Stimulation of NSF ATPase Activity by α-SNAP Is Required for SNARE Complex Disassembly and Exocytosis

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Abstract. *N*-ethylmaleimide–sensitive fusion protein (NSF) and α-SNAP play key roles in vesicular traffic through the secretory pathway. In this study, NH_2- and COOH-terminal truncation mutants of α-SNAP were assayed for ability to bind NSF and stimulate its ATPase activity. Deletion of up to 160 NH_2-terminal amino acids had little effect on the ability of α-SNAP to stimulate the ATPase activity of NSF. However, deletion of as few as 10 COOH-terminal amino acids resulted in a marked decrease. Both NH_2-terminal (1–160) and COOH-terminal (160–295) fragments of α-SNAP were able to bind to NSF, suggesting that α-SNAP contains distinct NH_2- and COOH-terminal binding sites for NSF. Sequence alignment of known SNAPs revealed only leucine 294 to be conserved in the final 10 amino acids of α-SNAP. Mutation of leucine 294 to alanine (α-SNAP(L294A)) resulted in a decrease in the ability to stimulate NSF ATPase activity but had no effect on the ability of this mutant to bind NSF. α-SNAP (1–285) and α-SNAP (L294A) were unable to stimulate Ca^{2+}-dependent exocytosis in permeabilized chromaffin cells. In addition, α-SNAP (1–285), and α-SNAP (L294A) were able to inhibit the stimulation of exocytosis by exogenous α-SNAP. α-SNAP, α-SNAP (1–285), and α-SNAP (L294A) were all able to become incorporated into a 20S complex and recruit NSF. In the presence of MgATP, α-SNAP (1–285) and α-SNAP (L294A) were unable to fully disassemble the 20S complex and did not allow vesicle-associated membrane protein dissociation to any greater level than seen in control incubations. These findings imply that α-SNAP stimulation of NSF ATPase activity may be required for 20S complex disassembly and for the α-SNAP stimulation of exocytosis.

In recent years, much interest has centered on the proteins and mechanisms required for vesicular trafficking within cells (Rothman, 1994). *N*-ethylmaleimide (NEM)[1] treatment of Chinese hamster ovary Golgi membranes results in an inhibition of membrane transport (Glick and Rothman, 1987). This led to the purification of NEM-sensitive fusion protein (NSF), which is able to reconstitute transport (Block et al., 1988). Subsequently it was found that NSF is required for many transport steps, such as from endoplasmic reticulum to *cis* Golgi (Beckers et al., 1989) and endosome–endosome fusion (Diaz et al., 1989; Rodriguez et al., 1994). NSF is an ATPase homologous to the yeast SEC18 gene product Sec18p, which is required in vivo for yeast secretion (Pryer et al., 1992). The widespread involvement of NSF in membrane trafficking suggests that it is a general, if not universal, component in the steps leading to vesicular fusion (Wilson et al., 1989).

NSF is able to associate with Golgi membranes in an ATP-dependent fashion. This association is dependent on three peripheral membrane proteins termed soluble NSF attachment proteins (α-, β-, and γ-SNAP), (Clary and Rothman, 1990; Whiteheart et al., 1993). When incubated with detergent-extracted brain membranes, in the absence of hydrolyzable ATP, α-SNAP and NSF are associated in a 20S complex with three membrane proteins: syntaxin, SNAP-25 (synaptosomal associated protein of 25 kD) and vesicle-associated membrane protein (VAMP), collectively termed SNAP receptors (SNAREs; Söllner et al., 1993*b*). Cleavage of syntaxin, SNAP-25, or VAMP by the clostridial neurotoxins results in an inhibition of calcium-regulated exocytosis in neuronal and neuroendocrine cells (Montecucco and Schiavo, 1994; Niemann et al., 1994), suggesting that these neurotoxin substrates, and by implication, NSF and α-SNAP, play a key role in regulated secretion. Assembly of the SNARE complex was postulated to allow the correct docking of vesicles to their target membrane (Söllner et al., 1993*a*). Hydrolysis of ATP, by

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1. *Abbreviations used in this paper*: NBB, NSF binding buffer; NEM, *N*-ethylmaleimide; NSF, NEM-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; SWB, SNAP wash buffer; VAMP, vesicle-associated membrane protein.

NSF, causes the 20S complex to disassemble, which was proposed to trigger fusion of vesicle and target membranes by an as yet unknown mechanism (Söllner et al., 1993*b*). More recently it has been suggested that NSF and α-SNAP may act before vesicle docking (Morgan and Burgoyne, 1995*b*; Mayer et al., 1996).

In vitro binding experiments have identified syntaxin as the major SNARE (Hanson et al., 1995; Hayashi et al., 1995; Kee et al., 1995). NSF can be recruited to α-SNAP bound to syntaxin alone. Upon ATP hydrolysis, this complex disassembles, and syntaxin is modified in a manner that prevents complex reassembly (Hanson et al., 1995). These actions of α-SNAP/NSF are consistent with those of cochaperone/chaperones, which in this case could modify the conformations of the SNARE proteins in a manner that would allow the progress of vesicle docking/fusion (Morgan and Burgoyne, 1995*b*). When α-SNAP is immobilized, it is able to bind NSF and stimulate its ATPase activity (Morgan et al., 1994), yet α-SNAP is unable to bind or stimulate NSF in solution. This suggests that stimulation of NSF ATPase activity might only occur when α-SNAP is correctly bound to the SNARE complex, thus acting as a molecular switch for NSF activity.

Direct evidence that NSF is required for exocytosis comes from the synaptic transmission defect in the *Drosophila comatose* mutant, which was recently found to be due to a temperature-sensitive mutation in *Drosophila* NSF-1 (Pallanck et al., 1995). α-SNAP is able to stimulate exocytosis when microinjected into the squid giant synapse (DeBello et al., 1995). In addition, it stimulates exocytosis when added to permeabilized chromaffin cells (Morgan and Burgoyne, 1995*a*), where it acts on an ATP-dependent priming step, which precedes calcium-triggered exocytosis (Chamberlain et al., 1995), or when added via a patch pipette (Kibble et al., 1996). α-SNAP is likely to act via the SNARE proteins in adrenal chromaffin cells that have been shown to be present in these cells (Hodel et al., 1994; Roth and Burgoyne, 1994) since its stimulatory effect is sensitive to clostridial neurotoxins (Morgan and Burgoyne, 1995*a*). α-SNAP presumably acts in concert with NSF in regulated secretion in chromaffin cells, although there is no direct evidence for this supposition.

