

# A Multisubunit Particle Implicated in Membrane Fusion

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**Abstract.** The N-ethylmaleimide sensitive fusion protein (NSF) is required for fusion of lipid bilayers at many locations within eukaryotic cells. Binding of NSF to Golgi membranes is known to require an integral membrane receptor and one or more members of a family of related soluble NSF attachment proteins ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -SNAPs). Here we demonstrate the direct interaction of NSF, SNAPs and an integral membrane component in a detergent solubilized system. We show that NSF only binds to SNAPs in the presence of the integral receptor, resulting in the formation of a multisubunit protein complex with a sedi-

mentation coefficient of 20S. Particle assembly reveals striking differences between members of the SNAP protein family;  $\gamma$ -SNAP associates with the complex via a binding site distinct from that used by  $\alpha$ - and  $\beta$ -SNAPs, which are themselves equivalent, alternative subunits of the particle. Once formed, the 20S particle is subsequently able to disassemble in a process coupled to the hydrolysis of ATP. We suggest how cycles of complex assembly and disassembly could help confer specificity to the generalized NSF-dependent fusion apparatus.

**M**EMBRANE bilayer fusion must be carefully controlled within the living cell. Failure to restrict fusion to the appropriate pair of membranes would disrupt the secretory and endocytic pathways, cause mislocation of proteins and lipids and destroy the cells' compartmental structure. Use of an in vitro system which reconstitutes vesicle-mediated transport between successive compartments of the Golgi stack (Balch et al., 1984a, b; Rothman and Orci, 1990) led to identification and purification of the N-ethylmaleimide (NEM)<sup>1</sup>-sensitive fusion protein NSF (Glick and Rothman, 1987; Block et al., 1988), a tetramer of identical 76,000 D (76 kD) subunits essential for fusion of transport vesicles with their target membrane (Malhotra et al., 1988) at multiple points in the secretory and endocytic pathways (Beckers et al., 1989; Rothman, 1987; Diaz et al., 1989). This fusion apparatus is highly conserved; in the budding yeast *Saccharomyces cerevisiae* NSF is encoded by the *SEC18* gene (Novick et al., 1981; Eakle et al., 1988; Wilson et al., 1989) and *Sec18p* is similarly required for multiple fusion events (Riezman, 1985; Kaiser and Schekman, 1990; Graham and Emr, 1991; Rexach and Schekman 1991; for review see Wilson et al., 1991).

How is the widely used activity of NSF directed to fuse any particular pair of bilayers? Association of NSF with Golgi membranes requires an integral membrane component (Weidman et al., 1989) and one or more of a family of

related soluble NSF attachment proteins;  $\alpha$ -,  $\beta$ -, and  $\gamma$ -SNAPs (Weidman et al., 1989; Clary and Rothman, 1990; Clary et al., 1990). We have previously suggested that NSF, SNAP(s), and the membrane-bound receptor associate (possibly in the presence of other factors) to form a multisubunit "fusion machine" capable of catalyzing bilayer fusion at its point of assembly (Malhotra et al., 1988; Clary et al., 1990). Here we demonstrate receptor-dependent formation and ATP hydrolysis-dependent disassembly of this multisubunit peripheral membrane complex in detergent-solubilized extracts, and assign distinct roles to the members of the SNAP family. We suggest a model in which cycles of programmed assembly and disassembly regulate the action of this generalized fusion machine.

## Materials and Methods

### Preparation of Anti-myc Antibody

For preparation of the anti-myc mouse monoclonal IgG 9E10 (Evan et al., 1985) the hybridoma cell line MYC 1-9E10.2 (No. 1729; American Type Culture Collection, Rockville, MD) was cultured in 90% RPMI medium supplemented with 10% FCS. 10 d after injection of pristane (2,6,10,14-tetramethyldecanoic acid)  $1 \times 10^6$  hybridoma cells were injected into the peritoneal cavity of BALB/c-BYJ mice and then ascitic fluid collected after 8 d. Anti-myc antibody was purified from ascitic fluid either by precipitation with 50% ammonium sulfate (wt/vol) followed by ion exchange chromatography using DEAE cellulose, or by use of a protein G agarose column (Pierce Chemical Co., Rockford, IL). In both cases purified antibody was dialyzed against 2,000 vol of  $2 \times$  PBS ( $2 \times$  PBS is 16 g NaCl, 0.4 g KCl, 2.9 g  $\text{Na}_2\text{HPO}_4$ , and 0.48 g  $\text{KH}_2\text{HPO}_4$  per litre, pH 7.6) using spectrapor 2 dialysis tubing before freezing aliquots at  $-80^\circ\text{C}$ .

1. *Abbreviations used in this paper:* NEM, N-ethylmaleimide; NSF, NEM-sensitive fusion protein; RLG, rat liver Golgi; SNAP, soluble NSF attachment protein.

## Preparation of Membrane Fractions

Golgi membrane-containing fractions were prepared from 160 g of minced rat livers by modification of the method of Tabas and Kornfeld (1979), as described for preparation of rabbit liver Golgi membrane (Malhotra et al., 1989). Rat livers were purchased from Pel-Freez Biologicals (Rogers, AR). Membranes were stripped of peripherally associated proteins (including endogenous SNAPs and NSF) by incubation either with 1 M KCl for 15 min on ice ("salt-washed" membranes) or with 100 mM sodium carbonate for 10 min on ice ("alkali-washed" membranes) then reisolated by centrifugation onto sucrose cushions as described (Clary et al., 1990). Membranes washed either with salt or alkali retained no detectable NSF or SNAP activity, however salt-washed membranes generally exhibited assembly factor of a slightly higher specific activity, and were preferred for sedimentation or gel exclusion chromatography studies (see Results). CHO Golgi membranes were isolated as described (Balch et al., 1984a).

