Biochemical Analysis of the *Saccharomyces cerevisiae SEC18* Gene Product: Implications for the Molecular Mechanism of Membrane Fusion†

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ABSTRACT: The *SEC18* gene product is 48% identical to mammalian NSF (*N*-ethylmaleimide-sensitive fusion protein), and both proteins encode cytoplasmic ATPases which are essential for membrane traffic in yeast and mammalian cells, respectively. A wealth of biochemical analysis has led to the description of a model for the action of NSF; through its interaction with SNAPs (soluble NSF attachment proteins), NSF can associate with SNAP receptors (SNAREs) on intracellular membranes, forming 20S complexes. SNAPs then stimulate the intrinsic ATPase activity of NSF, leading to the disassembly of the 20S complex, which is essential for subsequent membrane fusion. Although this model is based almost entirely on in vitro studies of the original clones of NSF and α-SNAP, it is nevertheless widely assumed that this mechanism of membrane fusion is conserved in all eukaryotic cells. If so, the crucial biochemical properties of NSF and SNAPs should be shared by their yeast homologues, Sec18p and Sec17p. Using purified recombinant proteins, we report here that Sec18p can specifically interact not only with Sec17p but also with its mammalian homologue, α-SNAP. This interaction leads to a stimulation of Sec18p D1 domain ATPase activity, with kinetics similar to those of α-SNAP stimulation of NSF, although differences in temperature and *N*-ethylmaleimide sensitivity were observed between NSF and Sec18p. Furthermore, Sec18p can interact with synaptic SNARE proteins and can synergize with α-SNAP to stimulate regulated exocytosis in mammalian cells. We conclude that the mechanistic properties of NSF and SNAPs are shared by Sec18p and Sec17p, thus demonstrating that the biochemistry of membrane fusion is conserved from yeast to mammals.

The molecular machinery required for vesicular transport is well-conserved between *Saccharomyces cerevisiae* and mammalian cells (*1*). One of the central components is the *N*-ethylmaleimide (NEM)-sensitive fusion protein (NSF[1]), an ATPase originally identified on the basis of its ability to reconstitute transport to NEM-treated Golgi membranes (*2*). Subsequent work has revealed a role for NSF in most, but not all, intracellular membrane fusion events (*3*). NSF is a soluble protein which requires other soluble factors to enable it to interact with membranes (*4*). Three such factors were identified from bovine brain cytosol, termed α-, *β*-, and *γ*-soluble NSF attachment proteins (SNAPs) (*5*). NSF and SNAPs do not interact in solution; SNAPs undergo a conformational change upon binding to membrane-bound SNAP receptors (SNAREs), thus enabling the interaction of NSF and formation of a 20S complex (*6*). The SNAREs were originally identified from detergent extracts of rat brain membranes as VAMP, syntaxin, and SNAP-25 (*7*), synaptic proteins which have been shown to be specific targets of clostridial neurotoxins (*8*). These findings emphasize the vital role of NSF, SNAPs, and SNAREs in intracellular membrane fusion (*3*, *9*).

After cloning and sequencing of the mammalian gene encoding NSF (*10*), it was found to be 48% homologous to the *SEC18* gene in yeast (*11*). Both NSF and Sec18p share the same domain structure: an N-terminal domain followed by two ATP-binding domains termed D1 and D2 (*11*, *12*). α-SNAP is homologous to the *SEC17* gene product in yeast (*13*), mutations in which display synthetic lethality with *sec18* mutants (*14*). Genetic screening experiments revealed that *SEC18* was essential for transport through the yeast secretory pathway (*15*), and temperature-sensitive mutants (*sec18-1*) block secretion and lead to an accumulation of transport vesicles (*14*, *16*). Like NSF, *SEC18* is required for membrane fusion at multiple stages of the biosynthetic pathway (*17*). More recently, studies of homotypic vacuolar fusion in yeast have shown that Sec18p and Sec17p are essential for fusion activity (*18*), and that both proteins are required at an early stage during the fusion reaction which precedes vacuole docking (*19*). NSF and Sec18p may not be directly interchangeable, as NSF is unable to complement the *sec18-1* mutation in yeast (*13*). Nevertheless, functional homology between NSF and Sec18p has been suggested after it was shown that yeast cytosols can reconstitute NSF activity to NEM-treated mammalian in vitro transport assays (*10*, *20*). Caution is needed when interpreting the findings of transport inhibition using the alkylating agent NEM, however, as it is clear that multiple NEM-sensitive components

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[1] Abbreviations: NEM, *N*-ethylmaleimide; NSF, NEM-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; AAA proteins, ATPases associated with a variety of cellular activities; DTT, dithiothreitol.

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contribute to overall transport activity (*21*). Surprisingly, there are few examples of transport steps which have been reconstituted using purified Sec18p rather than crude cytosolic fractions: vacuolar fusion (*19*) and ER to Golgi transport in yeast (*22*).

NSF has a low but measurable intrinsic ATPase activity (*12*). α-SNAP, as well as acting as an anchor for NSF, has been shown to stimulate the ATPase activity of NSF (*23*), by selectively stimulating the activity of the D1 domain (*24*). NSF ATPase activity (*25*, *26*) and its stimulation by α-SNAP (*27*) are vitally important as both are required for 20S complex disassembly. The disassembly of the 20S complex and concomitant rearrangement of SNARE proteins are thought to be required for the fusion of lipid bilayers (*7*). Although the proposed mechanism of NSF action has been debated (*28*), recent studies suggest that SNAPs and NSF act in vesicle priming and not fusion per se (*29*, *30*). Taken together, these data support a model in which SNAP-dependent recruitment and stimulation of NSF disassembles the 20S complex, thus priming the SNAREs for their downstream functions in docking and fusion (*9*, *28*, *31*). Such molecular models of membrane fusion are based on extensive biochemical analyses of recombinant CHO cell NSF and its interactions with recombinant bovine α-SNAP and (predominantly synaptic) SNAREs. In contrast, biochemical evidence describing the ability of yeast SNAREs to form 20S complexes (*32*, *33*) containing Sec17p (*34*) has emerged only recently, and the role of Sec18p in these systems is unknown. If, as is widely assumed, there is a universal mechanism used by all eukaryotes for intracellular transport (*3*), then fundamentally important biochemical functions will be conserved from NSF to Sec18p. This is the hypothesis we have set out to test in this report.

