1980; Balch et al., 1984a). This transport is measured between two populations of Golgi complex by a complementation assay. The Golgi apparatus in which the G protein originates (termed donor) is deficient in an enzyme, normally localized to the medial-Golgi, that adds a terminal *N*-acetylglucosamine (GlcNAc) to the G protein oligosaccharides. The target Golgi apparatus (acceptor) does not initially contain G protein, but is replete with the GlcNAc transferase. Therefore if G protein is transported from donor to acceptor, the G protein oligosaccharides will be modified by the addition of GlcNAc. This glycosylation event is used as a marker for transport from donor to acceptor.

1. *Abbreviations used in this paper:* NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide-sensitive factor; PF-1, priming factor 1; SNAP, soluble NSF attachment protein; VSV, vesicular stomatitis virus.

A variety of evidence indicates that transport between compartments of the Golgi apparatus is mediated by small transport vesicles (Rothman et al., 1984; Orci et al., 1986, 1989; reviewed in Rothman and Orci, 1992). Insight into the transport process therefore involves an understanding of how these vesicles are produced, how they are targeted to their correct destination, and how the membranes of the vesicles and the target compartment fuse to complete the delivery of the vesicle contents. An underpinning for this understanding has been achieved through a variety of kinetic and morphological studies which have defined and characterized several of the intermediate stages of the vesicle production and fusion cycle (Balch et al., 1984b; Wattenberg et al., 1986; Orci et al., 1989; Malhotra et al., 1988).

Perhaps the simplest of these observations is that there is a significant lag time (7–10 min) before transport proceeds at a linear rate (Balch et al., 1984b). This corresponds to the time required for G protein to progress through the intermediate steps of transport before arriving at its destination. If donor membranes are preincubated with cytosol and ATP before the addition of acceptor membranes, there is a small but consistent reduction in the lag time of transport (Balch et al., 1984b, also see Fig. 7 a of this report). This effect is termed donor priming. A striking change in the morphology of the donor membranes occurs under the same conditions. Initially, the Golgi stacks have relatively few vesicles associated with them. After incubation, a profusion of buds

and/or vesicles are seen in close proximity to the Golgi membranes. These new structures are coated with an apparently proteinaceous material (Orci et al., 1986). This coat is different from the clathrin coat best characterized in endocytic traffic. The kinetic effect of donor preincubation and the morphological transformation, show identical requirements for ATP, cytosol, elevated temperature, and sensitivity to primaquine (Orci et al., 1986; Hiebsch et al., 1991). It is therefore reasonable to conclude that the altered kinetics conferred by donor preincubation are due, at least in part, to the formation of buds, structures which will eventually pinch off to form transport vesicles.

Several proteins have been implicated in events leading up to the fusion of vesicles with their target once they have attached to the acceptor Golgi. The *N*-ethylmaleimide (NEM)-sensitive Factor (NSF) is presumed to act in such steps based on morphological evidence in both mammalian and yeast systems as well as biochemical data in a yeast system. In the absence of NSF, structures form which resemble attached but unfused transport vesicles (Glick and Rothman, 1987; Block et al., 1988; Malhotra et al., 1988; Orci et al., 1989). NSF is bound to membranes through three soluble proteins (the soluble NSF attachment proteins [SNAPs]) (Weidman et al., 1989; Clary et al., 1990; Clary and Rothman, 1990) and an as yet unidentified membrane receptor. Results using the yeast homologues of NSF and SNAP, Sec18p and Sec17p, respectively, indicate a somewhat different role for these proteins. Mutations in Sec17 and Sec18 lead to the accumulation of free vesicles in yeast (Kaiser and Schekman, 1990). This would suggest a role for SNAP and NSF in vesicle attachment, rather than fusion. Supporting this view are experiments using a broken cell assay in yeast which measures transport from the ER to the Golgi apparatus (Rexach and Schekman, 1991). In this assay also, free vesicles accumulate at the nonpermissive temperature when extracts are prepared from cells with a temperature sensitive mutation in sec18. It is as yet unresolved whether NSF and SNAPs are involved in vesicle attachment, vesicle fusion, or both.

The vesicle-producing reactions have been difficult to characterize biochemically. This is because the process is detected by the difference in kinetics of transport with or without donor preincubation (Balch et al., 1984b). However the consumption of the buds/vesicles formed on the donor is not synchronous. Therefore, in the time that the buds/vesicles from preincubated donor membranes go through the steps of vesicle targeting and fusion, new vesicles are formed and can also complete the transport process. Thus at no time is the difference between a preincubated and control incubation absolute. To alleviate this problem we have seized on the recent observation that the antimalarial drug primaquine is a selective inhibitor of donor priming (Hiebsch et al., 1991). Primaquine was shown to inhibit donor but not acceptor function. Primaquine was also shown directly to inhibit the kinetic priming of donor membranes, but not reactions that occur after the donor preincubation, such as vesicle targeting and fusion. Finally, an electron microscopic examination revealed that primaquine inhibited the formation of bud and vesicle structures in incubations of donor membranes. Here we use the ability of primaquine to inhibit vesicle formation to formulate an assay which specifically measures vesicle formation. Donor membranes are incubated under the conditions to be tested, and then primaquine is added along with

acceptor membranes. The buds and vesicles formed during the first incubation will progress through the remainder of the transport pathway, but little new vesicle formation will be initiated.

## Materials and Methods

### Materials

Unless otherwise specified all chemicals and biochemicals were from commercial sources and of the highest quality available.

### Chemicals

Primaquine, *N*-ethylmaleimide and Tween 20 were from Sigma Chemical Co. (St. Louis, MO). UDP-[3H]-GlcNAc (0.1 mCi/ml, 16.9 Ci/mMol) was from New England Nuclear (Boston, MA).

### Immunological Reagents

Antiserum to SNAP was a generous gift of W. Whiteheart and J. Rothman (Sloan-Kettering Cancer Institute, New York). The rabbit polyclonal was raised against a peptide (B4) common to  $\alpha$  and  $\beta$ SNAP and affinity purified against the same peptide. Purified SNAPs were also a gift from Drs. Whiteheart and Rothman. Nitrocellulose (0.45  $\mu$ m) was purchased from Schleicher and Schuell, Inc. (Keene, NH). Nonfat dry milk (Carnation, Los Angeles, CA) was used for blocking immunoblots. Immunoblots were stained with biotinylated goat anti-rabbit antiserum (Bethesda Research Laboratories, Gaithersburg, MD) and then avidin-conjugated alkaline phosphatase (Vector Laboratories, Burlingame, CA). Alkaline phosphatase activity was measured using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (both from Promega Biotec, Madison, WI).

### Methods

**Cell Culture and Preparation of CHO Cytosol and Membranes.** Wild-type CHO cells and CHO mutant lecl (Stanley et al., 1975) (ATCC), and VSV were grown and maintained as previously described (Balch et al., 1984a) as was growth, infection, and harvest of cells to produce donor and acceptor membranes. CHO cytosol was always prepared from lecl cells and was desalted by gel filtration on Sephadex G-25 into 25 mM Tris, 50 mM KCl, pH 8.0.

**Gel Electrophoresis and Immunoblotting.** SDS-gel electrophoresis was performed essentially as described (Laemmli, 1970), using 15% acrylamide separating gels. Minigel apparatus from BioRad Laboratories (Richmond, CA) were used for analysis of protein purification and 11-cm gels, run on Hoeffer apparatus (Hoeffer Scientific Instruments, San Francisco, CA), were used for the immunoblot shown in Fig. 5. For immunoblots, gels were transferred to nitrocellulose overnight (0.25 mA) in a blotting buffer of 3 g/l Trizma base, 14.4 g/l glycine in 20% methanol. The blots were blocked with 5% dry milk in PBS/0.2% Tween 20 (PBS/Tween) for 1 h at room temperature. The primary, anti-SNAP antibody at a dilution of 1:30 in PBS/Tween was incubated with the blots for 1 h at room temperature. Blots were washed three times in PBS/Tween and then incubated with secondary antibody, biotin-labeled goat anti-rabbit at 1:500, for 1 h at room temperature. These blots were then washed three times in PBS/Tween and incubated for 1 h with avidin-alkaline phosphatase conjugate (1:1,000 in PBS/Tween), washed three times with PBS/Tween and visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**Primaquine-based Vesicle Production Assay.** The assay is conducted in two stages. The first, vesicle production stage, consists of the cytosol fraction to be tested and 5  $\mu$ l of donor membranes in a buffer of 25 mM Tris, pH 7.5, 25 mM KCl, 2.5 mM Mg (C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub> and an ATP regenerating system (50  $\mu$ M ATP, 250  $\mu$ M UTP, 2 mM creatine phosphate, 7.3 U/ml creatine phosphokinase) in a total volume of 40  $\mu$ l. When testing for the NEM-sensitive priming factor 5  $\mu$ l (approximately 10  $\mu$ g) of NEM-treated lecl cytosol is also added. For NEM treatment, cytosol is incubated for 15 min on ice with 0.4 mM NEM, then the NEM is quenched with 0.8 mM DTT. The vesicle production incubation is for 20 min at 37°C. The second incubation, in which vesicle attachment and fusion occur, is initiated by adding a cocktail consisting of (for each tube) 2.5  $\mu$ l of crude lecl 1 cytosol, 5  $\mu$ l of acceptor membranes, 2.5  $\mu$ l of 6 mM primaquine, and 0.125  $\mu$ Ci of UDP-<sup>3</sup>H-GlcNAc. 10  $\mu$ l of this mix was added to each tube and the incubation