To elucidate the protein–protein interactions of α-SNAP with syntaxin or NSF, an initial series of α-SNAP truncation mutants has been generated (Hayashi et al., 1995; Barnard et al., 1996). Removal of 40–45 amino acids from the extreme NH_2 or COOH termini of α-SNAP abolished its ability to bind syntaxin (Hayashi et al., 1995; Barnard et al., 1996) and to stimulate exocytosis in permeabilized chromaffin cells (Barnard et al., 1996) and revealed that the COOH terminus of α-SNAP is important for its interaction with NSF (Barnard et al., 1996). The physiological significance of the α-SNAP stimulation of NSF ATPase activity, so far characterized only in in vitro assays, has not been established, and in previous work a truncation mutant unable to activate NSF was also unable to bind syntaxin (Barnard et al., 1996). In this study, therefore, we aimed to generate defined mutations within α-SNAP that would impair NSF ATPase activation without affecting α-SNAP interaction with the SNARE complex. We show a critical requirement for extreme COOH-terminal amino acids of α-SNAP in NSF activation, SNARE complex disassembly, and exocytosis, and we show that α-SNAPs with mutations in this domain are now inhibitors of α-SNAP–stimulated exocytosis.

Materials and Methods

Materials

Plasmids encoding His_6-NSF and His_6-α-SNAP were gifts from Dr J.E. Rothman (Memorial Sloan-Kettering Cancer Center, New York). High purity digitonin was obtained from Novabiochem (Nottingham, UK). FCS and DME with 25 mM Hepes were obtained from Gibco (Paisley, UK). Protein G–Sepharose was obtained from Pharmacia Biotech Sverige (Uppsala, Sweden). Nickel–nitriloacetic acid–agarose (Ni-NTA-agarose) was obtained from Qiagen (Dorking, UK). All other reagents were of analytical grade from Sigma (Poole, UK).

Buffers

Krebs-Ringer buffer: 145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, and 20 mM Hepes, pH 7.4. Culture medium: DME with 25 mM Hepes, 10% FCS, 8 μM fluorodeoxyuridine, 50 μg/ml gentamycin, 10 μM cytosine arabinofuranoside, 100 U/ml penicillin, 100 μg/ml streptomycin. Digitonin permeabilization buffer: 139 mM potassium glutamate, 20 mM Pipes, 5 mM EGTA, 2 mM ATP, 2 mM $MgCl_2$, 20 μM digitonin, pH 6.5. KGEP: 139 mM potassium glutamate, 20 mM Pipes and 5 mM EGTA, 2 mM ATP, 2 mM $MgCl_2$, pH 6.5. SNAP wash buffer (SWB): 25 mM Tris-HCl, 50 mM KCl, 1 mM DTT, 1 mg/ml BSA, pH 7.4. NSF binding buffer (NBB): 20 mM Hepes, 2 mM EDTA, 100 mM KCl, 500 μM ATP, 1 mM DTT, 1% (wt/vol) PEG_{4000}, 250 μg/ml soybean trypsin inhibitor, pH 7.4. ATPase assay buffer: 25 mM Tris-HCl, 0.5 mM DTT, 2 mM $MgCl_2$, 0.6 mM ATP, 10% (wt/vol) glycerol, pH 9.0. Immunoprecipitation buffer A: 20 mM Tris-HCl, 1 M KCl, 250 mM Sucrose, 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, pH 8.0. Buffer B: 10 mM Hepes, 100 mM KCl, 2 mM $MgC1_2$, 1 mM DTT, pH 7.8. Buffer C: 20 mM Hepes, 100 mM KCl, 1% (wt/vol) PEG_{4000}, 1% (vol/vol) glycerol, 0.9% (vol/vol) Triton X-100, 1 mM DTT, 0.5 mM ATP, 2 mM EDTA, pH 7.0. Wash buffer: 20 mM Hepes, 100 mM KCl, 0.5 mM ATP, 2 mM EDTA, 1% (vol/vol) Triton X-100, pH 7.0.

Plasmid Constructs

Truncated α-SNAP coding sequences were amplified by PCR with either Pfu polymerase (α-SNAP (1–160), α-SNAP (1–285), α-SNAP Δ (160–200), α-SNAP (L294A), α-SNAP "reverse" A294L) or Taq polymerase (all other SNAPs), from a plasmid encoding full-length α-SNAP. The primers contained restriction endonuclease sites to allow subcloning into the pQE-30 vector (Qiagen). Expression in the pQE-30 vector generates a protein with an NH_2-terminal MRGS-H_6 tag and a two–amino acid linker sequence fused to the amino terminus. Primers used for amplification were as follows: α-SNAP (1–270), sense BamHI 5′-CGGGATCCATGGACAACTCCGGGAAGG-3′, antisense HindIII, 5′-GTCAAGCTTGGAGATGGAGTCGTATT-3′; α-SNAP (1–250), sense, BamHI 5′-CGGGATCCATGGACAACTCCGGGAAGG-3′, antisense, 5′-TCAAGCTTGGCTTCTAACAGCTTTTTG-3′; α-SNAP (1–200), sense, BamHI 5′-CGGGATCCATGGACAACTCCGGGAAGG-3′, antisense HindIII, 5′-CTTAAGCTTATACTTGAGGAGTGGGCTGT-3′; α-SNAP (1–160), sense, BamHI 5′-CGGGATCCATGGACAACTCCGGGAAGG-3′, antisense Kpn I, 5′-CACTTGGTACCGAGCTGTTGGACTCCTCGC-3′; α-SNAP (81–295), sense, BamHI 5′-AGGGATCCGCAGCCACCTGCTTCGTGG-3′, antisense, HindIII 5′-CTGAAGCTTTTAGCGCAGGTCTTCCTCGT-3′; α-SNAP (121–295), sense, BamHI 5′-ACCGGATCCGCCAAGCACCACATCTCCAT-3′, antisense, HindIII 5′-CTGAAGCTTTTAGCGCAGGTCTTCCTCGT-3′; α-SNAP (160–295), sense, BamHI 5′-AACGGATCCGCCAACAAGTGTCTGCTGAA-3′, antisense, HindIII 5′-CTGAAGCTTTTAGCGCAGGTCTTCCTCGT-3′; α-SNAP (1–285), α-SNAP (L294A), and α-SNAP reverse (A294L) were created by site-directed mutagenesis of α-SNAP cloned into the pQE-9 expression vector, using the QuickChange™ (Stratagene, Cambridge, UK) SDM protocol. The primers used for this were α-SNAP (1–285), sense, 5′-CTGCGCATCAAGAAGTAAATCCAGGGTGACGAG-3′, antisense, 5′-CTCGTCACCCTGGATTTACTTCTTGATGCGCAG-3′. α-SNAP (L294A), sense, 5′-GACGAGGAAGACGCGCGCTAAGCCCCG-3′, antisense, 5′-CGGGGCTTAGCGCGCGTCTTCTTCGTC-3′. α-SNAP reverse (A294L),