EXPERIMENTAL PROCEDURES

Materials. The yeast strain RSY249 and affinity-purified anti-Sec18p antiserum were gifts from A. Haas (Lehrstuhl fur Mikrobiologie, University of Wurzburg, Wurzburg, Germany). The monoclonal anti-NSF antibody was a gift from M. Tagaya (Department of Biological Sciences, Kyoto University, Kyoto, Japan). The monoclonal anti-VAMP2 antibody was a gift from M. Takahashi (Mitsubishi Kasei Institute of Life Science, Tokyo, Japan). Plasmids encoding His_6-tagged NSF and α-SNAP were gifts from J. E. Rothman (Memorial Sloan Kettering Cancer Center, New York, NY). The plasmid pAX11-*SEC17* was a gift from A. Boyd (Department of Biochemistry, University of Edinburgh, Edinburgh, U.K.). Monoclonal anti-α/*β*-SNAP was obtained from Synaptic Systems (Göttingen, Germany). Unless otherwise specified, all other reagents were of analytical grade and obtained from Sigma (Poole, U.K.).

Cloning and Mutagenesis of SEC18 and SEC17. The wild-type *SEC18* coding sequence was PCR cloned from RSY249, which is the isogenic parent of the *sec18-1* mutant strain. Briefly, genomic DNA was isolated from RSY249 using a QIAamp tissue kit (Qiagen, Dorking, U.K.) and the *SEC18* coding sequence amplified using the following primers: sense, 5′-CAAAGGATCCATGTTCAAGATACCTCATTTTGG-3′; and antisense, 5′-TTTGAAGCTTTTATGCGGATTGGGTCATCAAC-3′. The *Bam*HI and *Hin*dIII restriction sites (underlined) present in the primers were used to directionally clone the 2.3 kb PCR product in-frame into the pQE30 expression vector (Qiagen). Automated DNA sequencing (Oswel) of the 5′ and 3′ ends of the insert gave an exact match to the *Saccharomyces* Genome Database open reading frame YBR080C over the 990 nucleotides that were sequenced.

The second exon of *SEC17* was subcloned from the pAX11-*SEC17* plasmid, which encodes protein A fused to amino acids 12−292 of Sec17p. The first exon is not required for in vivo function, since a construct encoding amino acids 18−292 of Sec17p fully complements the temperature-sensitive block of membrane fusion exhibited by the *sec17-1* mutant (*13*). Briefly, the *SEC17* insert was excised using *Bam*HI and *Hin*dIII endonucleases and directionally cloned in-frame into the pQE30 expression vector (Qiagen). Automated DNA sequencing (Oswel) of the entire insert gave an exact match to the *Saccharomyces* Genome Database open reading frame YBL050W, except for a silent A → G substitution at nucleotide 261 (numbering based on position 1 being A of the start codon of full-length *SEC17*). Point mutations in the *SEC18* sequence for producing the ATP hydrolysis defective mutant (E350Q) were created using the Quickchange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing (Oswel). The mutagenic primers that were used were as follows: sense, 5′-CATATTATTATTTTCGATCAGCTGGATTCTG-3′; and antisense, 5′-CAGAATCCAGCTGATCGAAAATAATAATATG-3′.

Purification of Recombinant Proteins. Recombinant His_6-tagged proteins were purified from the cytosolic fraction of XL-1Blue *Escherichia coli* (Stratagene), using Ni−NTA chromatography (Qiagen), as described previously (*26*). Additional purification was achieved by gel filtration chromatography on a Superdex 200 column (Pharmacia, 16/60; 120 mL bed volume) in buffer A [20 mM Hepes (pH 7.0), 200 mM KCl, 2 mM 2-mercaptoethanol, 0.5 mM ATP, 10% (w/v) glycerol, and 50 mM imidazole] by collecting 2 mL fractions. All chromatography was performed at room temperature using a Pharmacia FPLC system.

Binding Assay. This was based on the protocol developed by Clary et al. (*35*). Twenty microliters of 100 μg/mL α-SNAP or Sec17p was incubated for 20 min in 1.5 mL polypropylene microcentrifuge tubes at room temperature. Buffer containing unbound α-SNAP was removed, and the tubes were incubated with 100 μL of SNAP wash buffer [25 mM Tris-HCl, 50 mM KCl, 1 mM DTT, and 1 mg/mL BSA (pH 7.4)] for 2 min on ice. The SNAP wash buffer was removed; 20 μL of 100 μg/mL NSF or Sec18p in NSF binding buffer [20 mM Hepes, 2 mM EDTA, 100 mM KCl, 0.5 mM ATP, 1 mM DTT, 1% (w/v) PEG_{4000}, and 250 μg/mL soybean trypsin inhibitor (pH 7.4)] was then added to each tube, and the tubes were incubated for 10 min on ice. The supernatant was removed, and all the tubes were washed with 100 μL of NSF binding buffer. Fifty microliters of SDS dissociation buffer was added, and each tube was boiled for 5 min; duplicate 50 μL samples were then pooled. Samples were run on 10% polyacrylamide gels, and proteins were detected by either silver staining or immunoblotting using a monoclonal anti-His tag antibody. The protocol used for immunoblotting was as previously described (*36*).

ATPase Assay. ATPase assays were carried out as previously described (*23*) with the following modifications. Assays

were carried out in flat-bottomed microtiter plates (Fisher). Wells were preincubated with either 50 μL of α-SNAP, 50 μL of Sec17p (250 μg/mL in buffer A containing 250 mM imidazole), or buffer alone for 30 min at room temperature, before removal and incubation with NSF/Sec18p in a final volume of 40 μL in ATPase assay buffer [25 mM Tris (pH 9.0), 100 mM KCl, 10% (v/v) glycerol, 2 mM $MgCl_2$, 1 mM DTT, and 0.6 mM ATP] for 2 h at 37 °C. The amount of hydrolyzed phosphate was determined by a spectrophotometric assay (*37*). Values were corrected for breakdown of ATP in the absence of NSF or Sec18p by running duplicate assays without added proteins.