was continued for 90 min before terminating the assay. Cooling of the tubes after the donor preincubation resulted in loss of activity, so each tube was only briefly removed from the waterbath to add the second stage cocktail, and then immediately replaced. Immunoprecipitation of G protein, filtration of the immunoprecipitates and liquid scintillation counting were performed as previously described (Wattenberg et al., 1990).

**Assay for NSF-binding Activity.** NSF-membrane binding assays were performed using the two-stage assay previously described (Weidman et al., 1989) as modified by Clary et al., (1990). Each 25- $\mu$ l binding incubation (stage one) contained 1  $\mu$ g of KCl-extracted CHO Golgi membrane protein, 6 ng of CHO NSF, and the indicated volumes of purified priming factor 1 (PF-1)/SNAP (10  $\mu$ g/ml) or bovine brain cytosol (5.4 mg/ml) in NSF binding buffer (20 mM K<sup>+</sup>-HEPES, pH 7.4, 1 mM EDTA, 100 mM KCl, 200  $\mu$ M ATP, 1 mM DTT, 1% polyethylene glycol, 200  $\mu$ g/ml soybean trypsin inhibitor, and 0.2 M sucrose). Incubations were performed in 1.5 ml microtubes that had been preincubated with 10 mg/ml BSA to block nonspecific sites. After a 2-min incubation at 0°C, membrane-bound NSF was pelleted (12,000 g, 5 min) and the pellets washed with NSF binding buffer (30  $\mu$ l). The amount of NSF activity bound to the pellet was quantified in the second stage using the NSF-dependent Golgi transport reaction (assay conditions as described by Block et al., 1988).

**The SNAP-dependent Salt-washed Golgi Assay.** This assay was performed as previously described (Clary and Rothman, 1990). This system uses various fractions of mammalian cytosol to reconstitute transport with Golgi membranes that have been extracted with 1 M KCl to remove most of the peripheral membrane proteins (K-Golgi). Reconstitution is dependent on the addition of SNAP, NSF, and three protein fractions (fractions 1, 3, and 4) prepared from bovine brain cytosol. Each assay contained KCl-extracted donor and acceptor Golgi membranes, 6.2  $\mu$ g of fraction 1 protein, 5.5  $\mu$ g of fraction 3 protein, 3.5  $\mu$ g of protein 4 protein, 6 ng of purified CHO NSF, and the indicated volumes of PF-1/SNAP (10  $\mu$ g/ml) or bovine brain cytosol (5.4 mg/ml) in a 25- $\mu$ l reaction mixture. The concentrations of UDP-[<sup>3</sup>H]-GlcNAc, salts, ATP regenerating system, and palmitoyl CoA in the reaction mixture and the conditions of the incubation were exactly as described (Clary and Rothman, 1990).

**Purification of PF-1.** Brain cytosol was prepared from frozen bovine calf brain (Pel Freeze, Rogers, AR). 1 kg of brain was thawed in 2 l of breaking buffer (100 mM Tris, pH 8.0, 500 mM KCl, 250 mM sucrose, 1 mM DTT, 0.5 mM PMSF, 10  $\mu$ g/ml leupeptin, 10  $\mu$ M pepstatin, 1 mM 1,10 phenanthroline, 1 mM metabisulfite.) Homogenization was accomplished by two 30-s bursts in a Waring Blender (Waring Products, New Hartford, CN). The homogenate was centrifuged for 20 min at 8,000 rpm in a GSA rotor (Dupont/Sorvall Instruments Div., New Town, CT). The resulting supernatant was centrifuged for 1 h in either a 45Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) at 44,000 rpm or 50.2 Ti (Beckman Instruments, Inc.) at 49,000 rpm. The cloudy supernatant was then dialyzed overnight against 3 changes of 25 mM Tris/1 mM DTT, pH 8.0 (column buffer). The dialyzed homogenate was cleared by centrifugation (1 h at 44,000 rpm in a 45Ti rotor), resulting in  $\sim$ 900 ml of cleared cytosol.

**Anion Exchange Chromatography on Q Sepharose Fast Flow.** 900 ml of cytosol was loaded onto a 5  $\times$  10 cm column of Q Sepharose fast flow (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with column buffer. Flow rate was 400 ml/h. The column was washed with  $\sim$ 1 column volume of column buffer, then two column volumes of column buffer adjusted to 150 mM KCl. The column was then eluted with a linear gradient of 150–400 mM KCl in column buffer in a total volume of 2 l. 50-ml fractions were collected. A single activity peak was detected which eluted between 200–300 mM KCl with the peak at 250 mM KCl. A 200-ml pool was collected.

**Hydrophobic Chromatography on Phenyl-Sepharose.** The Q fast flow pool was dialyzed overnight against 25 mM Tris, pH 8.0, 0.7 M ammonium sulfate, 10% glycerol, 1 mM DTT. This was loaded onto a 5  $\times$  8.5 cm column of phenyl-Sepharose (Pharmacia Fine Chemicals) equilibrated with the same buffer. Flow rate was 325 ml/h. The column was washed with one column volume of the equilibration buffer and then eluted with a linear gradient of 0.7–0 M ammonium sulfate and 0–75% (vol/vol) ethylene glycol, 10 (vol/vol)–0% glycerol, all in 25 mM Tris, pH 8.0, 1 mM DTT, in a total volume of 1 l. 50-ml fractions were collected. A peak of activity was detected at 350 mM ammonium sulfate. Fractions were dialyzed against 25 mM Tris, pH 8.0, 1 mM DTT before assay. A 200-ml pool was collected.

**Gel Filtration Chromatography on Sephadex G-75.** The phenyl-Sepharose pool was concentrated from 200 to 28 ml on an Amicon (Danvers, MA) stirred concentration cell using a YM-10 membrane. This was applied to a 5  $\times$  88 cm column of Sephadex G-75 (Pharmacia Fine Chemicals) equilibrated with 25 mM Tris, pH 8.0, 200 mM KCl, 1 mM DTT. The flow rate was 200 ml/h and 50-ml fractions were collected. A broad peak

of activity was detected which was centered at an elution position corresponding to a globular protein of approximately 35 kD. A 250-ml pool was collected.

**Anion Exchange on Mono-Q.** The 250-ml G-75 pool was concentrated to 35 ml on an Amicon YM-10 membrane and dialyzed overnight with 25 mM Tris, 1 mM DTT, pH 7.0. This was loaded onto a Mono-Q HR 5/5 column (Pharmacia Fine Chemicals), equilibrated with 25 mM Tris, 1 mM DTT, pH 7.0. The flow rate was 0.3 ml/min. This was washed with 5 ml of equilibration buffer, then 5 ml of equilibration buffer adjusted to 150 mM KCl. Activity was eluted with a gradient of 150–400 mM KCl in the equilibration buffer in a total volume of 20 ml. 0.5-ml fractions were collected. Activity eluted as a relatively broad peak centered at 250 mM KCl. A 1.0-ml pool was made of the active fractions.

**Hydrophobic Chromatography on Propyl-Aspartamide.** The Mono-Q pool was dialyzed for 4 h against 25 mM Tris, 1 M ammonium sulfate, 1 mM DTT, pH 8.0, and then loaded onto a 4.6  $\times$  200-mm polypropyl Aspartamide column (The Nest Group, Southboro, MA), equilibrated with the same buffer used to dialyze the load. Flow rate was 0.5 ml/min. The column was washed with 3 ml of the equilibration buffer and then eluted with a simultaneous gradient of 1–0 M ammonium sulfate and 0–50% ethylene glycol, both in 25 mM Tris, 1 mM DTT, pH 8.0. 0.5-ml fractions were collected. Activity eluted at a sharp peak centered at 0.7 M ammonium sulfate. Activity co-eluted with the major protein peak. A 2.5-ml pool was collected.