sense, 5′-GTGACGAGGAAGACCTGCGCTAAGCCCCGC-3′, antisense, 5′-GCGGGGCTTAGCGCAGGTCTTCCTCGTCAC-3′. The plasmids were confirmed as correct by automated sequencing.

α-SNAP (Δ160–200) was produced by in frame cloning of DNA encoding amino acids 200–295 into the α-SNAP (1–160) plasmid, using KpnI and HindIII. The primers used for amino acids 200–295 were sense, Kpn I, 5′-TCCCTCGGTACCAGCGCCAAGGACTACTTTTT-3′, antisense, HindIII 5′-CTGAAGCTTTTAGCGCAGGTCTTCCTCGT-3′.

Purification of Fusion Proteins

Recombinant His_6-tagged proteins were purified from the cytosolic fraction of *Escherichia coli* XL-1 blue (Stratagene), or from *E. coli* M15 [pREP4] (Qiagen), on Ni-NTA-agarose based on previously published methods (Whiteheart et al., 1993).

Isolation and Culture of Chromaffin Cells and Assay of Catecholamine Secretion

Chromaffin cells were isolated from bovine adrenal medullae by enzymatic digestion as described previously (Burgoyne, 1992). Cells were washed in calcium-free Krebs-Ringer buffer, resuspended in culture medium, plated in 24-well trays at a density of one million cells per well, and maintained in culture for 3–7 d before use. To test the stimulatory activity of α-SNAP/SNAP mutant, each well was washed twice in PBS, and the cultured cells were permeabilized for 45 min with digitonin-permeabilization buffer. Cells were then stimulated for 30 min with KGEP containing 10 μM calcium and 25 μg/ml α-SNAP/α-SNAP mutant. To test the inhibitory activity of the mutant proteins, the cultured cells were permeabilized for 20 min with digitonin permeabilization buffer. 25 μg/ml of α-SNAP mutant was then added in KGEP buffer for 25 min and then stimulated with KGEP containing 10 μM free calcium for 30 min. Catecholamine release from the stimulation step in both experiments was measured using a standard fluorometric method. Total catecholamine content of cells was determined after lysis with 1% Triton X-100, and catecholamine secretion was calculated as a percentage of total cellular catecholamine. All experiments were performed at room temperature (22–25°C).

NSF Binding Assays

All samples for the NSF binding were run in duplicate and pooled at the end of the experiment. 20 μl of 100 μg/ml α-SNAP or α-SNAP mutant protein was incubated for 20 min in 1.5 ml polypropylene microcentrifuge tubes at room temperature. Buffer containing unbound α-SNAP was removed, and tubes incubated with 100 μl of SWB buffer for 2 min on ice. The SWB was discarded, and 20 μl of 100 μg/ml NSF in NBB was then added to each tube and incubated for 10 min on ice. The supernatant was discarded, and all tubes washed with 100 μl of NBB. 50 μl of SDS dissociation buffer was added, and each tube was boiled for 5 min; duplicate 50-μl samples were then pooled. The samples were run on 15% polyacrylamide gels, and proteins were detected by silver staining.

NSF ATPase Assay

α-SNAP and α-SNAP mutant proteins were immobilized by incubation in 1.5 ml polypropylene microcentrifuge tubes for 20 min at room temperature (final volume 20 μl). The SNAP solution was discarded and 50 μl of 20 μg/ml NSF in ATPase assay buffer added to each tube. Control tubes were incubated with 20 μg/ml NEM-inactivated NSF (2 mM NEM for 15 min on ice) in ATPase buffer. Samples were then incubated for 1 h at 37°C, and ATPase activity was determined using a spectrophotometric method (Lanzetta et al., 1979) with modifications (Lill et al., 1990). Corrections were made for preexisting phosphate in protein samples (since stored NSF requires ATP for stability), minor ATPase contaminants, and nonenzymatic breakdown of ATP by subtracting the NEM-inactivated control values from those obtained at 37°C.

