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 (α-, β-, and γ-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 sedimentation coefficient of 20S. Particle assembly reveals striking differences between members of the SNAP protein family; γ-SNAP associates with the complex via a binding site distinct from that used by α- and β-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.

Membrane 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 compartiments of the Golgi stack (Balch et al., 1984a, b; Rothman and Orci, 1990) led to identification and purification of the N-ethylmaleimide (NEM)-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; α-, β-, and γ-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 1E10.2 (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-tetramethyldecane) 1 x 10^8 hybridoma cells were injected into the peritoneal cavity of BALB/c-BYJ mice and 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 2x PBS (2 x PBS is 16 g NaCl, 0.4 g KCl, 2.9 g Na₂HPO₄, and 0.48 g KH₂PO₄ per litre, pH 7.6) using spectrapor 2 dialysis tubing before freezing aliquots at -80°C.

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

Golgol 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 ("lysozyme-washed" membranes) and then washed by briefly resolubilized by cesium chloride wash, onto sucrose cushions as described (Clary et al., 1990). Membranes washed either with salt or alkalai 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 α-SNAP

Typically, co-immunoprecipitation assays contained 0.5 μg of purified NSF/myc, 4.7 μg of anti-myc antibody, ~2.5 mg of in vitro translated [35S]radiolabeled α-SNAP (with a typical specific activity of 0.8 × 106 CPM/μg) and Golgi membranes (or membranes previously subjected to salt or alkalai washes) containing between 10 and 100 μg 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 μg/ml soya bean trypsin inhibitor) in a final volume of 200 μl. After incubation for 5 min on ice to permit binding, 10% Triton X-100 (wt/vol) was added to a final volume of 0.5% (wt/vol) and solubilization allowed to proceed for 20 min on ice. 20 μl 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-NF/myc-35S-α-SNAP complex recovered on glass microfiber filters (Whatman, Inc., Clifton, NJ). Filter washes 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 [35S] 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 Mg2+ we supplemented the incubations with 1 M MgCl2 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-γ-S, the non-hydrolyzable nucleotide was present at a concentration of 500 μM, in large excess over ATP (introduced with the addition of NSF/myc protein), present at a final concentration of 6 μM.

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

Western Immunoblotting Analysis of a γ-SNAP-NSF/myc Complex

For co-immunoprecipitation of γ-SNAP and NSF/myc, 2 μg of NSF/myc (or an equivalent volume of NSF buffer) were mixed with 200 ng of γ-SNAP, 200 ng of α-SNAP, and 18 μg of rat liver Golgi (RLG) membranes in a total volume of 50 μl and the same buffer conditions as described for α-SNAP co-immunoprecipitation (and similarly including or omitting Mg2+ ions). The mixture was incubated at 30°C for 5 min and then returned to ice and mixed with a further 50 μl of assay buffer containing 1% Triton-X 100 (wt/vol) resulting in a final detergent concentration of 0.5% (wt/vol) and 20 μg 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 Tris 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-γ-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 1:5000 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 μg of protein per gradient), previously dialyzed against 2,000 vol of 10 mM Tris/HCl, pH 7.4, using Spectra/por 2 tubing (to remove sucrose) were pelleted in a refrigerated microcentrifuge (set at 4°C) for 6 min. The membrane pellet was suspended 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 μl buffer per 100–150 μg 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-μl aliquots mixed with (or appropriate) 2 μg NSF/myc, NEM-treated NSF/myc or an equivalent volume of buffer, 1 μg of α-SNAP protein or ~25 ng of [35S]-α-SNAP protein (typically containing 2 × 105 CPM) and 65 ng-1 μg of γ-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-mi 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 ~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 0.13 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 α-unbound assembly factor activity, or of the NSF/myc-dependent peak of [35S]-α-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 α-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 W/v N,N,N,3(N-l-alanino)-N,l-alkanoylpropylamidomethyl (BigCHAP) (wt/vol). The extract was incubated with 1.2 μg of NSF/myc and ~20 ng of [35S]-α-SNAP (typically 1.6 × 103 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 [35S]-α-SNAP was determined by counting 50-μl aliquots of each fraction in a scintillation counter. We determined the Stokes radius of the NSF/myc-[35S]-α-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-[35S]-α-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 α-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 EKQLISE-EDL (single letter amino-acid code) derived from the human...
c-myc protein and which forms the epitope recognized by a mouse IgG, 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 ^35S-radiolabeled α-SNAP protein (prepared by in vitro transcription and translation of the α-SNAP gene; Brunner et al., manuscript in preparation) and NSF/myc immunoprecipitated using the anti-myc IgG mAb, ^35S-α-SNAP was coimmunoprecipitated (Fig. 1 a). α-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 ^35S-α-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°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°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 α-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 α-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°C for 15 min, conditions suf-
sufficient for inactivation of its transport activity (Glick and Rothman, 1987), and which causes disassembly of multimeric NSF (D. W. Wilson, unpublished observations) rendered it unable to compete with NSF/myc for α-SNAP binding (Fig. 1 c). Similarly, treatment of NSF/myc with NEM led to loss of its ability to coimmunoprecipitate 35S-α-SNAP (see below). By carrying out co-immunoprecipitation assays using 35S-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 35S-α-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, α-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, 35S-α-SNAP became incorporated into a particle with a sedimentation coefficient of 20S (Fig. 2 a). The 35S-α-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 35S-α-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,N-bis(3-D-gluconamido)propyl)cholamide (BigCHAP), which forms micelles of only 8 kD. An NSF/myc-dependent peak of 35S-α-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 α-, β-, or γ-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 35S-α-SNAP is low (typically 0.4 nM) the addition of γ-SNAP-stimulated particle formation. γ-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, α-SNAP, and assembly factor form a 20S complex. (A) Detergent extracts of salt-washed RLG membranes were incubated with 35S-α-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.
**ATP Hydrolysis and Complex Disassembly**