**Hydroxylapatite Chromatography.** The propyl Aspartamide pool was dialyzed overnight against 10 mM KP<sub>i</sub>, pH 7.0. This was loaded onto the HPLC hydroxylapatite column HCA (7.6  $\times$  100 mm, Rainin Instruments, Woburn, MA). Flow rate was 0.5 ml/min. The column was washed with 3 ml 10 mM KP<sub>i</sub>, pH 7.0, then 3 ml 25 mM KP<sub>i</sub>, pH 7.0, and eluted with a gradient of 25–70 mM KP<sub>i</sub>, pH 7.0, in a total volume of 40 ml. Activity co-eluted with a protein peak centered at 34 mM KP<sub>i</sub>.

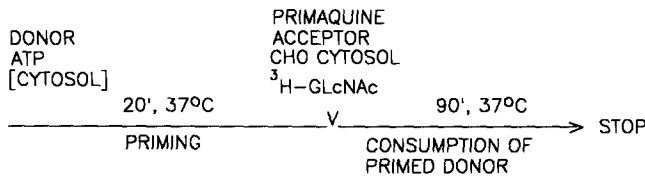
**Preparation of Crude NSF.** Lecl crude homogenate prepared as described (Balch et al., 1984a) was centrifuged for 1 h at 100,000 g in a 50ti rotor (Beckman Instruments, Inc.). The cloudy supernatant ( $\sim$ 8 ml) was loaded onto a 1.5  $\times$  50 cm column of Sephadex G-200 (Pharmacia Fine Chemicals) equilibrated and eluted with 20 mM Pipes-KOH (pH 7.0), 2 mM Mg<sub>2</sub>Cl<sub>2</sub>, 2 mM DTT, 0.5 mM ATP and 100 mM KCl. The flow rate was 50 ml/h. The void peak was collected, concentrated to 8.0 ml, frozen in liquid nitrogen, and stored at  $-80^{\circ}$ C before use.

**Electron Microscopy.** The visualization of membrane buds and vesicles was performed and quantitated as previously described (Hiebsch et al., 1991).

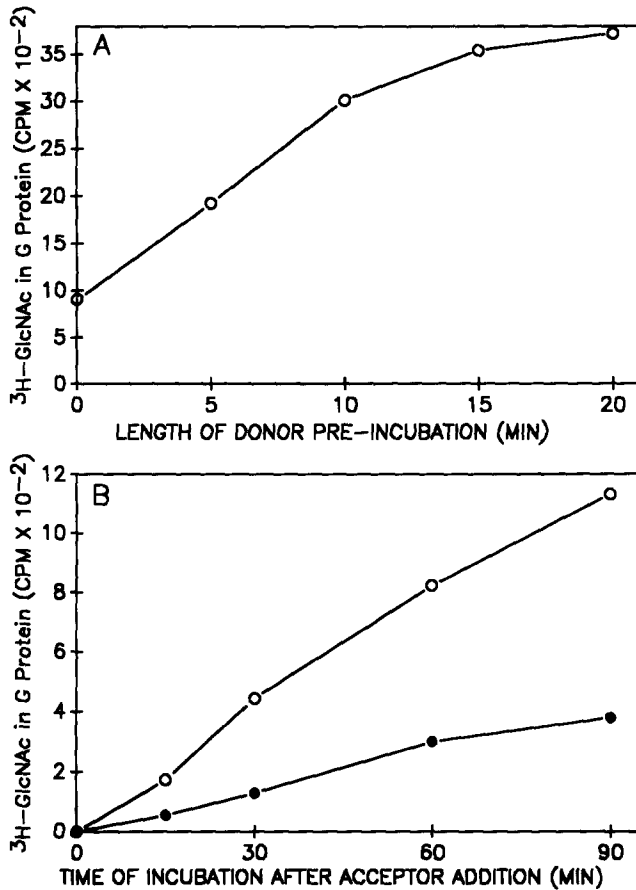
## Results

### *An Assay Measuring Early Events in the Production of Functional Transport Vesicles Identifies $\alpha$ SNAP as a Required Component*

It has previously been shown that primaquine will selectively inhibit the production of transport vesicles in a cell-free system reconstituting transport through the Golgi apparatus (Hiebsch et al., 1991). Using this property it was possible to construct an assay which measures the cytosolic requirements for vesicle production (Fig. 1). In this assay, donor membranes (those which produce transport vesicles containing the transported protein VSV G protein) are preincubated in the presence of an ATP regenerating system and the cytosol fractions to be tested for 20 min. During this time events in vesicle production take place. Primaquine is then added along with acceptor membranes (the membranes to which the vesicles will fuse), an excess of unfractionated cytosol and UDP-[<sup>3</sup>H]-N-acetylglucosamine, the sugar nucleotide substrate for the glycosyltransferase used to mark transport of G protein from donor to acceptor. By adding primaquine at the same time as acceptor little new vesicle formation will take place, and therefore only the influence of the cytosol fractions present during the donor preincubation will be measured. The excess unfractionated cytosol will provide the cytosolic requirements for reactions leading



**Figure 1.** The assay for cytosolic factors required for the production of functional transport vesicles. In the first stage donor membranes are incubated at 37°C (typically for 20 min) with the cytosol fraction being tested, in addition to an ATP regenerating system and buffer. In the second stage primaquine is added (to inhibit further vesicle production) as are acceptor membranes and unfractionated cytosol (to provide the cytosolic requirements of all transport steps subsequent to vesicle production) and UDP-<sup>3</sup>H-GlcNAc. The assay is then further incubated at 37°C (typically for 90 min) before terminating the assay and performing the G protein immunoprecipitation.



**Figure 2.** (A) Time course of vesicle production in the primaquine-based assay. Donor membranes were incubated for the indicated times in the presence of CHO cytosol (20  $\mu$ g/ml) and an ATP regenerating system. Acceptor membranes were then added along with 200  $\mu$ M primaquine and UDP-[<sup>3</sup>H]GlcNAc (5  $\mu$ Ci/ml) and the incubation was continued for 30 min before termination and immunoprecipitation. (B) The time course of transport after donor preincubation in the primaquine-based vesicle production assay. Donor membranes were incubated for 15 min at either 37°C (primed,  $\circ$ ) or on ice (unprimed,  $\bullet$ ) with CHO cytosol (40  $\mu$ g/ml) and an ATP regenerating system. Then acceptor membranes were added along with 250  $\mu$ M primaquine and UDP-[<sup>3</sup>H]GlcNAc (2.5  $\mu$ Ci/ml). Incubations were continued for the indicated times before termination and immunoprecipitation.

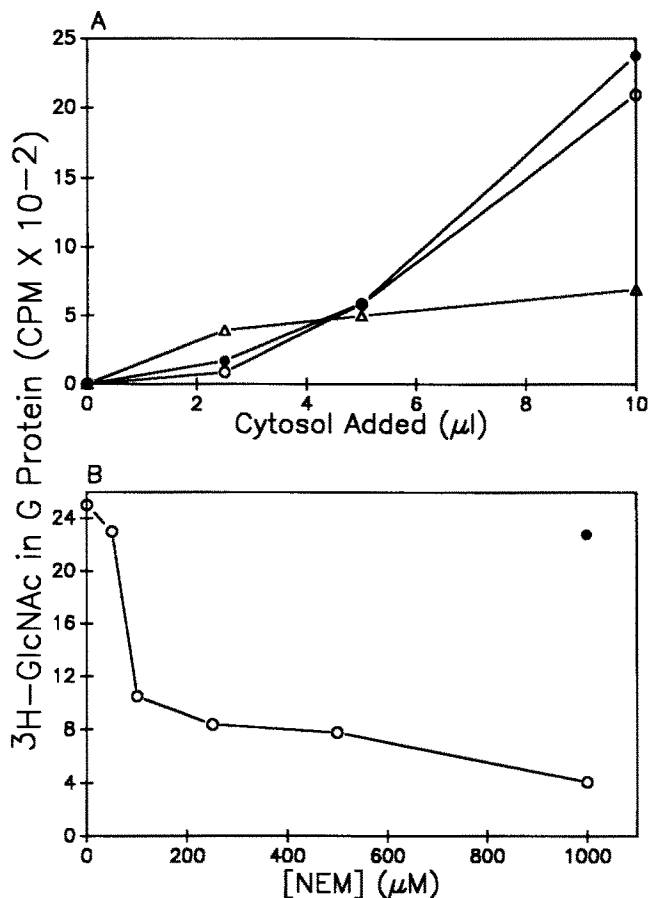
to the fusion of the transport vesicles. It must be emphasized that this assay does not precisely define the events in vesicle formation that are being measured; they may include only early events, such as the formation of membrane buds, or they may include both budding and pinching off of the vesicles. Further, only functional vesicles will be measured in this assay; if nonfunctional vesicle structures are produced, these will not be detected. With these characteristics in mind, for simplicity this assay will be referred to as a vesicle production assay in the remainder of this report.