20S Complex Disassembly Assay. One milligram of Triton X-100-extracted rat brain membrane proteins, prepared as previously described (*38*), was incubated with 15 μg of NSF or Sec18p and 10 μg of α-SNAP or Sec17p in the presence of 0.5 mM ATP or ATP-γ-S in a final volume of 1 mL in buffer C [20 mM Hepes (pH 7.0), 0.7% (v/v) Triton X-100, 100 mM KCl, 1 mM DTT, 1 mM PMSF, 1% (w/v) PEG_{4000}, 1% (w/v) glycerol, and 2 mM $MgCl_2$]. Samples were incubated head over head for 30 min at 4 °C. Forty micrograms of HPC-1 anti-syntaxin antibody was added and incubation continued for 2 h, followed by 30 μL of a 50% slurry of a protein G−sepharose conjugate (Pharmacia), and further incubation for 1 h at 4 °C. Sepharose beads were washed four times in 1 mL of wash buffer [20 mM Hepes (pH 7.0), 0.7% (v/v) Triton X-100, 100 mM KCl, 1 mM DTT, 1 mM PMSF, and 0.5 mM ATP], supplemented with either 2 mM $MgCl_2$ to promote 20S complex disassembly or 2 mM EDTA to prevent 20S complex disassembly. After the final wash, the beads were transferred to a fresh microfuge tube, and bound proteins were solubilized by adding 100 μL of SDS sample buffer. The samples were boiled for 3 min and separated on 10% polyacrylamide gels. The syntaxin immunoprecipitate was visualized by staining with Coomassie blue. In addition, association of Sec18p, NSF, VAMP2, and α-SNAP with the syntaxin immunoprecipitate was monitored by immunoblotting using specific antisera.

Regulated Exocytosis Assay. Chromaffin cells were isolated from bovine adrenal medullae by enzymatic digestion and cultured as described previously (*36*). Cells were plated in 24-well trays at a density of 1 million cells per well, and maintained in culture for 3−7 days before use. To test the stimulatory activity of added proteins, each well was washed twice in PBS, and the cultured cells were permeabilized for 6 min with KGEP [139 mM potassium glutamate, 20 mM Hepes, 5 mM EGTA, 2 mM ATP, and 2 mM $MgCl_2$ (pH 6.5)] containing 20 μM digitonin. This buffer was then removed, and the cells were incubated for 20 min with KGEP in the presence or absence of 8 μg/mL α-SNAP, 7 μg/mL Sec18p, or both. Upon removal of this buffer, cells were stimulated for 30 min in KGEP or KGEP containing 10 μM free Ca^{2+} in the presence or absence of 8 μg/mL α-SNAP, 7 μg/mL Sec18p, or both. The amount of catecholamine released by regulated exocytosis was measured using a standard fluorometric method. The total catecholamine content of the cells was determined after lysis with 1% Triton X-100, and the amount of catecholamine secretion was calculated as a percentage of the total cellular catecholamine. All experiments were performed at room temperature.

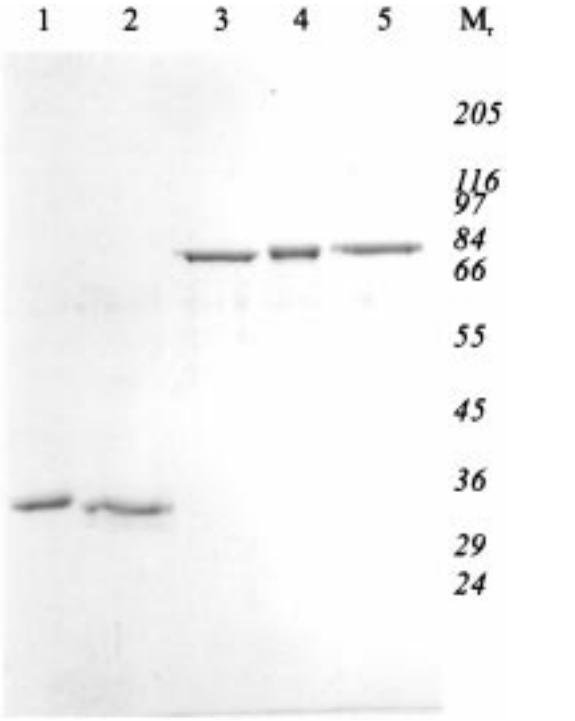

FIGURE 1: Purified recombinant proteins used in this study. Recombinant α-SNAP (lane 1), Sec17p (lane 2), NSF (lane 3), Sec18p (lane 4), and Sec18p E350Q (lane 5) were analyzed by SDS−PAGE on 10% polyacrylamide gels followed by Coomassie blue staining. Lanes 1 and 2 contained Ni−NTA-purified material, and lanes 3−5 contained hexameric proteins isolated after Superdex 200 sizing chromatography.

RESULTS

In this study, we have used bacterially expressed recombinant proteins bearing N-terminal six-histidine tags to enable purification using Ni−NTA affinity chromatography. This epitope tag has no effect on function as His_6-tagged NSF, Sec18p, α-SNAP, and Sec17p have all been shown previously to be functional in in vitro membrane fusion assays (*18*, *26*, *39*). The Sec17 protein used here is an N-terminally truncated form missing the first 11 amino acids of the protein; however, this form is fully functional since this construct can complement the *sec17-1* mutation in yeast (*13*). All the bacterially expressed proteins are greater than 90% pure after a single step (Figure 1); however, we routinely include a further purification step using Superdex 200 sizing chromatography for NSF and Sec18p to obtain quaternary structure data for these two proteins. Recent structural analysis has shown that NSF is a hexamer with the D2 domain being essential for oligomerization (*26*, *40*, *41*). NSF elutes from the Superdex 200 column as a single species with a molecular mass of 550 kDa, as estimated from molecular mass standards (*24*) (Figure 2), which fits well with the predicted hexameric structure. Sec18p, in contrast, eluted from the same column as two distinct populations, a hexameric pool with a molecular mass of 640 kDa and a second pool with an approximate molecular mass of 140 kDa, estimated to represent a dimeric form (Figure 2). The D1 ATP hydrolysis mutant, Sec18p E350Q, elutes with a pattern similar to that of wild-type Sec18p, with a hexameric pool and a second pool of material eluting at approximately 200 kDa (Figure 2). In this study, hexameric Sec18p, NSF, and Sec18p E350Q were used unless otherwise stated.