Immunoprecipitation of SNARE Complex

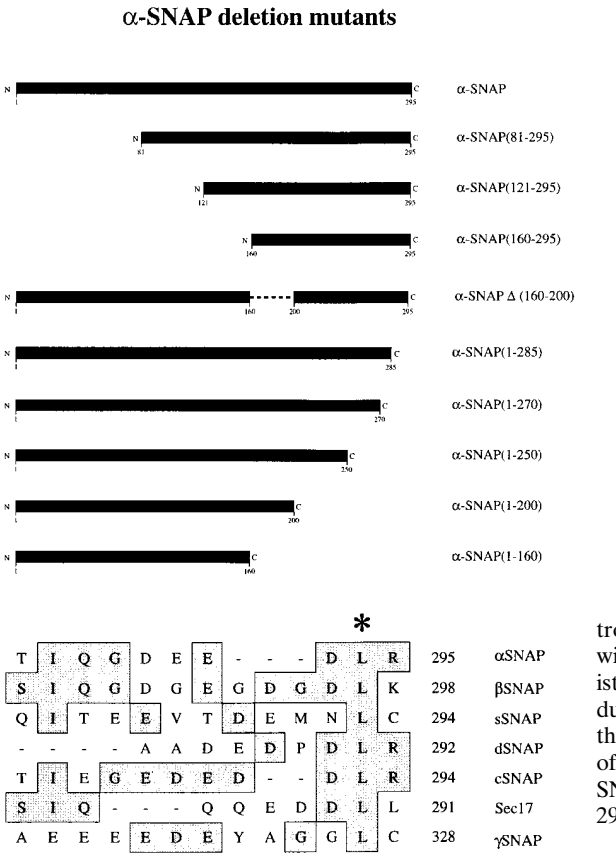
2 mg of Triton X-100–extracted rat brain membrane proteins, prepared as described by Söllner et al. (1993*a*), was incubated with 30 μg of NSF and 15 μg of α-SNAP/α-SNAP mutant with or without 2 mM $MgCl_2$, 0.5 mM ATP/ATP-γ-S in a final volume of 1.5 ml buffer C for 30 min at 4°C, rotating head-over-head. 100 μg of HPC-1 anti–syntaxin-Sepharose–conjugated antibody was then added to each tube and incubated for 2 h at 4°C, rotating head-over-head. The beads were washed five times in 1 ml wash buffer with or without MgATP, after which the beads were transferred to a new microfuge tube, and 80 μl of SDS sample buffer added. The samples were boiled for 5 min, separated on a 12.5% polyacrylamide gel, blotted with antisera to NSF, α-SNAP, syntaxin, and VAMP and developed using enhanced chemiluminescence (ECL; Amersham, Bucks, UK). For quantification, multiple exposures were generated, and only those within the linear range of the system were quantified by densitometric analysis.

Results

Plastic-immobilized α-SNAP is able to bind NSF (Clary et al., 1990) and stimulate its ATPase activity by 2–2.5-fold (Morgan et al., 1994). It has been shown previously that removal of up to 120 NH_2-terminal amino acids has no effect on the ability of α-SNAP to bind and stimulate NSF (Barnard et al., 1996). In the present study, further NH_2- and COOH-terminal α-SNAP truncation mutants were constructed to characterize in more detail the domains of α-SNAP required for stimulation of NSF ATPase activity to allow design of defined mutations within α-SNAP and assessment of the physiological role of NSF ATPase activation in regulated exocytosis.

α-SNAP mutants were initially tested for their ability to stimulate NSF ATPase activity. In such experiments, 1 μg of soluble NSF was added to 2 μg of α-SNAP (a submaximal concentration) that had been preimmobilized to microfuge tubes. NSF ATPase activity was measured using a spectrophotometric assay for free phosphate. Corrections were made for preexisting phosphate and contaminating ATPases by subtracting values obtained with NEM-inactivated NSF. The data for each mutant was then expressed as a percentage of the stimulation of NSF ATPase activity by wild-type α-SNAP. Removal of up to 160 NH_2-terminal amino acids had no major effect on the ability of α-SNAP to stimulate NSF ATPase activity, though some small differences between constructs were noted (Fig. 1). Deletion of a further 40 NH_2-terminal amino acids produced a mutant that could not be isolated in a soluble form, so to determine whether residues 160–200 were essential for the stimulation of NSF ATPase activity, an α-SNAP mutant was constructed with an internal truncation between 160–200 (Fig. 1). This mutant was also able to stimulate NSF activity, but by a reduced amount (Fig. 1). These data suggest that the 200 NH_2-terminal amino acids of α-SNAP are not essential for activation of NSF ATPase activity. In contrast, deletion from 135 to as few as 10 amino acids from the COOH terminus of α-SNAP resulted in a marked decrease in the ability of the mutants to stimulate the ATPase activity of NSF (Fig. 1).

To analyze whether COOH-terminal truncation mutants simply had a lower affinity for NSF ATPase activation, ATPase assays were performed with increasing amounts of α-SNAP mutants preimmobilized to the microfuge tubes. α-SNAP stimulation of NSF showed a dose-dependent increase to 5 μg/tube, at which the stimulation was saturated (Fig. 2 *A*). However, none of the COOH-terminal truncation mutants showed any more than a minor stimulatory activity, even at the supramaximal concentration of 15 μg/tube (e.g., see Fig. 2 *A* for α-SNAP (1–160), the COOH-terminal deletion construct with the largest effect on ATPase activity), suggesting that these mutants exhibit a decreased efficacy for NSF rather than a decreased affinity.



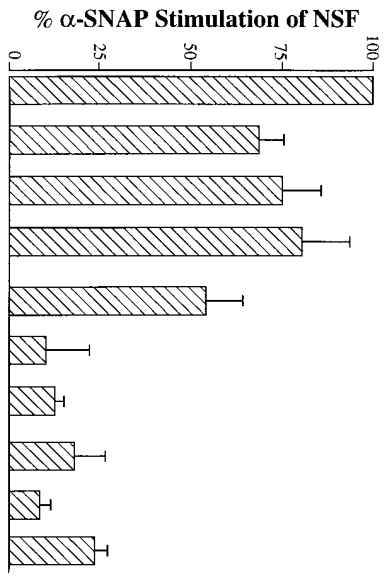


Figure 1. A schematic diagram of the α-SNAP truncation mutants used in this study and their ability to stimulate the ATPase activity of NSF compared to wild-type α-SNAP. (*Top left*) Schematic diagram of the α-SNAP truncation mutants used in this study. (*Top right*) Extent of stimulation of NSF ATPase activity as a percentage of stimulation by wild-type α-SNAP. 2 μg of full-length α-SNAP or α-SNAP truncation mutant were pre-immobilized to the surface of plastic microfuge tubes for 20 min on ice. α-SNAP was removed from the tubes, and 1 μg of NSF was added for 1 h at 37°C. NSF ATPase activity was measured using a spectrophotometric assay, and the results were expressed as a percentage of the wild-type α-SNAP stimulation of NSF. Corrections were made for preexisting phosphates and contaminating ATPases by subtracting values from duplicate samples run with NEM-inactivated NSF. Data was pooled from three to seven separate assays for each SNAP mutant. (*Bottom*) Alignment of database sequences of bovine α-SNAP, β-SNAP, γ-SNAP, *C. elegans* SNAP, squid SNAP, *Drosophila* SNAP, and yeast sec17p, showing leucine 294 to be the only conserved residue in the last 10 amino acids of α-SNAP.