Fig. 2 A illustrates the time course for production of the vesicles in the donor preincubation. PQ-resistant transport increases linearly with time, reaching a plateau after 15–20 min. The time course of glycosylation of G protein after addition of acceptor membranes in the secondary incubation is shown in Fig. 2 B. As expected, when donor membranes are preincubated for 20 min at 37°C ( $\circ$ ) there is a large enhancement of the signal produced in comparison with that where donor was not preincubated ( $\bullet$ ). The residual signal from unpreincubated donor membranes is probably due to a small amount of new vesicle formation initiated in the second incubation. Levels of PQ which completely inhibit vesicle formation were found to suppress acceptor function as well, so the level of PQ was empirically chosen to give the largest signal in response to donor preincubation.

As expected, the vesicle formation assay is cytosol dependent (Fig. 3 A). Cytosol derived from both CHO cells ( $\circ$ ) and bovine brain ( $\bullet$ ) are quite active, whereas yeast cytosol ( $\Delta$ ) is only weakly stimulatory. Activity of cytosol is eliminated by pretreatment of the cytosol with the alkylating agent NEM (Fig. 3 B), indicating that at least one required component is NEM sensitive.

To focus on the purification of the NEM-sensitive component(s) the assay was modified by the inclusion of NEM-treated CHO cytosol in the donor preincubation step. Therefore all NEM-resistant components are supplied and only the NEM-sensitive component(s) should elicit a response. This assay was applied to fractions derived from fractionation of bovine brain cytosol by ion-exchange, hydrophobic, gel filtration, and hydroxylapatite chromatographies (see Materials and Methods and Fig. 4) and a single peak of activity in each column was identified. The activity of this peak was completely dependent on the presence of NEM-inactivated CHO cytosol (not shown) indicating that vesicle formation depends on NEM-resistant cytosolic components in addition to the NEM-sensitive activity. This enrichment scheme resulted in the purification of a peptide of 35 kD (Fig. 4). We termed this protein PF-1. The specific activity of the purified fraction is increased up to 600-fold over that of the starting cytosol, although this varied from preparation to preparation. The yield of activity is modest, from 0.2–0.5% of starting activity.

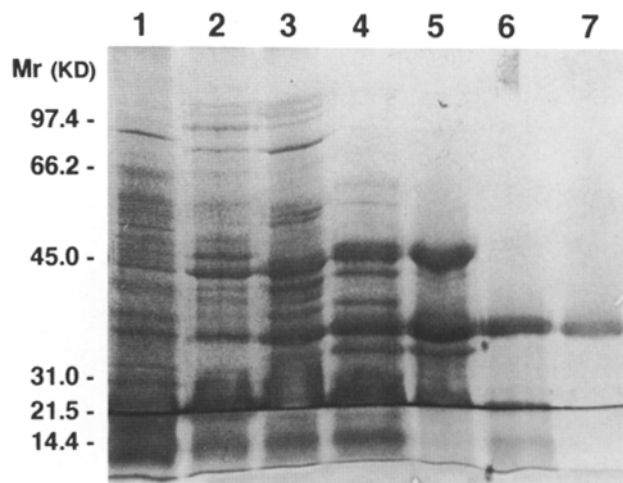
PF-1 exhibits two characteristics reminiscent of a previously identified transport component, the  $\alpha$ -form of the soluble NSF attachment protein ( $\alpha$ SNAP). First, it elutes from the ion exchange resin Q fast flow at 250 mM KCl, as does  $\alpha$ SNAP (Clary and Rothman, 1990). Secondly it has a molecular mass of 35 kD, similar to  $\alpha$ SNAP. To test the identity between PF-1 and  $\alpha$ SNAP a protein blot was probed with an antipeptide antibody directed against a peptide common to  $\alpha$ ,  $\beta$ , but not  $\gamma$ SNAP (Fig. 5). This antibody recognized PF-1 as well as  $\alpha$  and  $\beta$ SNAP. PF-1 migrates at the same molecu-



**Figure 3.** Cytosol dependence for the vesicle production assay. (A) Comparison of vesicle production activity of cytosol derived from brain (●), CHO cells (○) and yeast (Δ). Protein concentration of brain and CHO cytosols was 2.0 mg/ml and yeast cytosol was 4.0 mg/ml. The background in the absence of added cytosol (234 cpm) has been subtracted from each point. (B) CHO cytosol contains an NEM-sensitive vesicle production factor(s). CHO cytosol (2 mg/ml) was treated with the indicated levels of NEM for 15 min on ice. The NEM was quenched with a twofold excess of DTT before assay. 10 μl of treated cytosol was then tested in the assay for vesicle production (○). As a control for the carryover of NEM into the assay, one sample was treated first with 2 mM DTT, then 1 mM NEM (●). Shown is the mean of duplicate assays. Background in the absence of added cytosol (199 cpm) has been subtracted from each point.

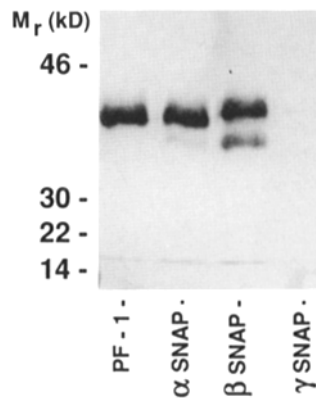
lar weight as αSNAP, slightly faster than βSNAP (the additional faster migrating band in the βSNAP lane is presumably a proteolytic product). As expected, the γSNAP sample was not detected by this antibody.

PF-1 was also tested in two assays for SNAP function (Fig. 6). The first of these assays measures the ability of fractions to mediate the binding of NSF to membranes (Fig. 6 B). This was originally the defining assay of SNAP function (Weidman et al., 1989). A second assay (Fig. 6 C) relies on the observation that SNAP-containing fractions will complement a set of partially fractionated cytosolic pools when salt washed Golgi membranes are assayed (Clary et al., 1990). In these assays, as well as the vesicle formation assay (Fig. 6 A) both native (●) and NEM-inactivated (Δ) PF-1 were titrated as was unfractionated brain cytosol (○). PF-1 exhibits strong activity in both assays for SNAP activity, and those