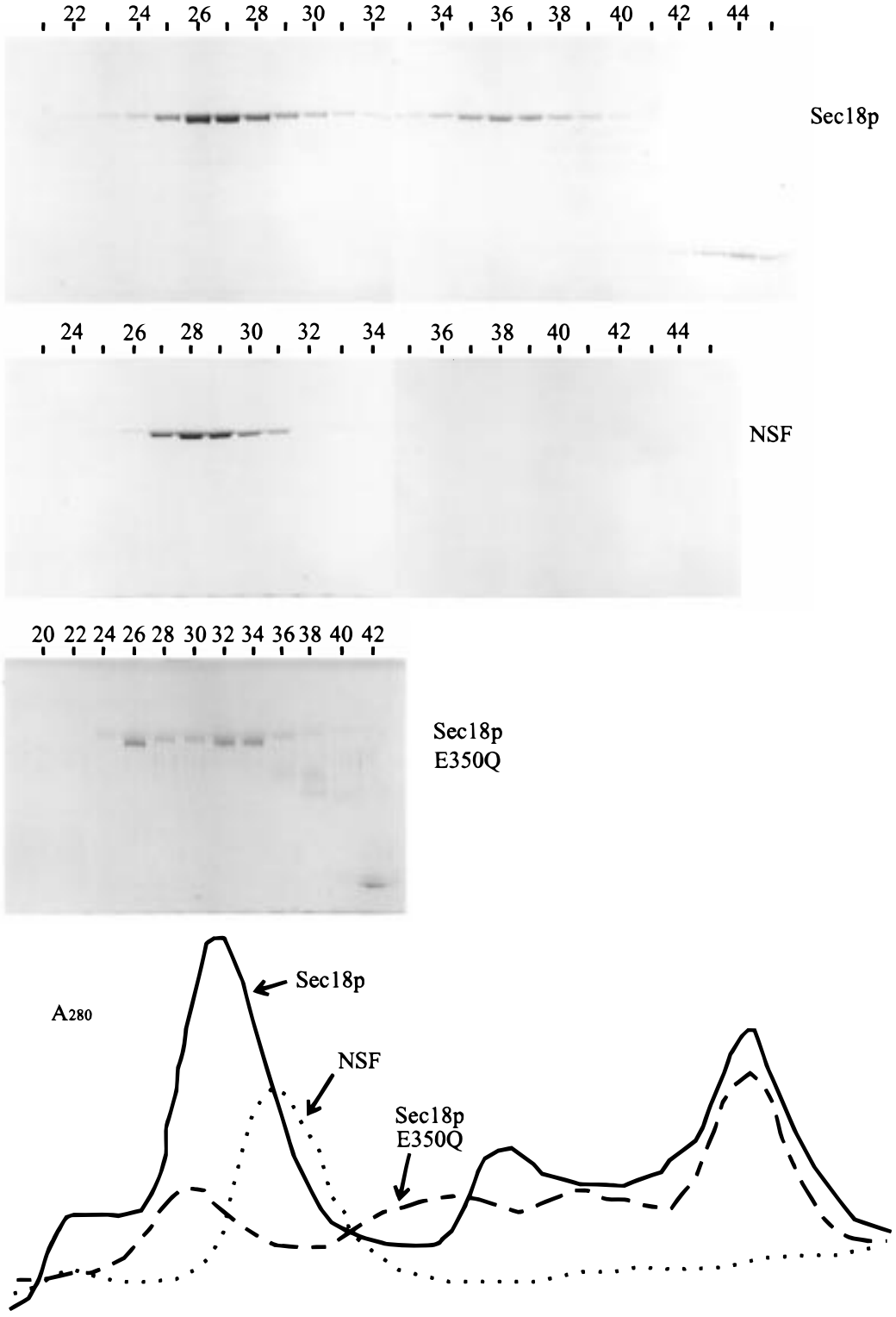

FIGURE 2: Chromatographic analysis of recombinant proteins. Ni−NTA-purified proteins (1−3 mg) were loaded onto the Superdex 200 column at a rate of 1 mL/min which had been pre-equilibriated in buffer A. Fractions (2 mL) were collected and analyzed by running samples on 10% polyacrylamide gels, and proteins were detected by Coomassie blue staining. Elution of Sec18p (solid line), NSF (dotted line), and Sec18p E350Q (dashed line) from the column was monitored using A_{280} absorbance measurements.

The interaction of NSF with membranes requires the presence of SNAP proteins bound to membrane SNAREs (*4*). Previous studies have found that SNAP proteins immobilized onto the surface of polypropylene tubes can recruit NSF (*35*, *42*). This binding of SNAP to plastic is postulated to induce a conformational change in the SNAP, which mimics binding to SNAREs, thus allowing NSF to bind. The direct interaction between proteins was demonstrated by immobilizing either α-SNAP or Sec17p to polypropylene tubes and briefly allowing NSF or Sec18p to bind. Although some NSF and Sec18p was able to bind in the absence of attachment proteins, it is clear that both NSF and Sec18p specifically bind to their respective binding partners (Figure 3). In addition, there is functional conservation between the

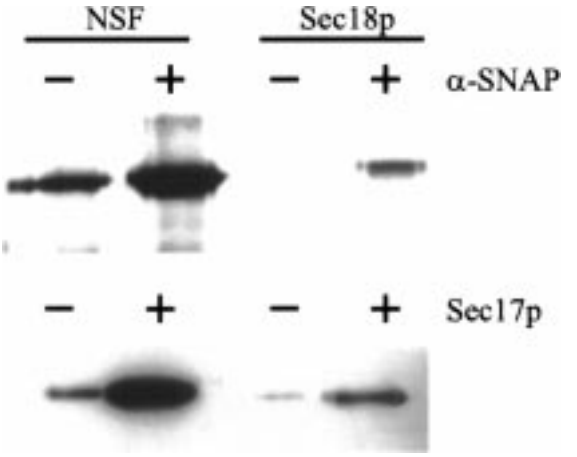

FIGURE 3: Binding of NSF/Sec18p to plastic-immobilized α-SNAP or Sec17p. α-SNAP or Sec17p (100 μg/mL in SNAP wash buffer without BSA) was immobilized onto polypropylene tubes for 20 min. After washing with SNAP wash buffer containing 1 mg/mL BSA, NSF or Sec18p (100 μg/mL in NSF binding buffer) was added for 10 min. Bound proteins remaining after a wash in NSF binding buffer were solubilized in SDS dissociation buffer and analyzed by SDS−PAGE on 10% polyacrylamide gels. α-SNAP-bound proteins were detected by silver staining, Sec17p-bound proteins were detected by immunoblotting using an anti-His tag antibody.