Addition of ATP and 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 Mg\(^{2+}\) ions. A solubilized membrane extract, \(^{35}\)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-

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**Figure 3.** \(\gamma\)-SNAP is a subunit of the complex and stimulates \(\alpha\)-SNAP incorporation: \(\alpha\)- and \(\beta\)-SNAPs are alternative subunits. (A) to 5 \(\mu\)g of alkali-washed RLG membranes were added purified \(\alpha\)-SNAP (●), \(\beta\)-SNAP (○), or \(\gamma\)-SNAP (■) and the co-immunoprecipitation assay conducted as usual. A background (RLG membrane independent) signal of 555 counts per minute has been subtracted from the data. (B) Assays as in A except titrations were in the presence of 100 ng \(\gamma\)-SNAP. A background signal of 1,093 counts per minute (determined as in A) was subtracted from the data. (C) NSF/myc and \(\gamma\)-SNAP were co-immunoprecipitated by anti-myc antibody, immunoprecipitates subjected to SDS-PAGE, and immunoblots performed using anti-\(\gamma\)-SNAP antibodies. Lane 1, Blotting control, 100 ng pure \(\gamma\)-SNAP; lane 2, complete co-immunoprecipitation reaction; lane 3, as lane 2 but omitting NSF/myc; lane 4, as lane 2 but including [Mg/ATP] during incubation. Positions corresponding to the migration of 47 and 33 kD prestationed molecular weight markers are indicated. Lanes 2 and 4 contained similar amounts of total protein, visualised by staining the nitrocellulose filter with 0.2% Ponceau-S (wt/vol) in 3% TCA (wt/vol) (not shown).

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**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]; ○) and the other half mixed with 2 mM, Mg\(^{2+}\) ions (final concentration after chelation by EDTA) and then sedimented through a +[Mg-ATP] gradient (●).
ther EDTA or Mg$^{2+}$. In the presence of [Mg·ATP] (but not ATP alone) the complex underwent disassembly (Fig. 4), leaving unbound $^{35}$S-$\alpha$-SNAP towards the top of the gradient. Similarly, when measured by co-immunoprecipitation of $^{35}$S-$\alpha$-SNAP (Fig. 5 a) or $\gamma$-SNAP (Fig. 3 c, compare lanes 2 and 4) addition of Mg$^{2+}$ and ATP reduced complex formation to background levels. The effect of Mg$^{2+}$ is via hydrolysis of [Mg·ATP] because in the presence of the non-hydrolyzable ATP analogue ATP-\gamma-S, complex formation was normal in the presence or absence of 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 in the presence or absence of Mg$^{2+}$. Incubation with ATP and Mg$^{2+}$ ions resulted in release of $^{35}$S-$\alpha$-SNAP from the immunoprecipitated complex (Fig. 5 b), showing that exogenous [Mg·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, 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 M. This relatively low affinity for nucleotide is consistent with the findings that 300 \mu 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 μM (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. β-SNAP was able to compete with α-SNAP for inclusion into the complex, indicating that α- and β-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 α- and β-SNAPs are isoforms of the same protein. We will therefore refer to this type of SNAP as α/β-SNAP, to distinguish this class of molecule from γ-SNAP. Clary et al. (1990) showed that purified α-SNAP protein, unlike β-SNAP, is able to complement the in vitro transport defect of yeast sec17Δ cytosol. Perhaps α-SNAP and Sec17p are the “archetypal” forms of α/β-SNAP which have been highly conserved during evolution, while β is a more recently diverged derivative. Since SNAP proteins are purified from crude bovine brain, β-SNAP may well be specialized to perform the same function as α-SNAP at a different cellular location or in a different brain cell type.

γ-SNAP plays a role distinct from that of α/β-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 γ-SNAP is able to assemble into the complex, yet did not compete with 35S-α-SNAP binding, suggesting γ-SNAP mediates NSF membrane association through binding sites distinct from those recognized by α/β-SNAP. This would explain the increased level of 35S-α-SNAP-complex association in the presence of γ-SNAP; although NSF can apparently become tethered to receptor/assembly factor by either α/β- or γ-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, α/β-SNAP, and γ-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 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 α-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, Sec17p 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 auto-regulation by fusion-coupled dispersal, may be the means by which the cell safely exploits a potentially catastrophic physical process.

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; α/β-SNAP, ovoid with light hatched bars; γ-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.
We thank Gregory Flynn and Lauren Silverman for helpful discussions and Ann Flower for the gift of purified bacterial inner membranes.

D. W. Wilson is an SERC/NATO postdoctoral fellow, S. W. Whiteheart is supported by the Jane Coffin Childs Memorial Fund for Medical Research, M. Brunner is an European Molecular Biology Organization fellow. This research was supported by a National Institutes of Health grant (DK27044) to J. E. Rothman.

Received for publication 4 December 1991 and in revised form 4 February 1992.

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