## Assay for Coimmunoprecipitation of NSF/myc and $\alpha$ -SNAP

Typically, co-immunoprecipitation assays contained 0.5  $\mu$ g of purified NSF/myc, 4.7  $\mu$ g of anti-myc antibody,  $\sim$ 2.5 ng of in vitro translated  $^{35}$ S-radiolabeled  $\alpha$ -SNAP (with a typical specific activity of  $0.8 \times 10^4$  CPM/ng) and Golgi membranes (or membranes previously subjected to salt or alkali washes) containing between 10 and 100  $\mu$ g of protein mixed together in assay buffer (20 mM Hepes/KOH, pH 7.4, 100 mM KCl, 2 mM DTT, 2 mM EDTA, 0.5 mM ATP, 1% polyethylene glycol 4,000, 250  $\mu$ g/ml soyabean trypsin inhibitor) in a final volume of 200  $\mu$ l. After incubation for 5 min on ice to permit binding, 10% Triton X-100 (wt/vol) was added to a final concentration of 0.5% (wt/vol) and solubilization allowed to proceed for 20 min on ice. 20  $\mu$ l of a 50% (vol/vol) slurry of protein G agarose beads (Calbiochem-Behring Corp., La Jolla, CA) were added and the mixture rotated for 60 min at 4°C. The beads were pelleted by spinning for several seconds in a microfuge, the supernatant carefully removed, and the protein G agarose-antibody-NSF/myc- $^{35}$ S- $\alpha$ -SNAP complex recovered on glass microfiber filters (Whatman, Inc., Clifton, NJ). Filters were washed three times with 0.5 ml of detergent wash buffer as previously described for the standard intra-Golgi transport assay (Balch et al., 1984a), dried using a heat lamp, and bound  $^{35}$ S counted in a liquid scintillation counter.

When assaying presolubilized membranes, the final Triton-X100 concentration was corrected to 0.5% (vol/vol). Assays were carried out exactly as described above, except for omission of the 20 min incubation before the addition of protein G agarose beads. For addition of Mg<sup>2+</sup> we supplemented the incubations with 1 M MgCl<sub>2</sub> to a concentration of 8 mM, resulting in a final concentration of 4 mM after chelation by EDTA already present. When assays were performed in the presence of ATP- $\gamma$ -S, the non-hydrolyzable nucleotide was present at a concentration of 500  $\mu$ M, in large excess over ATP (introduced with the addition of NSF/myc protein), present at a final concentration of 6  $\mu$ M.

We typically observe complex incorporation of  $\sim$ 15–20% of the TCA-precipitable  $^{35}$ S counts added to the assay. This is close to the maximum which could be expected, since a little <30% of the in vitro-translated  $^{35}$ S- $\alpha$ -SNAP is biologically active, as measured by its ability to bind to intact Golgi membranes in a specific manner. Moreover, addition of  $\gamma$ -SNAP stimulates  $^{35}$ S- $\alpha$ -SNAP incorporation (see Results), bringing the efficiency of the reaction closer to the theoretical maximum.

## Western Immunoblotting Analysis of a $\gamma$ -SNAP-NSF/myc Complex

For co-immunoprecipitation of  $\gamma$ -SNAP and NSF/myc, 2  $\mu$ g of NSF/myc (or an equivalent volume of NSF buffer) were mixed with 200 ng of  $\gamma$ -SNAP, 200 ng of  $\alpha$ -SNAP, and 18  $\mu$ g of rat liver Golgi (RLG) membranes in a total volume of 50  $\mu$ l and the same buffer conditions as described for  $\alpha$ -SNAP co-immunoprecipitation (and similarly including or omitting Mg<sup>2+</sup> ions). The mixture was incubated at 30°C for 5 min and then returned to ice and mixed with a further 50  $\mu$ l of assay buffer containing 1% Triton-X 100 (wt/vol) (resulting in a final detergent concentration of 0.5% wt/vol) and 20  $\mu$ g of anti-myc antibody. After 20 min on ice the complex was collected by incubation with protein G agarose beads as above, washed twice with 0.5 ml of assay buffer containing 0.5% Triton-X 100 (wt/vol) and then subjected to SDS-PAGE (Laemmli, 1970) using a 10% polyacrylamide gel. The gel was blotted to nitrocellulose for 1 h at 120 V (constant voltage) in a transfer buffer containing 25% methanol, 25 mM Trizma base, and 250 mM glycine and then the nitrocellulose by incubation in PBS and 1% BSA

(wt/vol) at 4°C for 1 h. After overnight incubation at 4°C with affinity-purified rabbit anti- $\gamma$ -SNAP antibodies (Whiteheart et al., in press) in PBS and 1% BSA (wt/vol), and then three successive 4-min washes in PBS and 0.1% Tween-20 the filter was incubated at room temperature for 45 min with peroxidase-conjugated goat anti-rabbit secondary antibody (United States Biochemical Corp., Cleveland, OH) diluted 1:5,000 in PBS and 1% BSA (wt/vol). The filter was washed as before, incubated for 1 min with ECL chemiluminescent detection reagent (Amersham Corp., Arlington Heights, IL) and exposed to film (XAR-5; Eastman Kodak Co., Rochester, NY) (typically for 5–30 s).

## Velocity Sedimentation Studies

Salt-washed RLG membranes (typically 100 to 150  $\mu$ g of protein per gradient), previously dialyzed against 2,000 vol of 10 mM Tris/HCl, pH 7.4, using Spectrapor 2 tubing (to remove sucrose) were pelleted in a refrigerated microcentrifuge (set at 4°C) for 6 min. The membrane pellet was resuspended in gradient buffer (20 mM Hepes/KOH, pH 7.4, 100 mM KCl, 2 mM DTT, 2 mM EDTA, 0.5 mM ATP, 0.5% wt/vol Triton-X 100) at a ratio of at least 300  $\mu$ l buffer per 100–150  $\mu$ g Golgi protein then stored on ice, with occasional agitation, for 45 min. After centrifugation for 45 min at 100,000 g in a TLA100 Benchtop ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) the supernatant was carefully recovered and 300- $\mu$ l aliquots mixed with (as appropriate) 2  $\mu$ g NSF/myc, NEM-treated NSF/myc or an equivalent volume of buffer, 1  $\mu$ g of  $\alpha$ -SNAP protein or  $\sim$ 25 ng of  $^{35}$ S- $\alpha$ -SNAP (typically containing  $2 \times 10^5$  CPM) and 65 ng-1  $\mu$ g of  $\gamma$ -SNAP protein. After incubation for 5 min on ice the mixture was layered onto the top of 10–35% (wt/vol) glycerol gradients, initially formed in steps by pouring eleven successive 0.4-ml cushions of gradient buffer (see above) containing 35, 32.5, 30, 27.5, 25, 22.5, 20, 17.5, 15, 12.5, and 10% glycerol (wt/vol) and then allowed to become continuous by storage at 4°C for  $\sim$ 1 h before loading. After centrifugation at 53,000 revolutions per minute for 5 h in a rotor (SW55; Beckman Instruments Inc.) using a slow acceleration and deceleration program, gradients were collected from the bottom as either 0.33 or 0.25 ml fractions, and the entire fraction or aliquots mixed with scintillant and counted in a liquid scintillation counter.