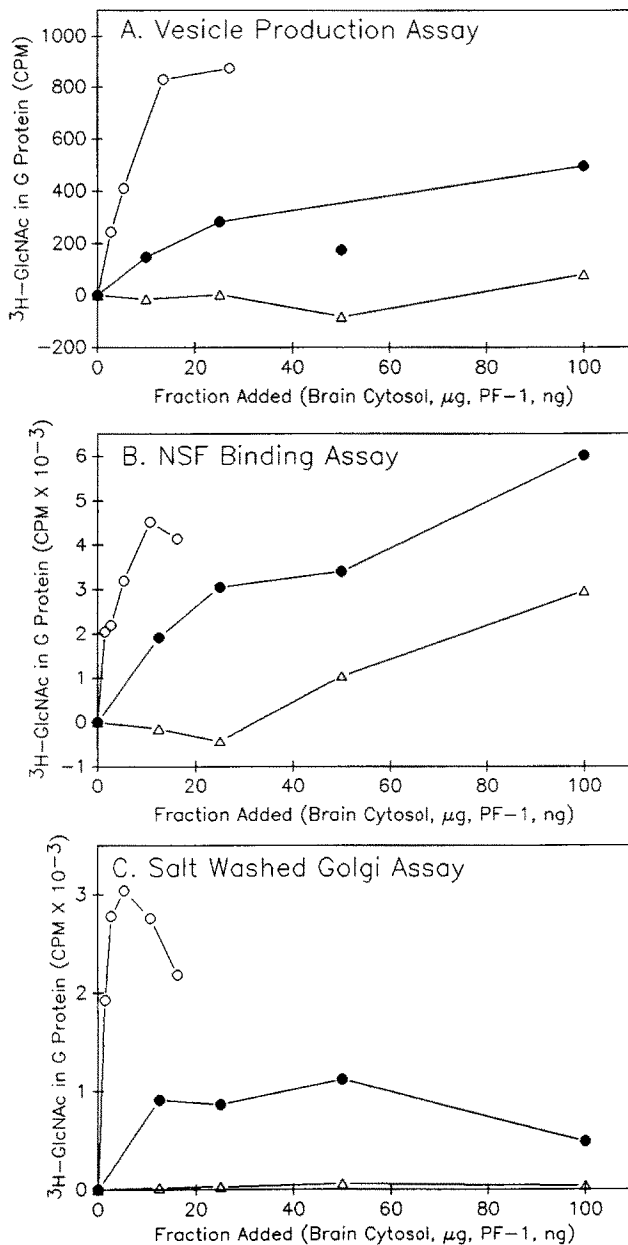


**Figure 4.** Purification of an NEM-sensitive protein required for vesicle production. Samples of pools generated during the purification (see Materials and Methods) were separated by SDS-PAGE on a 15% acrylamide separating gel as described in Materials and Methods. Lane 1, crude brain cytosol (20 μg); lane 2, pool resulting from Q fast flow anion exchange chromatography (20 μg); lane 3, pool from phenyl-Sepharose hydrophobic chromatography (20 μg); lane 4, pool from sizing on Sephadex G-75 (14 μg); lane 5, pool from Mono-Q anion exchange chromatography (8 μg); lane 6, pool from propyl-aspartamide hydrophobic chromatography (2 μg); and lane 7, pool from HCA HPLC hydroxylapatite chromatography (1.2 μg). Protein was visualized by silver staining. Migration of molecular weight standards is indicated on the left. A silver staining artifact is found to migrate at a molecular mass of 22 kD in all lanes containing sample buffer.

activities are inhibited by NEM treatment, as is activity in the vesicle production assay (Fig. 6 A). Interestingly, however, NEM treatment was only partially effective in inhibiting the ability of PF-1 to mediate NSF binding to membranes (Fig. 6 B). This partial NEM sensitivity parallels the ability of purified PF-1 to fully substitute for brain cytosol in the NSF binding assay, whereas in both the vesicle production and salt-washed Golgi SNAP assay the purified PF-1 was only partially active. We attribute this to some inactivation of the PF-1 fraction during purification and believe that this data indicates that the overall function of PF-1/SNAP in



**Figure 5.** PF-1 is immunologically indistinguishable from αSNAP. 100 ng each of PF-1, and α, β, and γSNAP were separated by PAGE, then blotted to nitrocellulose and probed with an antibody raised against a peptide common to α and βSNAP, but lacking in γSNAP. Molecular mass standards are shown at the left of the blot. Note that PF-1 stains equivalently with this antibody and comigrates with αSNAP, slightly faster than βSNAP. As expected, no staining is seen with γSNAP. The minor staining band in the α and βSNAP lanes is presumably a proteolytic product of the SNAPs.



**Figure 6.** PF-1 is active in the SNAP assay for NSF binding and the SNAP assay using salt-washed Golgi. The activity of bovine brain cytosol ( $\circ$ ) ( $5.4 \mu\text{g}/\text{ml}$  protein), control PF-1 ( $\bullet$ ) ( $10 \mu\text{g}/\text{ml}$ ) or NEM treated PF-1 ( $\Delta$ ) ( $10 \mu\text{g}/\text{ml}$ ) was tested in the primaquine-based vesicle production assay (A), the SNAP-dependent NSF membrane binding assay (B), or the SNAP-dependent salt-washed Golgi assay (C). The primaquine based-vesicle production assay (A) was performed as described in Materials and Methods. The SNAP-dependent NSF membrane binding assay (B), is a two-stage assay. In the first step cytosol fractions are incubated with NSF in the presence of membranes depleted of both SNAP and NSF. The NSF bound to those membranes, as mediated by SNAP activity in the cytosol, is then measured in a second, NSF-dependent transport assay (see Materials and Methods). The salt-washed Golgi assay (C) is based on the observation that when donor and acceptor are washed with  $1 \text{ M KCl}$ , transport is dependent on cytosolic components derived from four separable cytosolic pools. Pool 2 constitutes SNAP activity, so SNAP activity can be specifically measured by assaying salt washed membranes in the presence of pools 1, 3, and 4 (see Materials and Methods). Where indicated PF-1 was treated with  $0.4 \text{ mM NEM}$  on ice for  $15 \text{ min}$ , then  $0.8 \text{ mM DTT}$  was added. For control PF-1, the treatments with NEM and DTT were reversed.

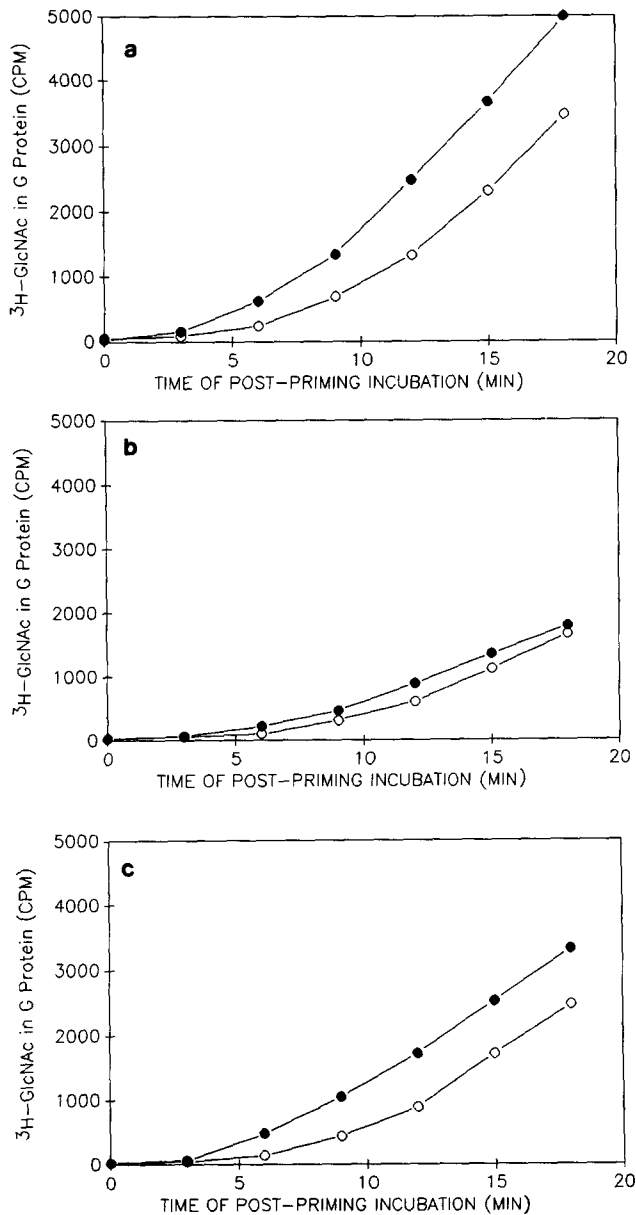
transport is more sensitive than that of simply mediating the binding of NSF to membranes.

Taken together the immunological and functional data strongly indicate that PF-1 and  $\alpha\text{SNAP}$  are identical or closely related. This is a surprising result as it has previously been suggested that SNAP has a role in membrane fusion, not vesicle production. For this reason it was important to document that the activity of PF-1/SNAP in the vesicle production assay was in fact due to an action of PF-1/SNAP on donor membranes during the vesicle production stage of the assay. To eliminate the possibility that activity was somehow due to the use of primaquine to monitor vesicle production, the activity of PF-1/SNAP was measured in the related phenomenon of donor "priming" (Fig. 7). It has been shown that preincubation of donor (but not acceptor) membranes results in a reduced lag time in the kinetics of G protein transport from donor to acceptor (Balch et al., 1984b, and Fig. 7 A, compare incubations without preincubation of donor [ $\circ$ ] with those including preincubated, "primed" donor [ $\bullet$ ]). This effect requires cytosolic factors and ATP (Balch et al., 1984b), and is sensitive to primaquine (Hiebsch et al., 1991), as is a morphological transformation of donor membranes resulting in the formation of membrane buds, the presumed precursors of transport vesicles. Priming is therefore thought to represent some stages in the formation of transport vesicles, presumably the same that are measured in the assay presented here. No priming was detected in the presence of NEM-treated cytosol (Fig. 7 b), however priming was restored when PF-1 was added in addition to NEM-inactivated cytosol (Fig. 7 c). Clearly PF-1 is acting during the priming preincubation, not merely enhancing the activity of the assay as a whole. In the latter case priming would have been detected with NEM-treated cytosol alone, albeit with lower overall counts. Moreover this data illustrates that the activity of PF-1/SNAP is not an artifact of the use of primaquine to measure vesicle production.