α-SNAP (Δ160–200) also exhibited only a partial stimulation of NSF at higher doses, suggesting that this mutant, while able to activate NSF ATPase activity, also has a decreased efficacy for NSF ATPase activation (Fig. 2 *A*). This could be due to the deletion of important residues involved in the interaction with NSF or alternatively due to perturbations in the tertiary structure of this mutant.

Since deletion of as few as 10 COOH-terminal amino acids from α-SNAP substantially reduces its ability to stimulate NSF, it is likely that the extreme COOH terminus contains key residues for this action of the protein. Sequence alignment of bovine α-SNAP, β-SNAP, γ-SNAP, *Caenorhabditis elegans* SNAP, squid SNAP, *Drosophila* SNAP, and yeast sec17p revealed only leucine 294 (L294) of α-SNAP to be absolutely conserved in the final 10 amino acids in the COOH-terminal region of these proteins, including γ-SNAP, the most divergent of the SNAPs (Fig. 1), which is also able to activate the ATPase activity of NSF (Morgan et al., 1994) as is β-SNAP (Sudlow et al., 1996). This raised the possibility that this is an essential amino acid. To assess whether L294 is a key residue for the stimulation of NSF ATPase activity, an α-SNAP mutant was constructed with L294 changed to an alanine (α-SNAP [L294A]). α-SNAP (L294A) was analyzed alongside α-SNAP for ability to stimulate NSF ATPase activity over a range of SNAP concentrations and compared to the deletion mutant α-SNAP (1–285). At 15 μg/tube, α-SNAP (L294A) was unable to stimulate NSF, as was the case for the smallest COOH-terminally truncated SNAP mutant, α-SNAP (1–285) (Fig. 2 *B*).

To ensure that L294 was the only significant amino acid mutated in α-SNAP (L294A), the site-directed mutation was "reversed" by mutating the alanine back to leucine (α-SNAP A294L). When tested alongside α-SNAP and α-SNAP (L294A), the control reversed α-SNAP (A294L) was now able to stimulate NSF ATPase activity with the same potency as α-SNAP (Fig. 3), confirming that conversion of leucine to alanine at position 294 was the sole mutation responsible for the inactivity of α-SNAP (L294A). An additional important point was that α-SNAP (L294A) did not inhibit the intrinsic NEM-sensitive ATPase activity of NSF (Fig. 3) but simply failed to produce the increase in activity seen with wild-type α-SNAP.

To analyze whether COOH-terminally truncated α-SNAP mutants or α-SNAP (L294A) were unable to stimulate NSF due to an inability to become immobilized on plastic or to bind to NSF, truncation mutants were analyzed in assays for NSF binding. Soluble NSF was unable to bind to polypropylene tubes alone but was able to bind to immobilized α-SNAP (Fig. 4). All deletion mutants were able to associate with the polypropylene tubes and bind NSF (Fig. 4). Some variability in the apparent amounts of the smaller constructs was seen, e.g., α-SNAP (160–295), but this was not consistent between experiments. α-SNAP (1–160) (Fig. 4 *B*, *fourth lane*) and α-SNAP (160–295) (Fig. 4 *A*, *top*, *seventh lane*), which were nonoverlapping NH_2- and COOH-terminal fragments, respectively, were both able to bind NSF. This data suggests that NSF binding sites are present in both the NH_2- and COOH-terminal domains of α-SNAP, but only interaction with the COOH-terminal domains leads to NSF ATPase activation. α-SNAP (L294A) was also able to bind NSF in this assay (Fig 4 *C*).

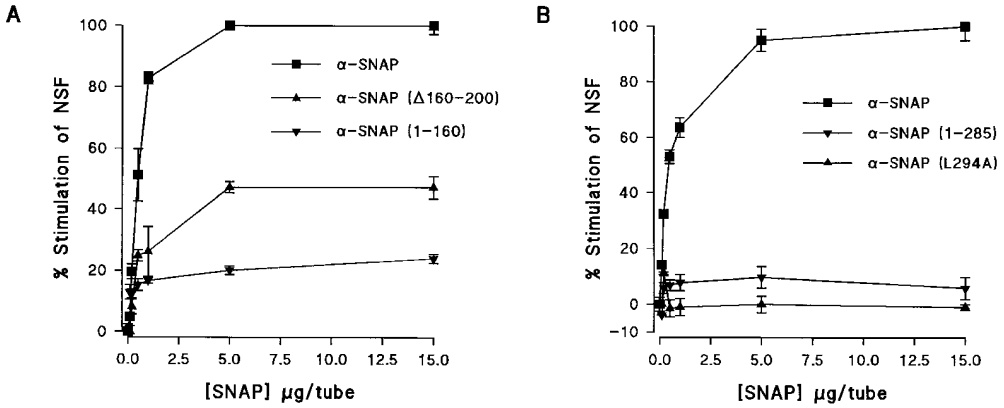


Figure 2. Effect on NSF ATPase activity of α-SNAP, α-SNAP (1–160), α-SNAP (Δ160–200), α-SNAP (1–285), and α-SNAP (L294A) over a range of concentrations. (*A*) Standard NSF ATPase assays were performed with several concentrations of full-length α-SNAP, α-SNAP (1–160), or α-SNAP (Δ160–200). (*B*) NSF ATPase assays with α-SNAP, α-SNAP (1–285), or α-SNAP (L294A). The data have been normalized to the maximal stimulation due to full-length α-SNAP. This was calculated by substracting NEM-insensitive ATPase activity and then calculating the relative differences between NSF ATPase activity in the presence or absence of α-SNAPs.