Sedimentation coefficients of NSF/myc-bound or -unbound assembly factor activity, or of the NSF/myc-dependent peak of  $^{35}$ S- $\alpha$ -SNAP, were determined by comparison of their gradient profiles with a calibration curve prepared from the sedimentation of marker proteins under identical conditions; standards used were BSA (4.6S), Catalase (11.4S), and  $\alpha$ -2 Macroglobulin (20S).

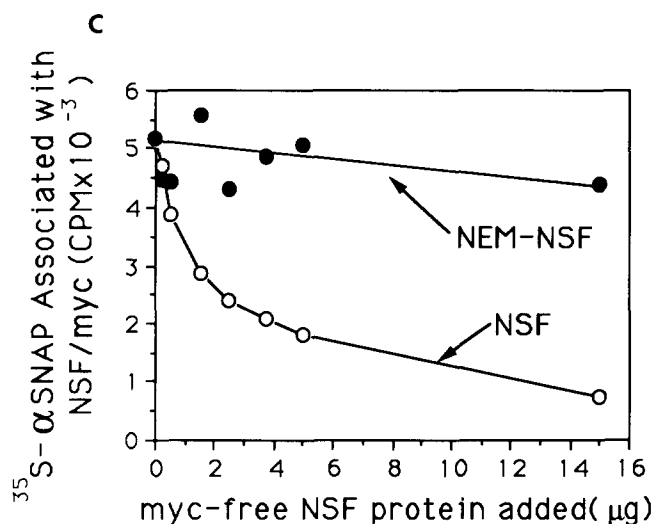
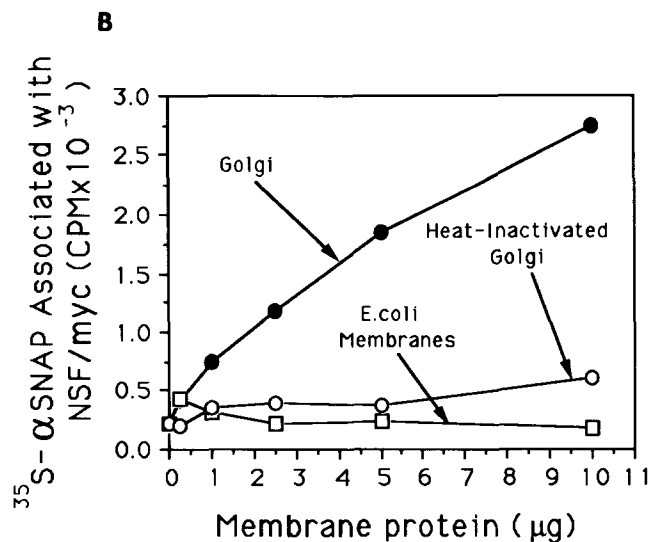
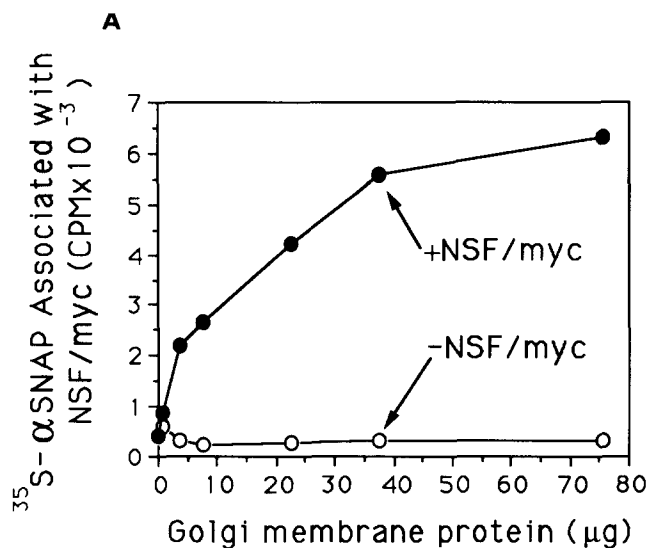
## Size Exclusion Chromatography

A 100,000 g detergent-extracted supernatant was prepared from RLG membranes exactly as described for velocity sedimentation studies (see above) except that instead of 0.5 w/v Triton-X 100 (wt/vol) we used 0.4% N,N,bis(3-D-gluconamidopropyl)cholamide (BigCHAP) (wt/vol). The extract was incubated with 1.2  $\mu$ g of NSF/myc and  $\sim$ 20 ng of  $^{35}$ S- $\alpha$ -SNAP (typically  $1.6 \times 10^5$  cpm) for 5 min on ice then loaded onto a Pharmacia Superose 6 FPLC gel filtration column (of included volume 23 ml) previously equilibrated with two column volumes of 20 mM Hepes/KOH, pH 7.4, 100 mM KCl, 2 mM DTT, 2 mM EDTA, 0.5 mM ATP, 10% glycerol, 0.4% BigCHAP. Elution was with the same buffer at a flow rate of 0.2 ml/min, and 0.5-ml fractions collected. The elution profile of  $^{35}$ S- $\alpha$ -SNAP was determined by counting 50- $\mu$ l aliquots of each fraction in a scintillation counter. We determined the Stokes radius of the NSF/myc- $^{35}$ S- $\alpha$ -SNAP complex by comparison with the elution profiles of known standards; Thyroglobulin (Stokes radius 85 Å), Ferritin (61 Å), and Catalase (52 Å). Marker proteins were subjected to gel filtration under conditions identical to those used for elution of the NSF/myc- $^{35}$ S- $\alpha$ -SNAP complex, except that ATP was omitted from the column buffer to permit monitoring of elution profiles by ultraviolet light absorption. The void volume of the column was determined using blue dextran.

## Results

### NSF Only Binds $\alpha$ -SNAP in the Presence of a Membrane-derived Assembly Factor

We make use of a recombinant derivative of NSF termed NSF/myc, bearing a carboxy-terminal extension EQKLISE-EDL (single letter amino-acid code) derived from the human



**Figure 1.** Rat liver Golgi membranes contain a specific NSF- $\alpha$ -SNAP assembly factor. (A) Increasing amounts of RLG membranes were added to  $^{35}\text{S}$ -radiolabeled  $\alpha$ -SNAP and anti-myc antibody in the presence ( $\bullet$ ) or absence ( $\circ$ ) of NSF/myc in a co-immunoprecipitation assay (see Materials and Methods). (B) A similar titration to A, using RLG membranes ( $\bullet$ ), RLG membranes heated to  $75^\circ\text{C}$  for 15 min and then returned to ice before assay ( $\circ$ ) or inner membranes from the bacterium *Escherichia coli* ( $\square$ ). All assays contained NSF/myc. Assembly factor activity measured in 10  $\mu\text{g}$  of RLG was unaffected when mixed with 10  $\mu\text{g}$  heat-treated RLG and diminished by 30% when mixed with 10  $\mu\text{g}$  of *E. coli* inner membranes (data not shown). (C) To 5  $\mu\text{g}$  of alkali-washed RLG membranes (free of endogenous SNAPs and NSF) were added NSF ( $\circ$ ) or NEM-treated NSF ( $\bullet$ ) and a co-immunoprecipitation assay performed in the presence of 0.5  $\mu\text{g}$  NSF/myc. An NSF/myc-independent signal of 566 cpm has been subtracted from the data.