This result was bolstered by testing whether the PF-1/SNAP activity could be detected when PF-1/SNAP was added after the donor preincubation step of the primaquine-dependent vesicle production assay (Fig. 8 A). There was no activity when PF-1/SNAP was added after the donor preincubation ( $\bullet$ ) where, as before, activity was observed when it was included during the donor preincubation ( $\circ$ ), consistent with the action of PF-1/SNAP on the donor membranes themselves. Furthermore the effect of PF-1/SNAP was only observed when it was preincubated with donor membranes (Fig. 8 B,  $\circ$ ) and not acceptor membranes (Fig. 8 B,  $\bullet$ ) illustrating that there is not simply a requirement that PF-1/SNAP be preincubated with a membrane fraction to exhibit activity.

### ***NSF Is Required during Vesicle Formation and the NEM-sensitive Function of NSF Is Fulfilled by Interaction with Donor Membranes***

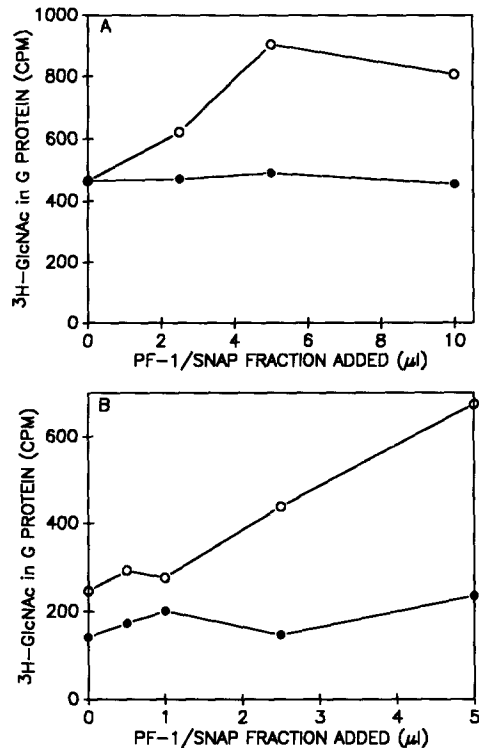
Because the function of the SNAP is to mediate the binding of NSF to Golgi membranes (Weidman et al., 1989), it was of interest to test whether NSF was also required in the vesicle formation assay. Fig. 9 shows that if NSF is inactivated with NEM before the donor incubation, no activity is observed, even when NSF is included in the second-stage incubation of the assay ( $\bullet$ ). Addition of NSF to the donor preincubation, however, completely restores activity ( $\circ$ ). This



**Figure 7.** PF-1 activity is required in a nonprimaquine-based, kinetic assay for the formation of functional vesicles. Donor membranes were first incubated for 20 min at either 37°C (primed) (●) or 4°C (unprimed) (○) in the presence of either 10 μg of untreated CHO cytosol (a), 10 μg of NEM-treated CHO cytosol (b) or 10 μg of NEM-treated cytosol plus 4 μg of partially purified PF-1 (pool from the Q fast flow column, see Materials and Methods). Acceptor membranes and UDP-<sup>3</sup>H-GlcNAc were then added and the incubations were continued for the indicated times before removing aliquots and freezing on dry ice. At the conclusion of the time course all samples were thawed and immunoprecipitated together. To the incubations depicted in b and c, 10 μg of untreated CHO cytosol was also added after the donor preincubation.

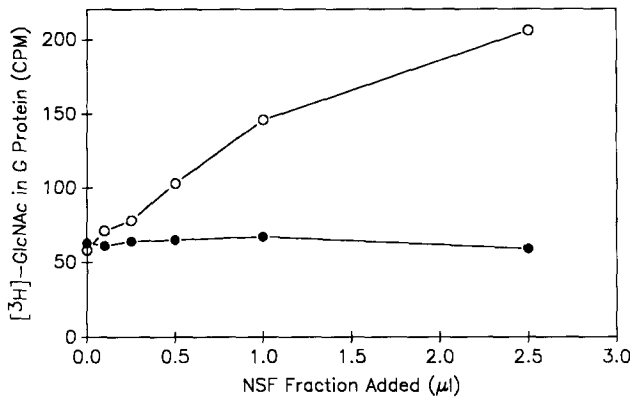
demonstrates that the NEM-sensitive function of NSF is required for the formation of functional vesicles in this assay.

To test whether the NEM-sensitive function of NSF is also required for fusion of transport vesicles (Fig. 10), donor membranes were preincubated to allow events in vesicle formation to occur. After various intervals the endogenous NSF was inactivated by NEM treatment for 5 min at 37°C. The NEM was then quenched with DTT and acceptor mem-

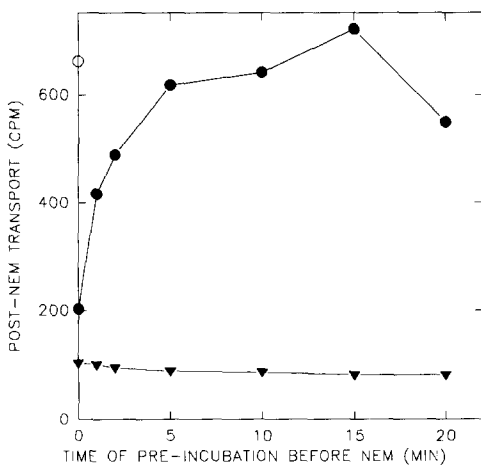


**Figure 8.** (A) PF-1 is active when added during the donor preincubation, but not afterwards, in the primaquine-based vesicle production assay. Donor membranes were first incubated for 20 min at 37°C with 10 μg of NEM-treated CHO cytosol. Then acceptor membranes were added along with 10 μg of unfractionated CHO cytosol primaquine, and UDP-<sup>3</sup>H-GlcNAc and the incubation was continued for 90 min before termination of the assay. The indicated amounts of PF-1 were added either during the donor preincubation (○), or in the second stage, postprimaquine incubation (●). Shown is the average of triplicate samples for each point. (B) PF-1 is active when preincubated with donor but not acceptor membranes in the primaquine-based vesicle production assay. The assay was performed as described in Materials and Methods with the modification that in one set of assays (●) acceptor membranes were substituted for donor membranes in the first preincubation. A second set (○) was assayed exactly as described, using donor in the first stage preincubation. Purified PF-1 (270 μg/ml) was added in the indicated amounts.

branes (pretreated with NEM to inactivate endogenous NSF) were added along with cytosol and UDP-<sup>3</sup>H-GlcNAc and the incubation was continued to allow transport. Note that primaquine is not used in this experiment. Preincubation of donor membranes (Fig. 10, ●) followed by NEM inactivation results in transport that is identical to the addition of exogenous NSF to membranes treated with NEM without preincubation (open circle on ordinate). In these experiments the donor membranes were continuously held at 37°C. It was found that NEM-resistant activity was lost if the donor membranes were placed on ice after preincubation (data not shown). In contrast to the results obtained with donor membranes, preincubation of acceptor membranes (▲), followed by the addition of NEM pretreated donor membranes, does not yield NEM-resistant transport activity. This striking result directly demonstrates that the NEM-sensitive function of NSF is no longer required once steps in the formation of transport vesicles have occurred.



**Figure 9.** NSF is active when added during the donor preincubation, but not afterwards, in the primaquine-based vesicle production assay. Donor membranes (treated with 1 mM NEM on ice for 15 min, the 2 mM DTT) were first incubated for 20 min at 37°C with 10 μg of NEM-treated CHO cytosol (priming). Then acceptor membranes (treated with NEM in the same manner as the donor) were added along with 10 μg of unfractionated CHO cytosol, primaquine, and UDP-<sup>3</sup>H-GlcNAc and the incubation was continued for 90 min before termination of the assay. The indicated amounts of crude NSF (see Materials and Methods) were added either during the donor preincubation (○), or in the second stage, postprimaquine incubation (●).



**Figure 10.** Preincubation of donor membranes, but not acceptor membranes, renders subsequent transport resistant to NEM inactivation of NSF. Donor (●) or acceptor membranes (▲) were incubated with brain cytosol and the ATP regenerating mix for the indicated times at 37°C. NEM was then added to each tube to a final concentration of 0.5 mM and the incubation was continued for 5 min. Then each tube received a mixture of 5 μl of brain cytosol and 5 μl of either acceptor membranes (in the case of preincubated donor membranes) or donor membranes (in the case of preincubated acceptor membranes), DTT to a final concentration of 1 mM and <sup>3</sup>H-UDP-GlcNAc. Membranes added at this stage had been pretreated with NEM (Glick and Rothman, 1987) to inactivate NSF. The incubation was then continued for 60 min before termination and immunoprecipitation. For comparison, to one sample of donor membranes that were NEM treated without preincubation, 5 μl of crude NSF (see Materials and Methods) was added (○). Results are the average of duplicate samples.