To determine the physiological significance of the α-SNAP stimulation of NSF ATPase activity in regulated exocytosis, α-SNAP (L294A) was assayed for the ability to stimulate Ca^{2+}-dependent exocytosis in permeabilized adrenal chromaffin cells. Digitonin-permeabilized chromaffin cells are stimulated maximally by 25 μg/ml α-SNAP (Morgan and Burgoyne, 1995*a*; Barnard et al., 1996). Cells were permeabilized with 20 μM digitonin for 45 min and then stimulated with 10 μM free Ca^{2+} for 30 min with or without 25 μg/ml α-SNAP or α-SNAP mutant, and released catecholamine was measured. The results of seven independent experiments were averaged, and secretion was expressed as a percentage of the 10 μM Ca^{2+} stimulation. Wild-type α-SNAP was able to stimulate Ca^{2+}-dependent exocytosis in permeabilized adrenal chromaffin cells by ~50% (Fig. 5 *A*), as previously reported (Morgan and Burgoyne, 1995*a*). However, neither α-SNAP (1–285) nor α-SNAP (L294A) were able to stimulate exocytosis in adrenal chromaffin cells at this maximal SNAP concentration. Reversal of the L294 mutation restored the α-SNAP activity for exocytosis, verifying (L294A) to be the only mutation responsible for the loss of stimulation of Ca^{2+}-dependent exocytosis (Fig. 5 *A*).

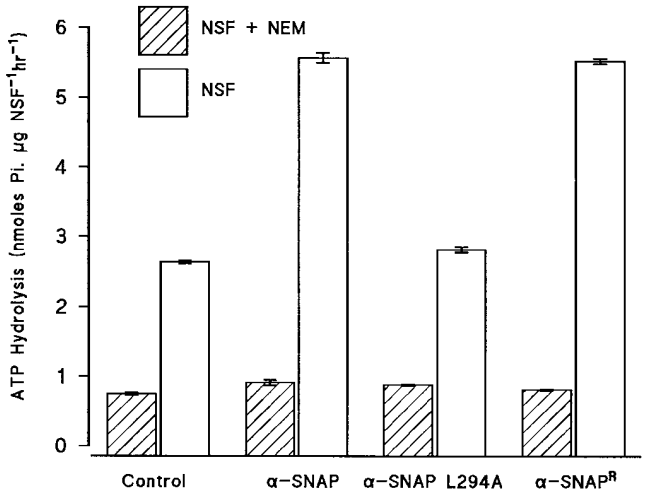


Figure 3. Reversal of the α-SNAP (L294A) mutation restores the ability to stimulate NSF ATPase activity. Standard assays of NSF ATPase activity were performed with 5 μg/tube of α-SNAP, α-SNAP (L294A), or α-SNAP (A294L), shown as α-SNAP^R. The data shows levels of ATP hydrolysis with untreated and with NEM-treated NSF. The intrinsic ATPase activity of NSF was not reduced by α-SNAP (L294A).

It appears that the removal of α-SNAP stimulation of NSF ATPase activity results in a mutant unable to stimulate exocytosis in permeabilized chromaffin cells. It is probable that exogenously added α-SNAP must first interact with an endogenous factor, probably SNAREs, before the stimulation of NSF. Addition of an α-SNAP mutant that is able to bind but not stimulate NSF would be predicted to inhibit the action of exogenously added α-SNAP in chromaffin cells. To test this possibility, digitonin-permeabilized chromaffin cells were preincubated with either buffer, α-SNAP (1–160) (as a control), α-SNAP (1–285), or α-SNAP (L294A) for 25 min and stimulated with 10 μM Ca^{2+} for 30 min with or without 25 μg/ml α-SNAP. The results of four separate experiments were averaged, and the results were expressed as a percentage of the 10 μM Ca^{2+} stimulation. When preincubated with buffer alone, α-SNAP stimulated exocytosis above control levels (Fig. 5 *B*). Preincubation with α-SNAP (1–160) had no inhibitory effect on α-SNAP action (Fig. 5 *B*), which would be predicted because such a large truncation would abolish the ability to interact with SNAREs (Hayashi et al., 1995; Barnard et al., 1996). However, addition of 25 μg/ml of either α-SNAP (1–285) or α-SNAP (L294A) resulted in the complete inhibition of the stimulatory activity of exogenous α-SNAP. The inhibitory effect of these mutants was still seen, even when an additional 10-min wash step was added between incubation of mutant proteins and the stimulation in the presence of α-SNAP (data not shown). It should be noted that the mutants α-SNAP (1–285) and α-SNAP (L294A) had little effect on endogenous exocytosis in the absence of added α-SNAP, nor did they do so even up to concentrations as high as 150 μg/ml (data not shown).

It has been shown previously that ATP hydrolysis by

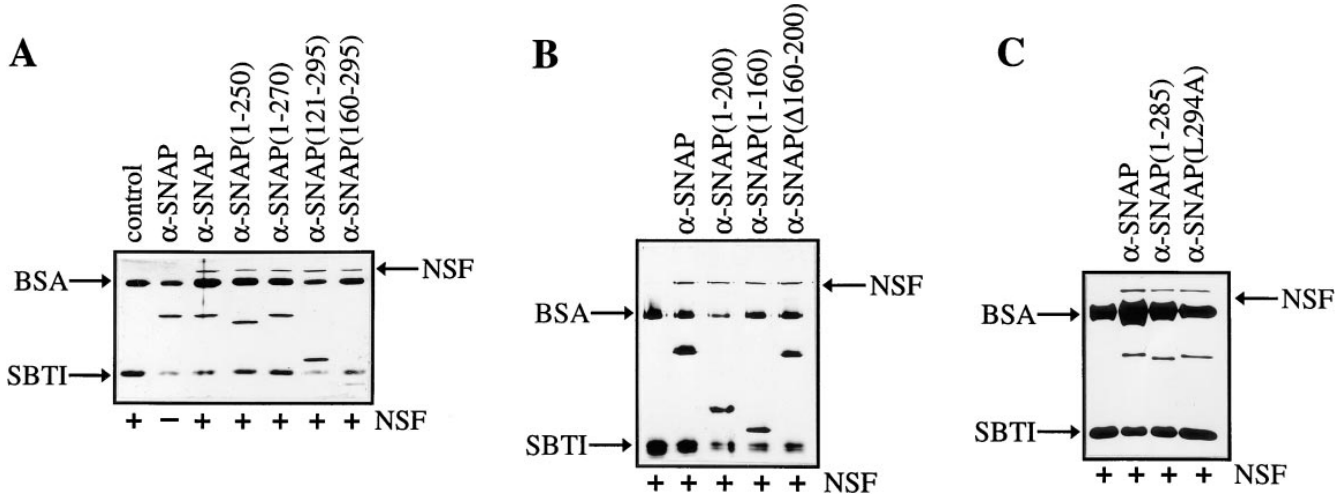


Figure 4. Binding of NSF to immobilized α-SNAP mutants. 2 μg of full-length SNAP or α-SNAP mutant was preimmobilized to the surface of polypropylene tubes for 20 min on ice. After washing with 1 ml SWB, the tubes were incubated with 2 μg of NSF in NBB for 10 min. The tubes were washed with 1 ml NBB, and bound proteins solublized with 50 μl of SDS buffer. The samples were then analyzed by SDS-PAGE and detected by silver-staining. *A–C* show results from different experiments. Note that no NSF binding was detected in control tubes without added SNAPs (first lane of each part).