*c-myc* protein and which forms the epitope recognized by a mouse IgG<sub>1</sub> mAb termed 9E10 (Evan et al., 1985; Munro and Pelham, 1987; Semenza et al., 1990). Details of the expression, characterization, and purification of NSF/myc protein from *Escherichia coli* will be described elsewhere (Wilson and Rothman, in press). When purified NSF/myc was mixed with  $^{35}\text{S}$ -radiolabeled  $\alpha$ -SNAP protein (prepared by in vitro transcription and translation of the  $\alpha$ -SNAP gene; Brunner et al., manuscript in preparation) and NSF/myc immunoprecipitated using the anti-myc IgG mAb,  $^{35}\text{S}$ - $\alpha$ -SNAP was coimmunoprecipitated (Fig. 1 a).  $\alpha$ -SNAP prepared in this manner is biologically active as measured by SNAP-dependent in vitro intra-Golgi transport assays (M. Brunner, S. W. Whiteheart, D. W. Wilson, and J. E. Rothman, manuscript in preparation) and by membrane-binding studies (Whiteheart et al., in press). Immunoprecipitation of  $^{35}\text{S}$ - $\alpha$ -SNAP required NSF/myc and showed dose-dependence upon RLG membrane protein, suggesting the presence of a saturable membrane-derived "assembly factor." Golgi-derived assembly factor was inactivated by heating RLG membranes at  $75^\circ\text{C}$  for 15 min (Fig. 1 b), conditions shown to

destroy the NSF-binding capacity of CHO Golgi membranes (Weidman et al., 1989) and 10 to 30% of assembly factor activity was lost after incubation of RLG membranes at  $37^\circ\text{C}$  for 60 min (data not shown). Golgi membranes prepared from rabbit liver or CHO cells and crude membrane fractions obtained from bovine brain or *S. cerevisiae* were similarly able to promote the specific association of  $\alpha$ -SNAP and NSF/myc (data not shown) whilst the inner membrane of *E. coli* was devoid of this activity (Fig. 1 b). Assembly factor is an integral component of Golgi membranes since its activity is present following removal of peripheral membrane proteins by incubation with 1 M KCl or alkaline sodium carbonate (see below). We therefore carried out subsequent studies using membranes prepared in this way, since they were free of the activity of contaminating endogenous SNAPs or NSF.

The interaction between NSF/myc and  $\alpha$ -SNAP is specific since it could be competed by addition of increasing amounts of NSF lacking the *myc* epitope tag (Fig. 1 c) with half maximal inhibition at an NSF concentration of 35 nM. Incubation of NSF with 1 mM NEM at  $4^\circ\text{C}$  for 15 min, conditions suf-

ficient for inactivation of its transport activity (Glick and Rothman, 1987), and which causes disassembly of multi-metric NSF (D. W. Wilson, unpublished observations) rendered it unable to compete with NSF/myc for  $\alpha$ -SNAP binding (Fig. 1 c). Similarly, treatment of NSF/myc with NEM led to loss of its ability to coimmunoprecipitate  $^{35}\text{S}$ - $\alpha$ -SNAP (see below). By carrying out co-immunoprecipitation assays using  $^{35}\text{S}$ -SNAP of known specific activity we estimated the abundance of assembly factor by Scatchard analysis; 1 mg of alkali-washed RLG membranes could attach a maximum of 77 pMols of  $^{35}\text{S}$ - $\alpha$ -SNAP to excess NSF/myc with an overall affinity constant of 28 nM (data not shown). This abundance agrees well with that described for the SNAP/NSF receptor in 1 mg of CHO Golgi (50 pMols; Weidman et al., 1989) and RLG membranes (34 pMols; Whiteheart et al., in press). We conclude that Golgi membranes are able to provide an integral membrane NSF/SNAP assembly factor, with the characterizations and abundance expected of the NSF/SNAP receptor.

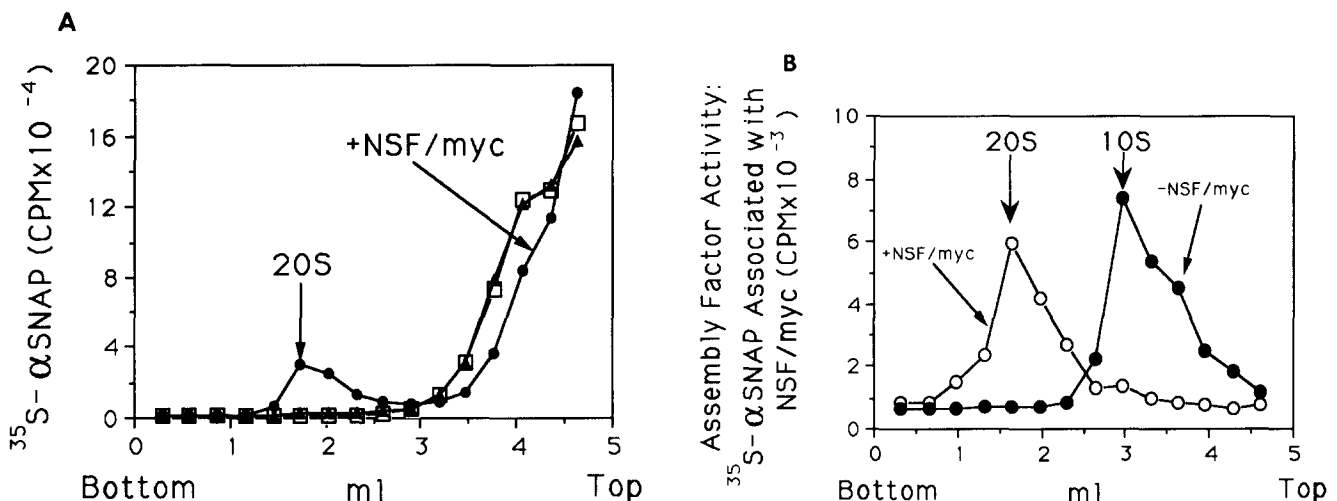
### NSF/myc, $\alpha$ -SNAP, and Assembly Factor Associate to Form a Complex