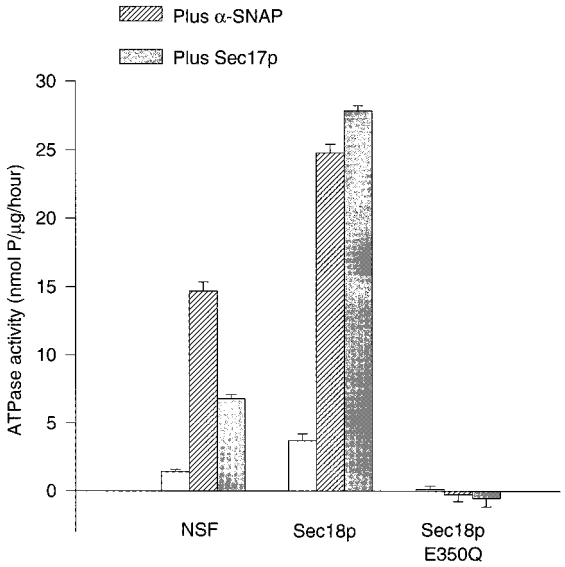

FIGURE 4: ATPase activity of wild-type NSF, Sec18p, and Sec18p E350Q. Standard ATPase reactions were carried out at 37 °C using NSF at a final concentration of 2.5 μg/mL and Sec18p and Sec18p E350Q at a final concentration of 1 μg/mL in the assay. The proteins were incubated following preincubation with buffer A (white bars), 400 μg/mL α-SNAP (hatched bars), or 400 μg/mL Sec17p (black bars). The data shown represent means ± standard deviations (n = 4) from a representative experiment.

yeast and mammalian proteins, with NSF being able to bind to Sec17p and Sec18p being able to bind to α-SNAP (Figure 3).

As well as being able to interact with the attachment proteins, we further investigated the effect this has on the ATPase activity of the proteins as it is known that α-SNAP can stimulate the basal ATPase activity of NSF (*23*). From 13 experiments, the basal activity of NSF was 1.86 ± 0.13 nmol of P μg^{-1} h^{-1}, and Sec18p had a higher basal activity of 4.13 ± 0.54 nmol of P μg^{-1} h^{-1}. The ATPase activity of Sec18p displayed approximately linear time and dose dependence in assays of up to 4 h using up to 200 μg/mL Sec18p. Unlike NSF, which exhibits a high pH optimum (*12*), Sec18p ATPase activity was found to be similar at pHs ranging from 6 to 9 (data not shown). To facilitate heterologous studies, ATPase assays were carried out using the pH 9 NSF buffer devised by Tagaya et al. (*12*), but results were confirmed using a more physiological pH 7.3 buffer (data not shown). To maintain SNAP- and Sec17p-mediated stimulation activities within the linear range of the assay, the basal activity of NSF and Sec18p must be reduced to close to background levels, resulting in significant variability between assays. However, over a series of experiments, the ATPase activity of NSF was stimulated by α-SNAP [317 ± 54.9% (n = 6)] and to a lesser extent by Sec17p [184 ± 14.0% (n = 4)], whereas Sec18p was stimulated similarly by both α-SNAP and Sec17p [303 ± 45.1% (n = 8) and 315 ± 41.2% (n = 13), respectively; see also Figure 4]. From four independent preparations, hexameric Sec18p generally displayed higher basal activity than the putative dimeric Sec18p, which exhibited significant variability in ATPase activity (data not shown).

A mutation in *SEC18* (E350Q) was constructed which, by analogy with the corresponding mutation in NSF (*25*, *26*), allows ATP to bind to the D1 domain, but prevents hydrolysis. This construct had no measurable ATPase activity in the presence or absence of attachment proteins (Figure 4), suggesting that, like for NSF (*24*), wild-type Sec18p is stimulated by α-SNAP and Sec17p by activating the D1 domain. It has been shown that NSF displays classical Michaelis−Menten kinetics with an increase in V_{max} upon SNAP stimulation (*12*, *24*). Analysis of Sec18p over a wide range of ATP concentrations revealed that there is an increase in the V_{max} upon Sec17p stimulation (4.95 nmol of P μg^{-1} h^{-1} in the absence and 12.82 nmol of P μg^{-1} h^{-1} in the presence of Sec17p), with little change in the K_m (0.86 mM ATP in the absence and 0.56 mM ATP in the presence of Sec17p). These values are similar to those found previously for NSF by Tagaya et al. (V_{max} = 4.0 nmol of P μg^{-1} h^{-1} and K_m = 0.65 mM) (*12*) and by this laboratory (V_{max} = 2.77 nmol of P μg^{-1} h^{-1} in the absence and 12.33 nmol of P μg^{-1} h^{-1} in the presence of α-SNAP; K_m = 0.16 mM ATP in the absence and 0.27 mM ATP in the presence of α-SNAP), implying that the mechanism of stimulation of these two enzymes is highly conserved.

Although the ATPase activities of NSF and Sec18p are similar, there are two distinct differences. First, the temperature of incubation profoundly affects the stimulation of Sec18p. Although having lower activities on ice, as expected, both NSF and Sec18p had detectable levels of ATPase activity (Figure 5; basal activity has been normalized to 100%). When ATPase activity was measured at 4 or 37 °C, in the presence of prebound α-SNAP, the extent of stimulation for NSF was similar at both temperatures (266 ± 22% at 4 °C and 227 ± 8% at 37 °C). In contrast, the ability of Sec18p to be stimulated by prebound α-SNAP was severely reduced at the lower temperature (129 ± 3% at 4 °C and 236 ± 4% at 37 °C). Second, NSF is sensitive to inhibition by the alkylating agent NEM; incubation of NSF on ice with low concentrations of NEM completely inhibited α-SNAP-mediated stimulation and significantly reduced the basal ATPase activity (Figure 6). In contrast, treatment of Sec18p with concentrations of NEM of up to 10 mM had no effect

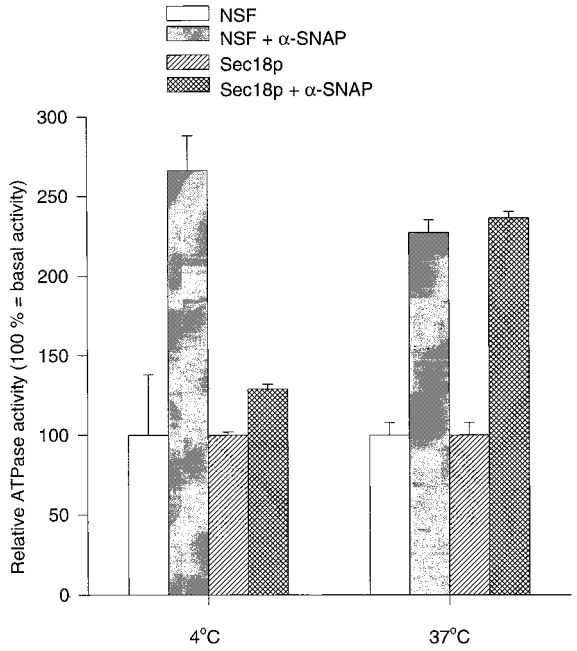

FIGURE 5: Effect of temperature on the ATPase activation of NSF/Sec18p. Standard ATPase reactions (40 μL) were carried out using NSF at a final concentration of 2.5 μg/mL and Sec18p at a final concentration of 1 μg/mL at either 4 or 37 °C. NSF was added following preincubation with buffer A (white bars) or 400 μg/mL α-SNAP (black bars). Sec18p was added following preincubation with buffer A (hatched bars) or 400 μg/mL α-SNAP (cross-hatched bars). Basal activities for NSF at 4 and 37 °C were 0.520 and 1.703 nmol of P μg^{-1} h^{-1}, respectively. Basal activities for Sec18p at 4 and 37 °C were 0.887 and 3.920 nmol of P μg^{-1} h^{-1}, respectively. For each temperature, the basal activity of each protein is expressed as 100%. The data shown represent means ± standard deviations ($n = 4$) from a representative experiment.