### Vesicle/Bud Structures Are Produced in the Absence of SNAP

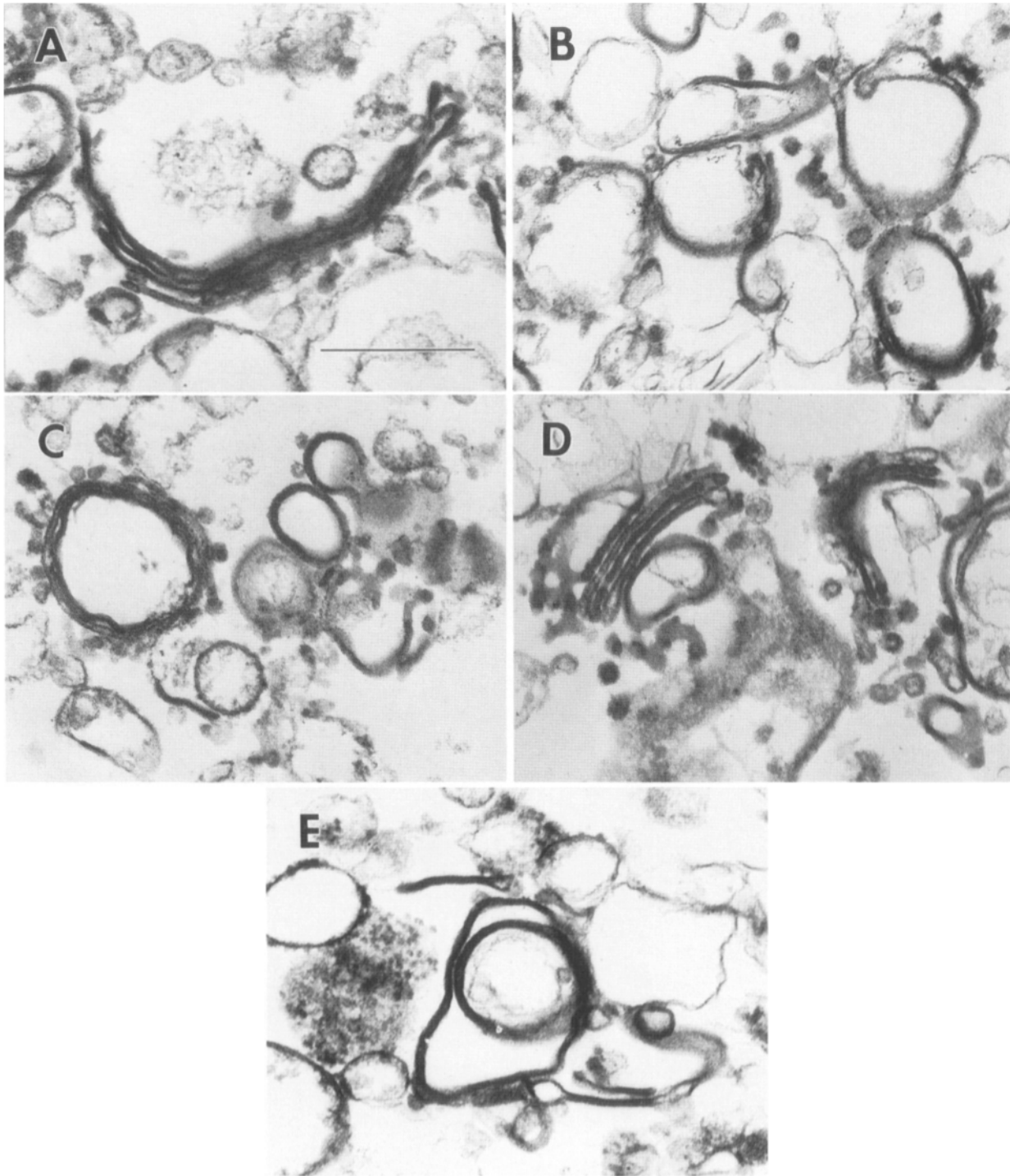
Because the experiments described above measure the formation of vesicles by a functional criteria it was important to determine whether the formation of vesicle structures also requires SNAP. After incubation under the appropriate conditions Golgi preparations were visualized by EM (Fig. 11) and the formation of membrane buds, which are thought to be the precursors to transport vesicles (Balch et al., 1984b; Orci et al., 1986, 1989), were quantitated (Fig. 12). Donor membranes were incubated either on ice (Fig. 12 A) or at 37°C (Fig. 12, B-E), with complete cytosol (Fig. 12 B), NEM-treated cytosol to deplete PF-1/SNAP (Fig. 12 C), NEM-treated cytosol plus PF-1/SNAP (Fig. 12 D), or without cytosol (Fig. 12 E). Relatively few buds/vesicles were found when the membranes were incubated on ice or without cytosol, as previously reported (Balch et al., 1984b). The number of buds/vesicles produced did not appear to depend on the presence of PF-1/SNAP as similar numbers were noted with untreated cytosol and NEM-treated cytosol. These data indicate that whereas SNAP and NSF appear to be required for the formation of functional vesicles, nonfunctional vesicle structures may still be manufactured in the absence of SNAP and NSF. It is also formally possible that buds are formed, but that SNAP and NSF are required for the pinching off of those buds to produce fully formed transport vesicles.

### Discussion

The data presented here indicate that two proteins, SNAP and NSF, which had previously been thought to function during vesicle fusion steps of transport, are required during the formation of functional transport vesicles. Initially, we arrived at this conclusion by the use of an assay which measures the activity in driving donor membranes, the membranes which produce transport vesicles in this assay, into a primaquine-resistant state. We also used a kinetic "priming" assay, in which preincubation of donor membranes reduces the lag time of transport to acceptor membranes. The supposition that these assays measure events in the formation of transport vesicles is based on the observation that both types of transformation (primaquine resistance, and kinetic alterations) are dependent on ATP and cytosol, as is the morphological appearance of membrane buds, the presumed precursors of transport vesicles (Balch et al., 1984b; Orci et al., 1986). In addition, a priori reasoning suggests that events in vesicle production are being affected because these alterations are occurring in donor membranes, whose sole function in the transport system is to produce transport vesicles.

These conclusions were strongly supported by the striking observation that after preincubation of donor (but not acceptor) membranes the NEM-sensitive function of NSF is no longer required to drive transport (Fig. 10). This result demonstrates that NSF must interact specifically with donor membranes to perform its function, and that the NEM-sensitive portion of that function is complete after that interaction. The most plausible explanation for this result is that NSF is required for the formation of functional transport