If assembly factor is the NSF/SNAP receptor, responsible for anchoring these proteins to the surface of Golgi membranes, it follows that binding of NSF and SNAP occurs as a result of complex formation between NSF, SNAP, and assembly factor. To directly demonstrate the existence of this complex and the presence of assembly factor we carried out velocity sedimentation studies. In the presence of NSF/myc (but not NEM-treated NSF/myc) and a detergent extract of salt-washed RLG membranes,  $^{35}\text{S}$ - $\alpha$ -SNAP became incorporated into a particle with a sedimentation coefficient of 20S (Fig. 2 a). The  $^{35}\text{S}$ - $\alpha$ -SNAP in this rapidly sedimenting peak was immunoprecipitated by the anti-myc IgG mAb (but not by a control monoclonal IgG), confirming the peak contains NSF/myc bound to  $^{35}\text{S}$ - $\alpha$ -SNAP (data not shown). We next measured the distribution of assembly factor; in the

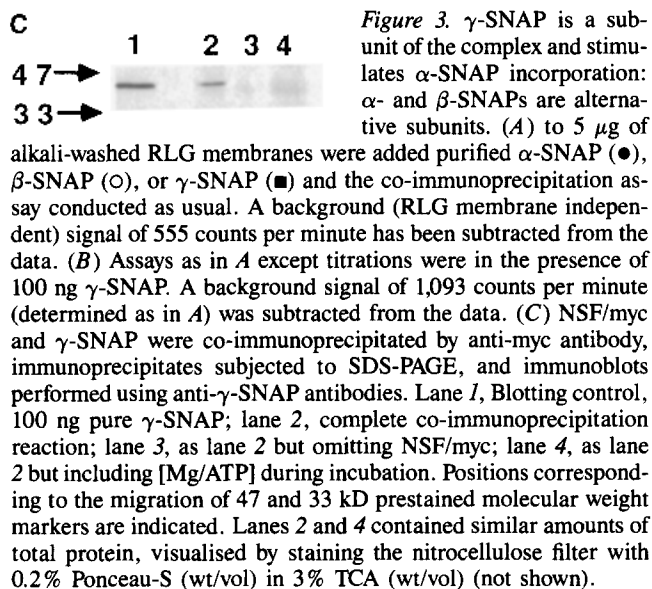
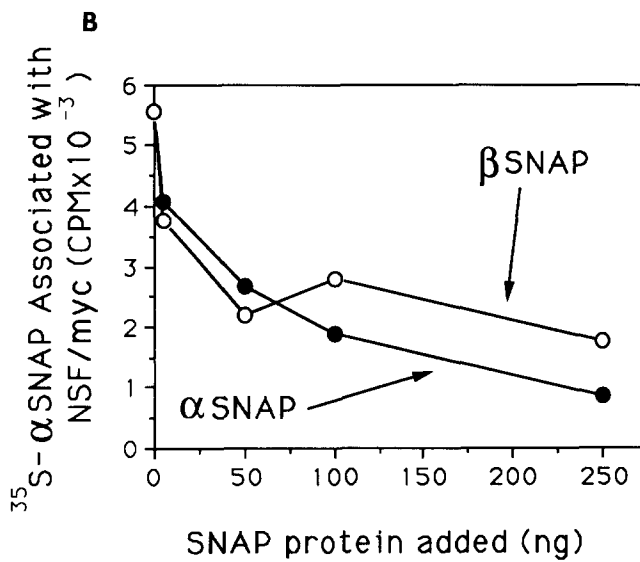
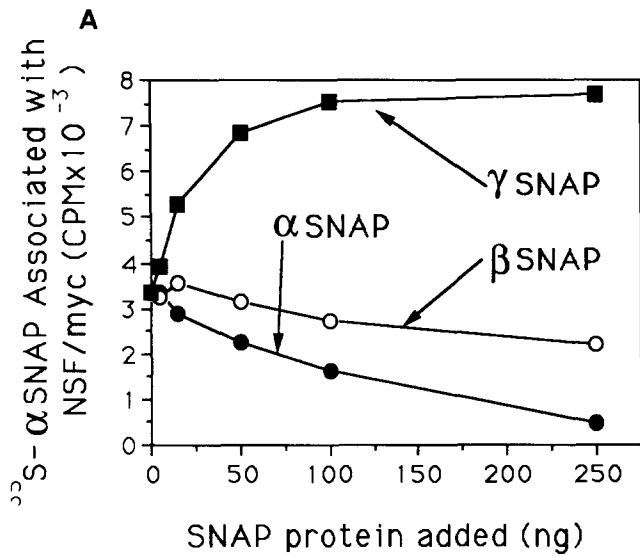
presence of NSF/myc, assembly factor (as determined by testing gradient fractions in a standard co-immunoprecipitation assay) also sedimented with a coefficient of 20S (Fig. 2 b), suggesting that NSF/myc, SNAP, and assembly factor are associated together in the complex. When NSF/myc was absent, uncomplexed assembly factor sedimented instead at 10S. As an independent measure of complex size we subjected it to FPLC size exclusion chromatography using a Superose 6 column (Pharmacia Fine Chemicals, Piscataway, NJ) in the presence of the detergent N,N,bis(3-D-gluconamidopropyl)cholamide (BigCHAP), which forms micelles of only 8 kD. An NSF/myc-dependent peak of  $^{35}\text{S}$ - $\alpha$ -SNAP eluted from the column at a position corresponding to a Stokes radius of 90 Å, and subsequently sedimented at 20S (data not shown). NEM-treated NSF/myc was unable to drive formation of this particle, and a preformed complex was destroyed by mild NEM treatment (data not shown). Combination of the sedimentation coefficient and Stokes radius predicts a molecular weight of approximately 700 kD for the complex. Since tetrameric NSF/myc would have a mass of 340 kD (Wilson and Rothman, 1992) the complex is likely composed of one NSF tetramer associated with SNAPs and assembly factor(s). As the amount of bound detergent remains unknown, these size estimates are at best approximate.