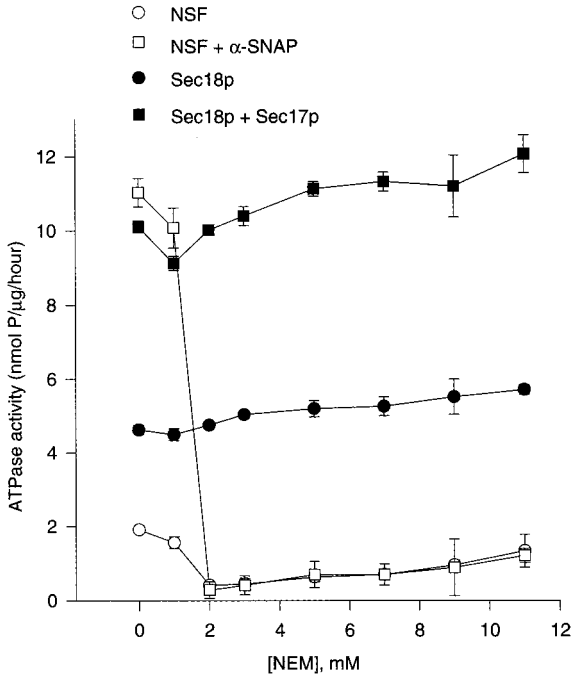

FIGURE 6: Sensitivity of the ATPase activity of NSF or Sec18p to *N*-ethylmaleimide (NEM). Mixtures containing NSF or Sec18p were incubated with various concentrations of NEM for 1 h on ice before quenching with DTT. Aliquots were used for the standard ATPase reaction using NSF (white symbols) at a final concentration of 2.5 μg/mL and Sec18p (black symbols) at a final concentration of 1 μg/mL in the assay. The proteins were incubated following preincubation with buffer A (○ and ●), 400 μg/mL α-SNAP (□), or 400 μg/mL Sec17p (■).

on the basal or Sec17p-stimulated ATPase activity (Figure 6).

The ability of Sec18p to incorporate into the mammalian 20S complex was examined. In the presence of detergent-solubilized rat brain membranes, under conditions where the hydrolysis of ATP is prevented (ATP-γ-S), NSF and α-SNAP can assemble into a 20S particle (*38*). Disassembly of this complex occurs under conditions where ATP hydrolysis proceeds (MgATP). Using a syntaxin immunoprecipitation technique (*43*), it was found that NSF could assemble into a complex containing syntaxin, VAMP2, and recombinant α-SNAP and that this complex was disassembled in the presence of MgATP (Figure 7, compare lanes 1 and 2). Likewise, Sec18p was able to assemble into the 20S complex, with α-SNAP as the cofactor, but was unable to disassemble the complex in the presence of MgATP (Figure 7, compare lanes 5 and 6). In contrast to α-SNAP, Sec17p was found to be inefficient in the recruitment of both NSF and Sec18p into the 20S complex (Figure 7, compare lanes 3 and 7).

To further examine the interaction of Sec18p with synaptic SNARE proteins, we decided to investigate whether Sec18p could stimulate Ca^{2+}-dependent exocytosis in permeabilized adrenal chromaffin cells. Exocytosis in this cell type is mediated by the same protein machinery (including SNAREs) that is utilized for synaptic vesicle exocytosis (*44*), and previous reports have shown that α-SNAP can stimulate exocytosis in this system (*36*). As Sec18p can interact with α-SNAP (Figure 3) and this interaction leads to a significant stimulation of its ATPase activity (Figure 4), which has been suggested to be essential for exocytosis (*27*), Sec18p was assayed either on its own or in combination with α-SNAP for its effects on regulated exocytosis. As shown in Figure 8, Sec18p alone caused an increase in the extent of Ca^{2+}-dependent exocytosis, but when in combination with α-SNAP exhibited a more than additive stimulation. This stimulatory effect on regulated exocytosis was observed using three separate batches of Sec18p on three different chromaffin cell preparations. In contrast, Sec17p gave no reproducible enhancement of regulated exocytosis either alone or in combination with Sec18p (data not shown).

DISCUSSION

NSF and Sec18p are members of a heterogeneous group of proteins known as ATPases associated with a variety of cellular activities (AAA) proteins. Members of this family have widely divergent functions and are characterized by having one or two copies of a conserved ~230-amino acid ATPase domain (*45*, *46*). AAA proteins show a tendency to oligomerize, and this is supported in this study with both NSF and Sec18p migrating as hexameric proteins. Unlike NSF, which migrates predominantly as a hexameric species, Sec18p has a heterogeneous distribution between hexamer and dimer, suggesting that Sec18p is more unstable than NSF or alternatively that it exists in two conformations. Such an equilibrium between oligomeric states has recently been observed in the AAA family member Vps4p, which also functions in yeast membrane traffic. Recombinant Vps4p can exist in dimeric and decameric states, but the active conformation involved in Vps complex disassembly is