NSF is required to disassemble the 20S complex of NSF, α-SNAP and the SNAREs syntaxin, SNAP-25, and VAMP (Söllner et al., 1993*b*). As α-SNAP (1–285) and α-SNAP (L294A) are unable to stimulate either the ATPase activity of NSF or exocytosis in adrenal chromaffin cells, they were analyzed for their ability to support assembly and disassembly of the 20S complex. A detergent extract of rat brain membrane proteins was incubated with NSF and with α-SNAP, α-SNAP (1–285), or α-SNAP (L294A) in the presence of MgATP or the nonhydrolyzable analogue MgATPγS. The 20S complex was immunoprecipitated with an antisyntaxin antibody, and immunoprecipitated syntaxin and proteins bound to syntaxin were visualized by immunoblotting. In the presence of ATP-γ-S, added α-SNAP, α-SNAP (1–285), and α-SNAP (L294A) were able to bind to the SNARE complex and recruit NSF, forming the 20S complex (Fig. 6 *A*). In the presence of MgATP, α-SNAP was able to disassemble the 20S complex, and the levels of VAMP, α-SNAP, and NSF in the syntaxin immunoprecipitate were significantly reduced (Fig. 6 *A*, *second lane*). The level of NSF dissociation from the 20S complex was similar with both wild-type and mutant α-SNAPs (Fig. 6 *A*). However, in the presence of MgATP, both α-SNAP (1–285) and α-SNAP (L294A) were still significantly associated with syntaxin, and in addition, VAMP did not fully disassemble from the 20S com-

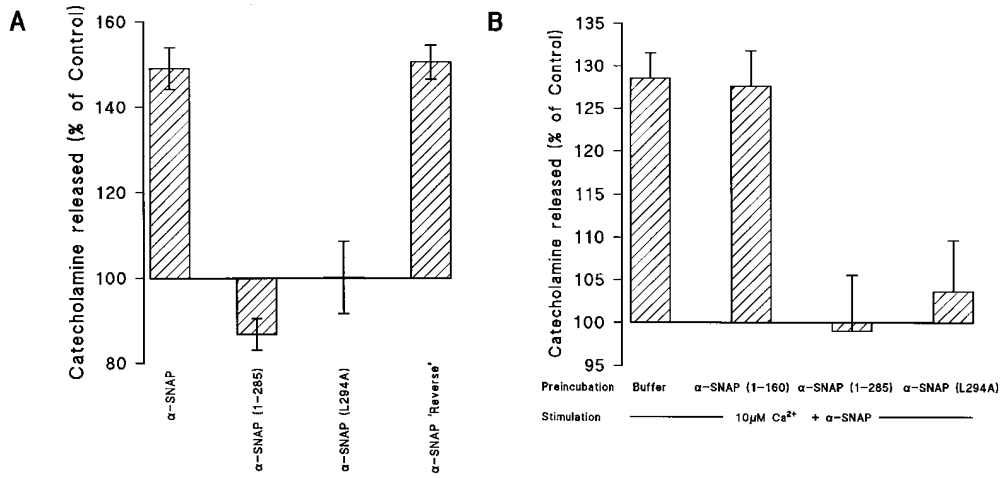


Figure 5. α-SNAP (1–285) and α-SNAP (L294A) are unable to stimulate catecholamine release from digitonin-permeabilized adrenal chromaffin cells but inhibit the α-SNAP stimulation of exocytosis. (*A*) Adrenal chromaffin cells were permeabilized for 45 min with permeabilization buffer and stimulated with 10 μM Ca^{2+} with or without 25 μg/ml of full-length α-SNAP, α-SNAP (1–285), α-SNAP (L294A), or the reverse mutation α-SNAP (A294L) for 30 min, and released catecholamine was assayed. The data were pooled from seven separate experiments, and the means were expressed as a percentage of the 10 μM Ca^{2+} control stimulation ± SEM. (*B*) Adrenal chromaffin cells were permeabilized for 20 min with permeabilization buffer, incubated with KGEP buffer with or without 25 μg/ml of recombinant α-SNAP (1–160), α-SNAP (1–285), or α-SNAP (L294A) for 25 min, and then subsequently stimulated with 10 μM Ca^{2+} with or without 25 μg/ml full-length α-SNAP for 30 min, and released catecholamine was assayed. The data was pooled from four separate experiments, and the means were expressed as a percentage of the 10 μM Ca^{2+} control stimulation ± SEM.