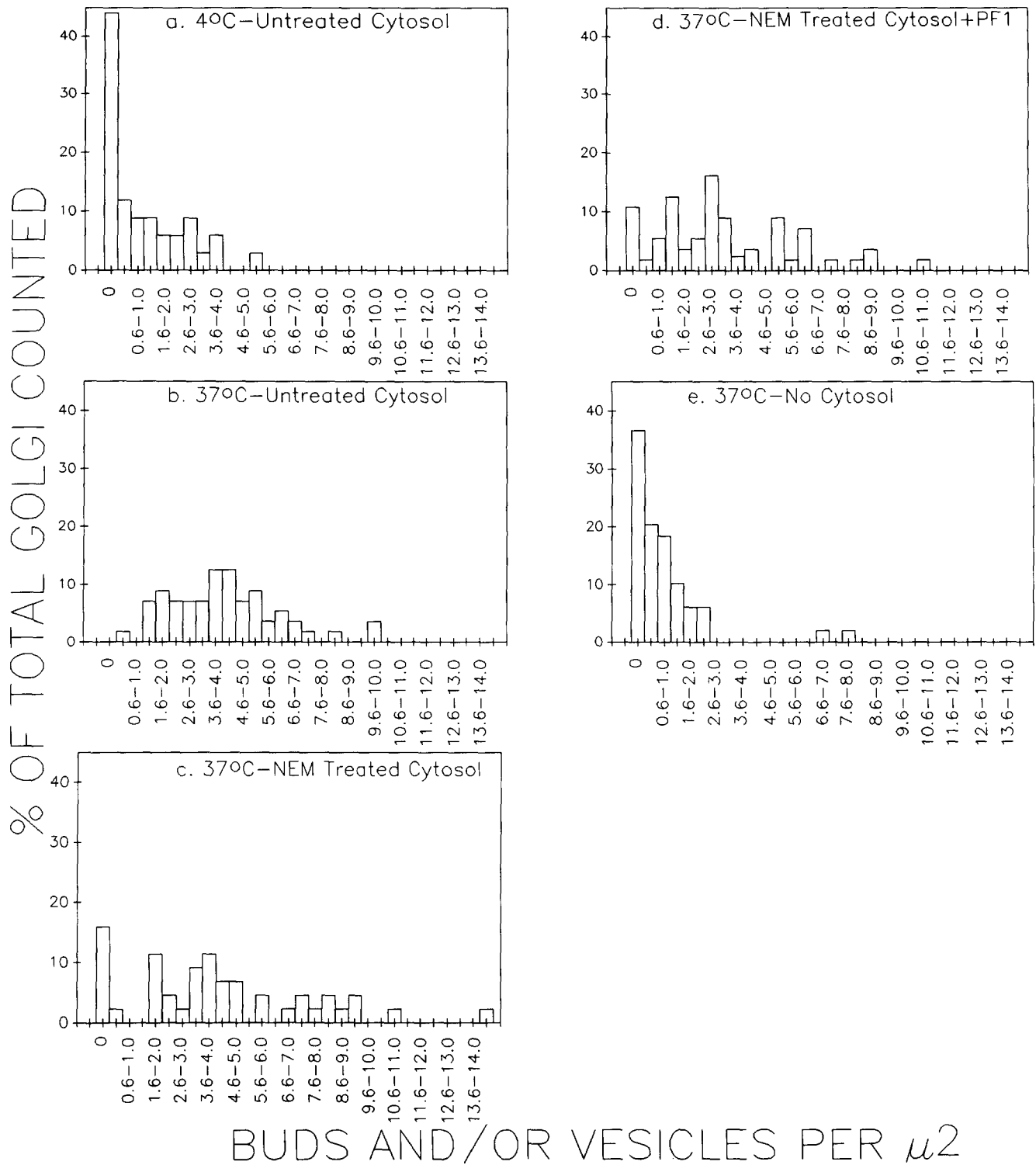


**Figure 11.** The structural formation of buds/vesicles does not depend on PF-1/SNAP. Transport reactions were incubated for 20 min either at 4°C (*A*) or 37°C (*B–E*). The cytosol additions were: unfractionated, 0.5 mg/ml untreated CHO cell cytosol (*A* and *B*), 0.5 mg/ml NEM-treated CHO cytosol (PF-1/SNAP deficient) (*C*), 0.5 mg/ml NEM-treated CHO cytosol plus 58 μg/ml partially purified PF-1/SNAP (through Mono-Q) (*D*), no cytosol (*E*). The samples were then processed for EM as described in Materials and Methods.

vesicles. This was an unexpected finding as the ability of preincubation of donor and acceptor membranes to fulfill NSF function had been tested previously (Malhotra et al., 1988), and no such effect had been found. This inconsistency is explained by a crucial technical difference between the two experiments. In retesting the conditions used by Malhotra and colleagues, where the NEM treatment was performed on

ice after membrane incubation, we found that cooling the membranes resulted in a loss of NEM resistance (data not shown). In contrast, our NEM treatments were performed at 37°C, and the membranes were never cooled. Apparently whatever function is performed by NSF in the donor membranes is reversed by incubation at low temperature.

Previous models suggesting a role for NSF and SNAP in



**Figure 12.** Quantitation of bud/vesicle formation in the presence and absence of PF-1/SNAP. Micrographs resulting from transport reactions corresponding to those shown in Fig. 11 were quantitated for the formation of buds and/or vesicles as described (Hiebsch et al., 1991). (a) Unfractionated CHO cytosol, incubation at 4°C. (b) Unfractionated CHO cytosol, incubation at 37°C. (c) NEM-treated CHO cytosol (PF-1/SNAP deficient). (d) NEM-treated CHO cytosol plus partially purified PF-1/SNAP (purified through Mono-Q). (e) No cytosol. Details of transport reactions are given in the legend to Fig. 11. Histograms were constructed by counting individual Golgi complex areas and dividing the total number of buds/vesicles counted by the area counted. Each Golgi apparatus counted was then placed into a density class. The results are expressed as percentage of total Golgi complexes to compensate for the unequal number of Golgi complex areas counted in the different samples. Number of Golgi complexes counted were: a, 34; b, 56; c, 44; d, 56; and e, 49.

vesicle attachment and/or fusion are based on studies both in mammalian and yeast systems. When NSF was inactivated by NEM treatment in the mammalian Golgi transport system, the morphological accumulation of attached vesicles was noted (Malhotra et al., 1988; Orci et al., 1989). The incapacitation of the yeast SNAP and NSF homologues Sec17 and Sec18 by mutation results in the accumulation of free transport vesicles in vivo (Kaiser and Schekman, 1990). In a permeabilized yeast ER to Golgi transport system, inactivation of the sec18 gene product by mutation also leads to the accumulation of free transport vesicles (Rexach and Schekman, 1991). These data are consistent with our finding that the absence of PF-1/SNAP does not impair the formation of membrane buds as quantified by EM. Although each of these results indicate that the formation of vesicle structures does not depend on SNAP or NSF function, the functionality of the vesicles produced was not ascertained.

The simplest model reconciling these data is that SNAP and NSF are required during vesicle formation because they become incorporated into the vesicle membrane. These proteins would then be ferried on the vesicles to the fusion site where they would perform a role in vesicle attachment and/or fusion. This could be a mechanism to ensure that these proteins are specifically targeted to the fusion sites. It should be noted that in an assay measuring fusion of endocytic vesicles (a situation in which vesicle formation has already occurred) NSF can be added to NEM-inactivated membranes to restore fusion activity (Diaz et al., 1989), whereas in our assays we could not detect activity when SNAP or NSF were added after the vesicle formation step. This may be due to differences between endocytic fusion and transit through the Golgi, or may indicate that with appropriate experimental manipulations SNAP and NSF can be forced to bind to vesicles after their formation. However since SNAP and NSF are always present in the cytosol, and thus available during vesicle formation, this is unlikely to be physiologically relevant. One implication of this scheme is that the incorporation of NSF into forming vesicles is NEM sensitive, whereas the attachment/fusion function of NSF is not. In fact, a recent report indicates that the NEM treatment of NSF cripples its ability to bind to the SNAP/membrane receptor complex (Wilson et al., 1992). The binding of NSF and SNAP to forming vesicles followed by their action at the site of fusion is similar to a model proposed for the cycling of small molecular weight GTP binding proteins that act in vesicular transport (Bourne, 1988). Morphological and biochemical data indicate that Sec4p, a yeast GTP binding protein required for vesicle fusion to the plasma membrane, is incorporated into the vesicle membrane and then released after successful delivery of the vesicle to its target site (Goud et al., 1988).

One interesting observation from this work is that SNAP function is sensitive to alkylation by NEM, but that the degree of NEM sensitivity is dependent on the assay used to measure SNAP function. In an assay measuring the ability of SNAP to mediate the binding of NSF to the membrane binding sites (Fig. 6 B) NEM only partially inactivated PF-1/SNAP. However in a transport assay where SNAP is required to complement other cytosolic pools as well as in the vesicle production assay, SNAP activity was completely eliminated by NEM treatment. This indicates that SNAP has

a catalytic function in transport that goes beyond its ability simply to mediate NSF binding. Such a view supports an earlier contention that SNAP functions with NSF as part of a multisubunit functional particle (Malhotra et al., 1988).

The assertion that the Golgi protein transport assay measures vesicular transport has recently come under scrutiny. It has been suggested, for example, that the system measures "homotypic" fusion, that is, the fusion between the medial-cisternae of donor and acceptor membranes (Mellman and Simons, 1992). We show here that there are very specific conditions of preincubation of donor but not acceptor membranes which confer a number of special properties to subsequent transport (i.e., primaquine resistance and NEM resistance). Quite apart from the morphological examinations that have been performed on this system, this asymmetry is a strong biochemical argument that vesicular transport is indeed occurring in this assay. If the transport was simply due to cisternal fusion, then there would be no differentiation between donor and acceptor membranes. We therefore believe that results derived from the Golgi transport assay can be reliably interpreted as reflecting vesicular transport. As exemplified by the unexpected findings we have reported here, the primaquine-based vesicle production assay should prove to be a very useful tool in examining the biochemical mechanisms of vesicle production and function.

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