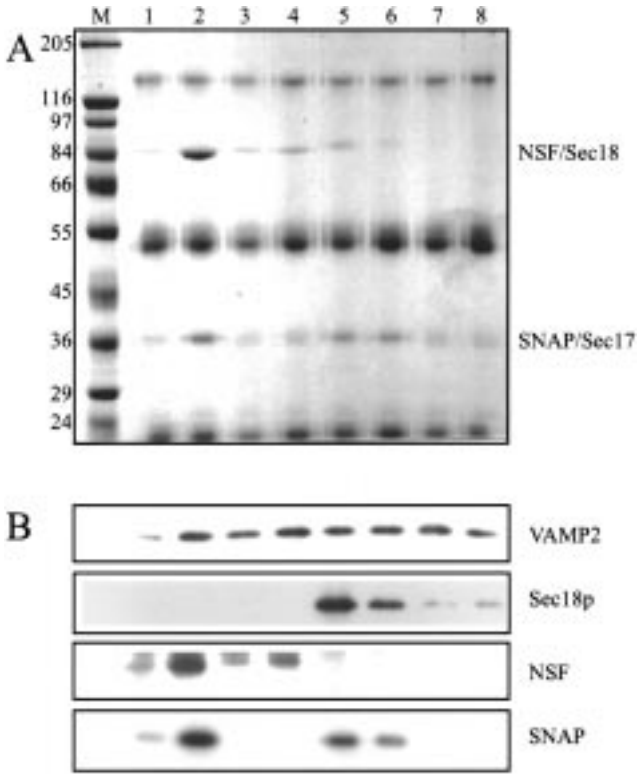

FIGURE 7: Sec18p is able to associate with mammalian SNAREs to form a 20S complex. One milligram of a detergent extract of rat brain membrane proteins was incubated with either 10 μg of NSF (lanes 1−4) or Sec18p (lanes 5−8) and 10 μg of α-SNAP (lanes 1, 2, 5, and 6) or Sec17p (lanes 3, 4, 7, and 8) for 30 min with 0.5 mM MgATP (lanes 1, 3, 5, and 7) or MgATP-γ-S (lanes 2, 4, 6, and 8) at 4 °C. Proteins were immunoprecipitated with an anti-syntaxin antibody (HPC-1) and protein G−sepharose, and bound proteins were analyzed by SDS−PAGE on 10% polyacrylamide gels. The syntaxin immunoprecipitate was detected by Coomassie blue staining (A). Sec18p, NSF, SNAP, and VAMP2 were detected by immunoblot analysis using specific antibodies (B).

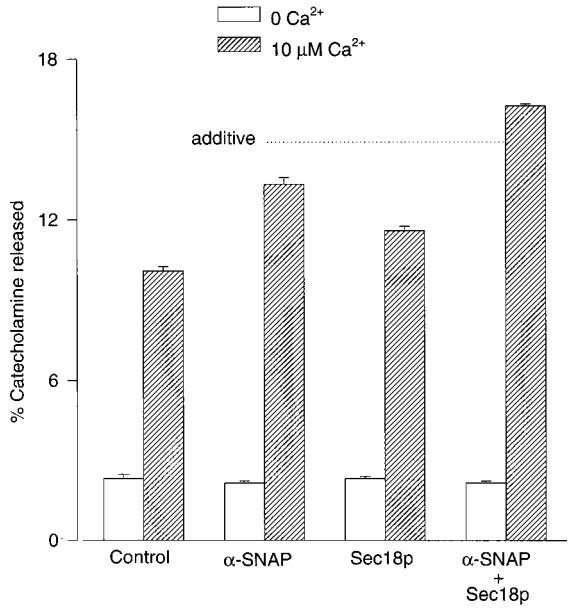

FIGURE 8: Sec18p stimulates Ca^{2+}-dependent exocytosis in chromaffin cells. Cells were permeabilized for 6 min with KGEP buffer containing 20 μM digitonin before being incubated for 20 min in KGEP buffer in the presence or absence of 8 μg/mL α-SNAP, 7 μg/mL Sec18p, or both. After this period, the buffer was removed and replaced with either KGEP buffer with no added Ca^{2+} (0 Ca^{2+}, white bars) or containing 10 μM free Ca^{2+} (hatched bars) in the presence or absence of 8 μg/mL α-SNAP, 7 μg/mL Sec18p, or both. After 30 min, released catecholamine was assayed and expressed as a percentage of total cellular catecholamine content. Data shown are means ± standard error of the mean ($n = 6$).

thought to be the decamer, which exhibits much higher ATPase activity than the dimer (*47*). Similarly, we observed that the hexameric form of Sec18p generally had higher basal ATPase activity than the dimeric form, suggesting that the hexameric form is the active conformation. In support of this idea, incubation of low-molecular mass in vitro-translated Sec18p with Golgi membranes leads to oligomerization of the protein and a significant increase in its transport activity (*13*). Some factor on the Golgi membrane or the lipid environment may promote oligomerization or stabilize the hexameric form, which may be important for the activation of Sec18p in allowing cycles of membrane fusion.

The interaction of NSF and α-SNAP has been well-documented both on plastic and as part of the 20S complex. It has also been shown that Sec17p, when bound to plastic or as part of a complex, will bind to Sec18p (*13*). However, some confusion remains as to whether the yeast and mammalian proteins can interact with each other. In an NSF binding assay, yeast cytosol was unable to bind NSF to mammalian Golgi membranes (*35*), and this finding was originally interpreted to mean that Sec17p could not interact with NSF. However, we have shown here that Sec17p, when immobilized on plastic, not only can bind to NSF but also can stimulate the ATPase activity of NSF. This is consistent with the observation that purified Sec17p can replace SNAP activity in mammalian intra-Golgi transport assays (*13*).

NSF has two ATP binding sites, D1 and D2 (*12*, *26*), and it has been shown that activity associated with the D1 domain of NSF is absolutely required for in vitro transport activity (*26*). The ATPase activity of NSF is stimulated by α-SNAP (*23*) by selective stimulation of the D1 domain (*24*). Furthermore, α-SNAP-mediated stimulation of NSF ATPase activity has been shown to be essential for both 20S complex disassembly and membrane fusion (*27*). If this ATPase activation is a universal requirement for SNARE priming, then the molecular basis of this process will be conserved in Sec18p. Indeed, we have shown that the ATPase activity of Sec18p is stimulated equally well by either α-SNAP or Sec17p, and NSF is stimulated by α-SNAP or Sec17p. The lower level of stimulation of NSF by Sec17p represents a lower efficacy, and not affinity of Sec17p, as the level of stimulation seen with α-SNAP is not reached when a large excess of Sec17p is used (data not shown). These findings demonstrate that the molecular interactions required for ATPase activation are conserved between NSF and its yeast homologue, indicating that ATPase activation is a ubiquitous requirement for SNARE priming.

To confirm that the stimulation of ATPase activity was due to the D1 domain of Sec18p, a mutant protein defective in D1 ATP hydrolysis was constructed (Sec18p E350Q) and shown to have no basal or stimulated ATPase activity even at high doses (up to 50 μg/mL; data not shown). These data strongly indicate that the D2 domain of Sec18p has negligible ATPase activity, similar to NSF (*24*, *26*). Sec18p E350Q exhibits chromatographic properties similar to those of wild-type Sec18p, and hexameric Sec18p E350Q was used in this study, indicating that the lack of ATPase activity is not simply due to the inability to form active multimers. While

this work was in progress, it was reported that Sec17p-mediated stimulation of Sec18p ATPase activity can be enhanced by incubation with LMA1, a heterodimer of thioredoxin and protease B inhibitor 2 (*48*). One speculative interpretation of this finding is that LMA1 promotes the hexameric conformation of Sec18p, which as discussed above may represent the active conformation of the protein.