### Distinct Roles for SNAP Proteins in Complex Formation

We added unlabeled  $\alpha$ -,  $\beta$ -, or  $\gamma$ -SNAPs to a co-immunoprecipitation assay containing limiting amounts of alkali-washed Golgi membranes (Fig. 3 a). Under standard assay conditions, when the concentration of  $^{35}\text{S}$ - $\alpha$ -SNAP is low (typically 0.4 nM) the addition of  $\gamma$ -SNAP-stimulated particle formation.  $\gamma$ -SNAP similarly stimulated complex assembly when measured by sedimentation or gel filtration (data not shown). Nevertheless, complex formation was still abso-



**Figure 2.** NSF/myc,  $\alpha$ -SNAP, and assembly factor form a 20S complex. (A) Detergent extracts of salt-washed RLG membranes were incubated with  $^{35}\text{S}$ - $\alpha$ -SNAP, NSF/myc (●), NEM-inactivated NSF/myc (▲) or no NSF/myc (□) and then sedimented through 10–35% glycerol (wt/vol) gradients. The position corresponding to a sedimentation coefficient of 20S is indicated. (B) Non-radioactive SNAP proteins (purified as described by Clary and Rothman, 1990), a detergent extract of salt-washed RLG membranes and NSF/myc (○) or buffer (●) were incubated to form a complex then subjected to sedimentation as in A. Gradient fractions were tested for assembly factor using a standard coimmunoprecipitation assay. Positions corresponding to sedimentation coefficients of 10S and 20S are indicated.



lutely dependent upon the presence of assembly factor (data not shown). As expected, unlabeled  $\alpha$ -SNAP competed with  $^{35}\text{S}$ - $\alpha$ -SNAP for co-immunoprecipitation with NSF/myc, with a  $K_d$  of 15 nM (Fig. 3 a), in good agreement with the affinity constant determined by Scatchard analysis (see above) and that measured for the binding of  $^{35}\text{S}$ - $\alpha$ -SNAP to its membrane receptor (Whiteheart et al., in press).  $\beta$ -SNAP also competed for incorporation of  $^{35}\text{S}$ - $\alpha$ -SNAP, though with severalfold lower affinity (Fig. 3 a).

Addition of  $\gamma$ -SNAP compensated for this reduced affinity; under these conditions both  $\alpha$ - and  $\beta$ -SNAP exhibit a  $K_d$  of 7 nM for complex association (Fig. 3 b). The ability of  $\gamma$ -SNAP to stimulate incorporation of  $\alpha$ -SNAP, together with its capacity to bind NSF to its receptor in the absence of other SNAPs (Clary et al. 1990) suggests  $\gamma$ -SNAP is itself a subunit of the complex. Indeed, under conditions similar to those used for the co-immunoprecipitation assay,  $\gamma$ -SNAP is immunoprecipitated in an NSF/myc-dependent manner (Fig. 3 c, compare lanes 2 and 3).

### ATP Hydrolysis and Complex Disassembly

Addition of ATP and  $\text{Mg}^{2+}$  ions to Golgi membranes led to release of NSF activity (Glick and Rothman, 1987), suggesting hydrolysis of a [Mg·ATP] substrate might affect the assembly/disassembly of the 20S complex. Since the presence of ATP is required to stabilize soluble NSF and NSF/myc (Block et al., 1988; Wilson and Rothman, 1992) and prevents disassembly of multimeric NSF (D. W. Wilson, unpublished observations) we sought to distinguish the role of ATP hydrolysis from the stabilizing effect of ATP binding by adding or withholding  $\text{Mg}^{2+}$  ions. A solubilized membrane extract,  $^{35}\text{S}$ - $\alpha$ -SNAP and NSF/myc were mixed and sedimented as previously shown in Fig. 2 a. Fractions containing the 20S particle were harvested and then subjected to a second round of sedimentation through gradients containing ATP and ei-

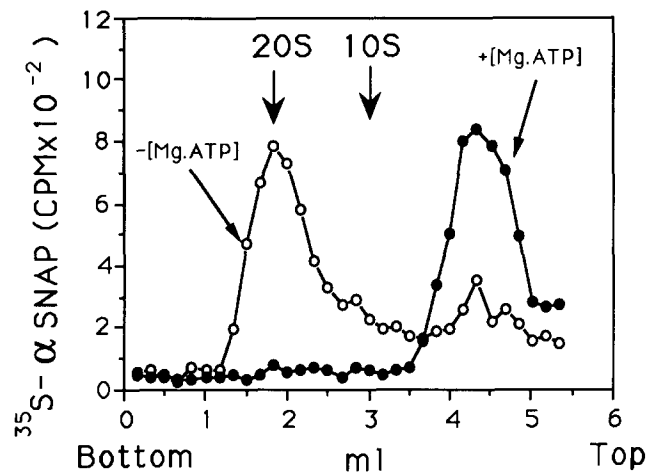
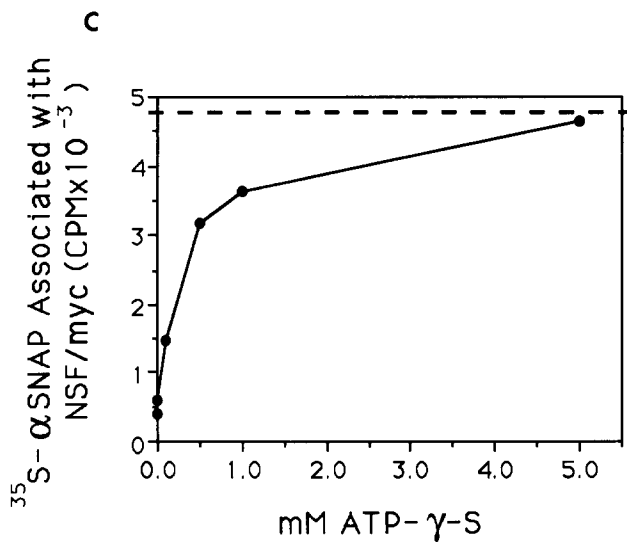
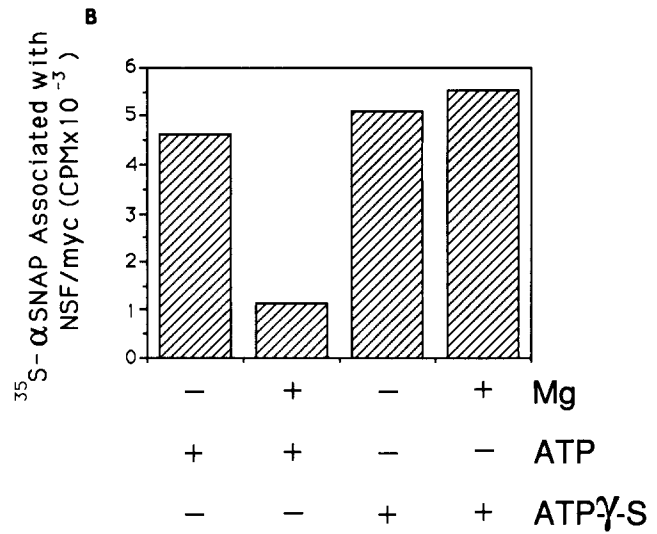
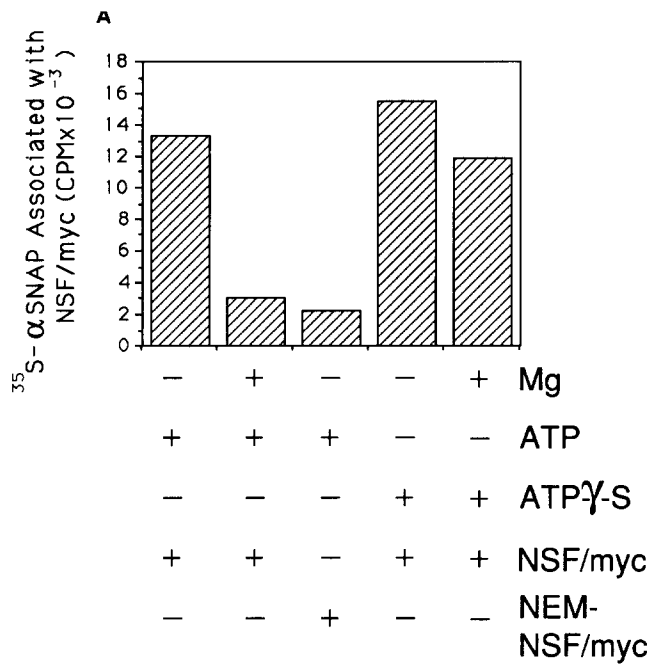


Figure 4. The 20S complex disassembles in the presence of [Mg·ATP]. A 20S complex was recovered from a velocity sedimentation gradient similar to that shown in Fig. 2 a, half resedimented through an identical gradient (i.e., in the absence of [Mg·ATP];  $\circ$ ) and the other half mixed with 2 mM,  $\text{Mg}^{2+}$  ions (final concentration after chelation by EDTA) and then sedimented through a +[Mg·ATP] gradient ( $\bullet$ ).



ther EDTA or  $\text{Mg}^{2+}$ . In the presence of  $[\text{Mg} \cdot \text{ATP}]$  (but not ATP alone) the complex underwent disassembly (Fig. 4), leaving unbound  $^{35}\text{S}$ - $\alpha$ -SNAP towards the top of the gradient. Similarly, when measured by co-immunoprecipitation of  $^{35}\text{S}$ - $\alpha$ -SNAP (Fig. 5 a) or  $\gamma$ -SNAP (Fig. 3 c, compare lanes 2 and 4) addition of  $\text{Mg}^{2+}$  and ATP reduced complex formation to background levels. The effect of  $\text{Mg}^{2+}$  is via hydrolysis of  $[\text{Mg} \cdot \text{ATP}]$  because in the presence of the non-hydrolyzable ATP analogue ATP- $\gamma$ -S, complex formation was normal in the presence or absence of  $\text{Mg}^{2+}$  ions (Fig. 5 a). We next uncoupled assembly and disassembly by forming the complex in the presence of ATP- $\gamma$ -S then, after washing to remove nucleotide, incubating with ATP- $\gamma$ -S or ATP

**Figure 5.** Complex formation proceeds in the presence of ATP- $\gamma$ -S and the complex can be reprogrammed after formation. (A) Lane 1, standard co-immunoprecipitation assay (buffer contains EDTA and ATP); lane 2, buffer contains  $[\text{Mg} \cdot \text{ATP}]$ ; lane 3, as lane 1 but using NEM-treated NSF/myc; lane 4, buffer contains EDTA and ATP- $\gamma$ -S; lane 5, buffer contains  $[\text{Mg} \cdot \text{ATP-}\gamma\text{-S}]$ . Assays in the presence of ATP- $\gamma$ -S contained 500  $\mu\text{M}$  ATP- $\gamma$ -S and 6  $\mu\text{M}$  ATP (introduced with NSF/myc). (B) Protein G-bound complexes were prepared in the presence of ATP- $\gamma$ -S and EDTA, washed twice and then resuspended in 200  $\mu\text{l}$  of buffer and incubated for 5 min at 4°C before collection on filters as usual. Buffers were assay buffer containing 0.5% Triton-X 100 (wt/vol) and the following: Lane 1, EDTA and ATP; lane 2,  $[\text{Mg} \cdot \text{ATP}]$ ; lane 3, EDTA and ATP- $\gamma$ -S; lane 4,  $[\text{Mg} \cdot \text{ATP-}\gamma\text{-S}]$ . Values plotted are the mean of duplicates. (C) Complex disassembly is competed by ATP- $\gamma$ -S. Protein G-bound complexes were prepared in the presence of ATP and EDTA, washed twice in 500  $\mu\text{l}$  of assay buffer containing 0.5% Triton-X 100 (wt/vol) but lacking nucleotide and then supplemented with 100  $\mu\text{M}$  ATP, 2 mM  $\text{Mg}^{2+}$  (after chelation by EDTA), and ATP- $\gamma$ -S. After incubation for 2 min on ice, co-immunoprecipitated  $^{35}\text{S}$ - $\alpha$ -SNAP was collected on filters and counted as usual. The broken line shows  $^{35}\text{S}$ - $\alpha$ -SNAP coimmunoprecipitated when the 2-min incubation was in the presence of 100  $\mu\text{M}$  ATP but no  $\text{Mg}^{2+}$ , representing the maximum signal obtainable.

in the presence or absence of  $\text{Mg}^{2+}$ . Incubation with ATP and  $\text{Mg}^{2+}$  ions resulted in release of  $^{35}\text{S}$ - $\alpha$ -SNAP from the immunoprecipitated complex (Fig. 5 b), showing that exogenous  $[\text{Mg} \cdot \text{ATP}]$  is able to freely enter the ATP hydrolysis site and “reprogram” the particle for disassembly. Conversely when a washed, preformed complex was incubated with ATP,  $\text{Mg}^{2+}$  ions, and an increasing concentration of ATP- $\gamma$ -S, the breakdown of the particle was progressively inhibited (Fig. 5 c) with half maximal inhibition of disassembly at an ATP- $\gamma$ -S concentration of 300  $\mu\text{M}$ . This relatively low affinity for nucleotide is consistent with the findings that 300  $\mu\text{M}$  ATP is required to fully stabilize NSF against thermal inactivation (Block et al., 1988) and that the apparent  $K_m$

for ATP hydrolysis by NSF is 650  $\mu\text{M}$  (Tagaya et al., manuscript in preparation). Competition for ATP binding by the non-hydrolyzable analogue shows that both nucleotides are able to bind to the hydrolysis/disassembly site under these conditions.