The classic work of Rothman and co-workers established that transport between Golgi cisternae could be reproduced in an in vitro assay (*49*) which could be blocked by preincubation with 1 mM NEM (*50*). Indeed, NSF was purified on the basis of its ability to restore transport activity to NEM-inactivated reactions (*2*). NSF's ATPase activity is severely reduced, but more importantly, the α-SNAP-mediated stimulation is abolished by treatment with 1 mM NEM on ice (*23*), conditions which block intra-Golgi transport (*10*), endocytosis (*21*), and exocytosis (*36*). In contrast, we have shown that both the basal and Sec17p-stimulated ATPase activities of Sec18p are unaffected by NEM at concentrations of up to 10 mM. NSF activity provided by yeast cytosol requires a concentration of 5 mM to abolish its supportive role in intra-Golgi transport (*10*), and treatment with similar levels of NEM abolishes the fusion-promoting activity of purified Sec18p in vacuolar fusion reactions (*18*). These findings indicate that some other function distinct from ATPase activation is the target of NEM inactivation of Sec18p. The principle site of NEM inhibition of NSF has been suggested to be cysteine 264 in the ATP-binding pocket of the D1 domain (*12*). The corresponding amino acid in the Sec18p polypeptide is threonine 285, which may explain the lack of NEM sensitivity observed in this study.

NSF has been shown to disassemble the 20S complex formed between detergent-solubilized extracts of rat brain membranes, NSF, and SNAPs (*38*). This complex has been faithfully reproduced using recombinant versions of the synaptic SNARE proteins, syntaxin, SNAP-25, and VAMP (*51*). In this study, we have shown that Sec18p not only can bind to plastic-immobilized α-SNAP but also can interact with a complex of mammalian SNARE proteins derived from detergent-solubilized rat brain membranes and recombinant α-SNAP. This finding indicates that the structural elements of NSF required for interacting with the synaptic fusion machinery are conserved in Sec18p. From a functional point of view, it may be that there are indeed differences, as the ATP-dependent disassembly of the synaptic SNARE complex by Sec18p could not be demonstrated; however, this may be due to other influencing factors. First, the loss of SNAP-mediated ATPase stimulation of Sec18p at 4 °C reported here would potentially protect SNARE disassembly, and Sec17p is not released from vacuolar membranes which have been incubated on ice, indicating that the Sec18p action cannot occur at low temperatures (*19*), whereas the mammalian 20S complex is readily dissociated at 4 °C (*38*, *43*, *51*−*53*). Second, other factors such as LMA1 or its putative mammalian counterpart may be required for Sec18p to be able to disassemble the mammalian SNARE complex, since it has recently been stated that LMA1 is essential for disassembly of a vacuolar SNARE complex in vitro (*48*). Third, unlike ATP-dependent NSF and α-SNAP release from SNARE proteins (*38*), ATP hydrolysis by Sec18p on vacuolar membranes leads to release of Sec17p, whereas the concentration of Sec18p on vacuolar membranes appears to be undiminished (*19*). Furthermore, it has been shown that Sec18p binds equally well to vacuoles which do not contain SNARE proteins (*54*). These observations suggest that even after successful ATP hydrolysis and activation of the SNARE proteins, Sec18p remains bound, findings that are consistent with the data presented here.

It is becoming accepted that α-SNAP and NSF function together to disassemble the SNARE complex in the stages which precede membrane fusion (*44*). This can be reconstituted using whole cells by examining Ca^{2+}-dependent exocytosis in digitonin-permeabilized adrenal chromaffin cells. α-SNAP stimulates regulated exocytosis by acting at an early ATP-dependent priming step (*29*). Recombinant NSF was unable to stimulate exocytosis in this system, and it was suggested that leakage-insensitive endogenous NSF may be sufficient to provide the necessary NSF enzymatic activity (*36*). During the course of these experiments, we observed that NSF, but not Sec18p, precipitated in the buffers used for our secretion studies, suggesting that this may be one explanation for the lack of an effect of recombinant NSF seen previously (*36*). Although Sec18p is unable to disassemble the synaptic SNARE proteins in the context of detergent-solubilized rat brain membranes, Sec18p was found to stimulate Ca^{2+}-dependent exocytosis in these cells. One simple interpretation of the data is that the stimulatory action of Sec18p in regulated exocytosis is independent of SNARE complex disassembly. However, in view of the caveats discussed above, it may be that Sec18p does indeed promote disassembly of the synaptic SNAREs in the permeabilized cell environment. In the absence of exogenously added α-SNAP, Sec18p presumably stimulates exocytosis by interacting with endogenous α-SNAP which remains after permeabilization [it has been shown (*36*) that even prolonged permeabilization results in the leakage of no more than 50% of the total pool of α-SNAP in chromaffin cells]. This notion is consistent with the observations described here that α-SNAP not only can bind Sec18p but also can stimulate its ATPase activity to the same level as its natural attachment protein, Sec17p. The more than additive effect shown when Sec18p and α-SNAP are added together strengthens the interpretation that Sec18p is acting by a SNARE-dependent mechanism, since the stimulatory action of α-SNAP in this system is inhibited by botulinum neurotoxin (*36*).

Early work using powerful yeast genetic studies and reconstitution of mammalian membrane traffic in vitro led to the suggestion that there is a universal mechanism responsible for intracellular membrane fusion in all eukaryotic cells (*3*). Further studies examined in detail the interactions of proteins involved at the biochemical level, thus allowing the construction of molecular models for describing the actions of NSF, SNAPs, and SNAREs. More recently, biochemical evidence has emerged indicating that yeast SNARE proteins can form complexes which resemble synaptic 20S complexes (*32*, *33*), with stoichiometric binding of Sec17p (*34*), although the role of Sec18p was not determined. In this study, we have demonstrated that NSF and Sec18p are biochemically similar, but display clear differences with respect to temperature and NEM sensitivity. Nevertheless, the basic elements of function (interaction between proteins, stimulation of enzymic activity, and downstream events involved in the priming of membrane

fusion) are all conserved. This conservation of function is exemplified by the demonstration here for the first time that a yeast protein can participate in that most highly adapted and precisely controlled membrane fusion event, regulated exocytosis. These findings support the idea that the molecular events which allow fusion of lipid bilayers are biochemically conserved between yeast and the human brain.

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