## Discussion

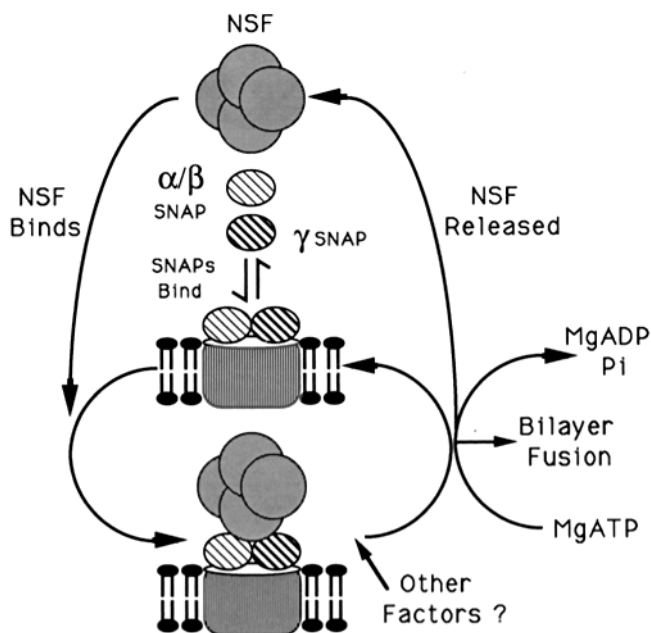
### The SNAP Protein Family

A key question raised by earlier studies (Clary and Rothman, 1990; Clary et al., 1990) is, why should there be three distinct SNAP proteins? Complex assembly reveals the similarities and differences between members of this related family.  $\beta$ -SNAP was able to compete with  $\alpha$ -SNAP for inclusion into the complex, indicating that  $\alpha$ - and  $\beta$ -SNAPs recognize the same binding site. Given this functional redundancy, their very similar size and specific activities and the relatedness of their partial proteolysis maps (Clary and Rothman, 1990; Clary et al., 1990) it is probable that  $\alpha$ - and  $\beta$ -SNAPs are isoforms of the same protein. We will therefore refer to this type of SNAP as  $\alpha/\beta$ -SNAP, to distinguish this class of molecule from  $\gamma$ -SNAP. Clary et al. (1990) showed that purified  $\alpha$ -SNAP protein, unlike  $\beta$ -SNAP, is able to complement the in vitro transport defect of yeast *sec17-1* cytosol. Perhaps  $\alpha$ -SNAP and *Sec17p* are the "archetypal" forms of  $\alpha/\beta$ -SNAP which have been highly conserved during evolution, while  $\beta$  is a more recently diverged derivative. Since SNAP proteins are purified from crude bovine brain,  $\beta$ -SNAP may well be specialized to perform the same function as  $\alpha$ -SNAP at a different cellular location or in a different brain cell type.

$\gamma$ -SNAP plays a role distinct from that of  $\alpha/\beta$ -SNAP, consistent with its markedly different partial proteolysis map and very much lower specific activity (Clary and Rothman, 1990; Clary et al., 1990). Co-immunoprecipitation with NSF/myc demonstrated that  $\gamma$ -SNAP is able to assemble into the complex, yet did not compete with  $^{35}\text{S}$ - $\alpha$ -SNAP binding, suggesting  $\gamma$ -SNAP mediates NSF membrane association through binding sites distinct from those recognized by  $\alpha/\beta$ -SNAP. This would explain the increased level of  $^{35}\text{S}$ - $\alpha$ -SNAP-complex association in the presence of  $\gamma$ -SNAP; although NSF can apparently become tethered to receptor/assembly factor by either  $\alpha/\beta$ - or  $\gamma$ -SNAP alone, the complex is presumably most stable when NSF association is mediated by both SNAP species.

### Assembly and Disassembly of the Complex

Fusion must occur efficiently between selected pairs of membranes, yet be completely suppressed at all other times. NSF,  $\alpha/\beta$ -SNAP, and  $\gamma$ -SNAP bind together when able to associate with an integral membrane assembly factor; limiting the accessibility or activity of this factor would maintain the subunits of the particle in a soluble state, rendering the fusion apparatus inactive. Whenever an appropriate membrane pair became selected for fusion, NSF (and possibly soluble SNAPs) would be recruited from a generally available cytosolic pool to form a membrane-bound complex at the desired location. Since the subunits of the complex disperse upon ATP hydrolysis (see Results) utilization of ATP during fusion (Balch et al., 1984b) could couple fusion to disassembly of the apparatus responsible; an efficient means of ensuring



**Figure 6.** Subunit composition of the NSF/SNAP/receptor (assembly factor) complex and its assembly and disassembly. For clarity, only one bilayer is depicted. NSF tetramer, stippled spheres;  $\alpha/\beta$ -SNAP, ovoid with light hatched bars;  $\gamma$ -SNAP, ovoid with heavy hatched bars; receptor (assembly factor), cylinder with vertical bars. Lipid bilayer is in black. The nature of the NSF/SNAP/receptor stoichiometry is unknown.

suppression of the fusion apparatus once it has performed its task. Whether the same ATP molecule is hydrolyzed for both processes, and whether these hydrolysis events are by NSF itself remain to be shown.

Direct proof that NSF carries out its role as part of the 20S complex awaits its incorporation into liposomes and subsequent reconstitution of the fusion event. Nevertheless, several lines of evidence are strongly suggestive; NSF becomes unable to participate in complex formation following the same mild NEM treatment which inactivates its fusion activity and defective alleles of *SEC17* (a good candidate for yeast  $\alpha$ -SNAP; Clary et al., 1990) and *SEC18* (yeast NSF; Wilson et al., 1989) exhibit synthetic lethality, suggesting the physical interaction of the two gene products is an essential aspect of their function (Kaiser and Schekman, 1990). Furthermore, a role for the 20S particle in fusion predicts that other subunits of the complex (in addition to NSF) should be essential for the fusion pathway. Indeed, in the absence of SNAPs in vitro intra-Golgi transport arrests at the same point at which NSF is required (Clary et al., 1990) and a *ts* allele of *SEC17* accumulates unfused vesicles in vivo (Kaiser and Schekman, 1990). Finally, *Sec18p* performs an essential role in promoting or maintaining the attachment of ER-derived transport vesicles with the yeast Golgi apparatus in vitro (Rexach and Schekman, 1991), indicating there exists some means of targeting this soluble protein to the vesicle/membrane junction.

Our view of the composition and regulation of the NSF-containing particle is summarized in Fig. 6. Imposition of strict criteria for assembly of the complex, and its autoregulation by fusion-coupled dispersal, may be the means by which the cell safely exploits a potentially catastrophic physical process.

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