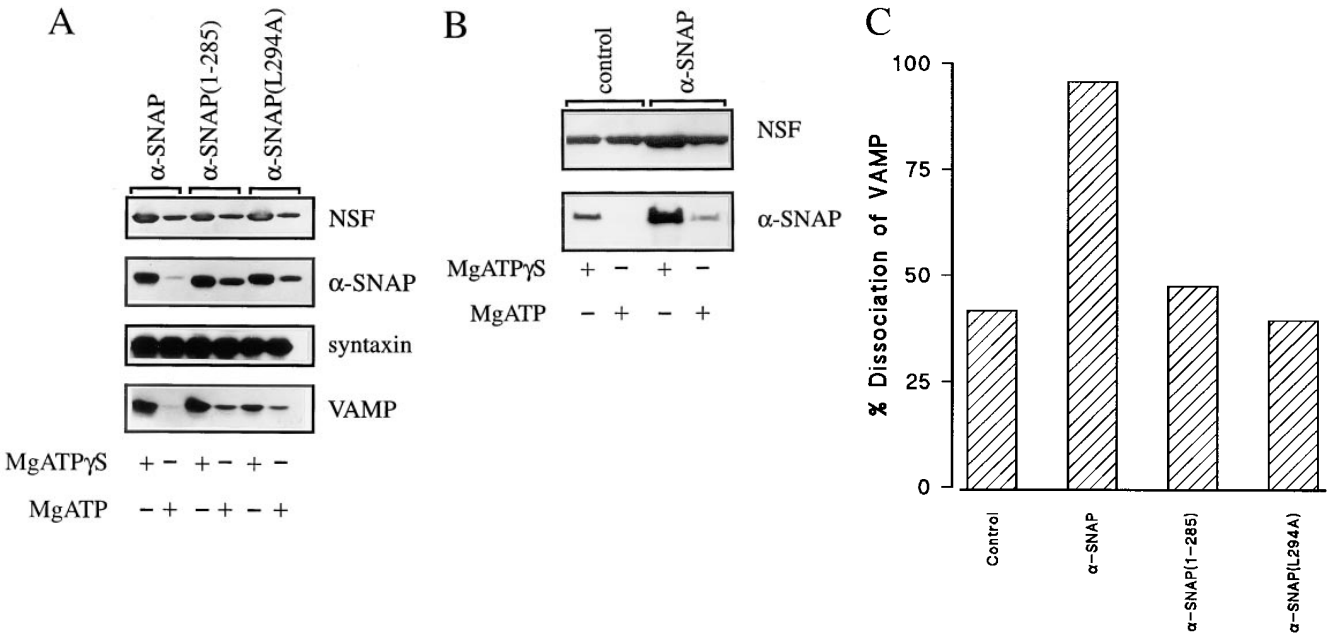


Figure 6. α-SNAP (1–285) and α-SNAP (L294A) associate with but are unable to support dissociation of the 20S complex. (*A*) A detergent extract of rat brain membrane proteins was incubated with 15 μg of NSF, 30 μg α-SNAPs for 30 min with 0.5 mM MgATP or MgATPγS as indicated at 4°C. Proteins were immunoprecipitated with an antisyntaxin antibody conjugated to protein G–Sepharose, and bound proteins were solubilized with SDS sample buffer and separated on a 12.5% polyacrylamide gel. Proteins were detected using specific antisera to NSF, α-SNAP, syntaxin, and VAMP. (*B*) Extracts were incubated without (control) or with added α-SNAP and with 15 μg NSF in the presence of 0.5 mM MgATP or MgATPγS as indicated. Endogenous α-SNAP in control incubations was sufficient to recruit exogenous NSF. (*C*) Extracts were incubated with NSF with no added α-SNAP (control) or with added α-SNAPs as indicated. The presence of VAMP in syntaxin immunoprecipitates was determined by immunoblotting, and the amount of VAMP dissociated in the presence of MgATP was calculated as a percentage of the amount of bound VAMP in MgATPγS incubations.The data shown are the mean values from two experiments.

plex formed with these mutants (Fig. 6 *A*, *fourth and sixth lanes*). In five experiments under varying conditions, no consistent differences were seen in the extent of VAMP dissociation with α-SNAP (1–285) or α-SNAP (L284A), which was always less than with α-SNAP. In the absence of added exogenous α-SNAP, low levels of endogenous α-SNAP, sufficient to recruit added NSF, could be detected by immunoblotting (Fig. 6 *B*). Since some VAMP dissociation was seen with the mutants, additional experiments were carried out in which the extent of VAMP dissociation was compared to incubations in which NSF but no α-SNAP was added. Quantification was based on densitometric analysis of blots within the linear range of the assay, and to account for variations between individual samples, the data shown is based on two separate experiments. Partial VAMP dissociation occurred in an ATP-dependent manner in the control incubation and to the same extent as with the mutants (Fig. 6 *C*), indicating that α-SNAP (1–285) and α-SNAP (L294A) are unable to support any VAMP dissociation.

Discussion

Four major findings emerge from the work described here. First, we have demonstrated that extreme COOH-terminal amino acids of α-SNAP are required for its ability to stimulate the ATPase activity of NSF and that the penultimate amino acid of α-SNAP, leucine 294, is an essential residue for this phenomenon. Second, we have demonstrated that α-SNAP mutants unable to stimulate the NSF ATPase activity do not support disassembly of the 20S SNARE complex. Third, mutants unable to stimulate NSF ATPase activity do not increase Ca^{2+}-dependent exocytosis in permeabilized chromaffin cells, suggesting that stimulation of NSF ATPase activity by α-SNAP may be an essential step in exocytosis. Fourth, the mutant protein α-SNAP (L294A) prevents the stimulation of exocytosis by exogenous α-SNAP and can, therefore, act as a dominant negative inhibitor of α-SNAP/NSF function.

We have shown that truncation of up to 160 NH_2-terminal amino acids or internal deletion of amino acids 160–200 from α-SNAP does not abolish the stimulation of NSF ATPase. In contrast, COOH-terminal truncations down to only 10 amino acids dramatically decrease the NSF ATPase stimulation by α-SNAP, showing that only amino acids within the domain 200–295 are essential for this activity. In contrast, nonoverlapping NH_2-terminal (α-SNAP (1–160)) and COOH-terminal (α-SNAP (160–295)) fragments are both able to bind NSF, showing that the binding of NSF to α-SNAP is not itself sufficient for significant ATPase activation in the assay used and that α-SNAP must possess at least two independent NSF binding sites. In a previous study, an α-SNAP (1–250) construct was used that contained nine additional COOH-terminal vector-derived residues (Barnard et al., 1996). This was not able to bind NSF, but this appeared to be caused by the additional resi-

dues. The α-SNAP (1–250) construct used here did not possess the extra COOH-terminal amino acids and could bind NSF. The single mutation in amino acid 294 (in α-SNAP (L294A)) was sufficient to abolish the ability of α-SNAP to stimulate NSF ATPase activity. α-SNAP (L294A) was able to bind NSF, when immobilized to plastic, and more significantly could bind within the 20S SNARE complex and recruit NSF as efficiently as wild-type α-SNAP. While we cannot entirely rule out an effect on protein folding, it seems likely that α-SNAP (L294A) is impaired only in its ability to activate NSF ATPase activity. The (L294A) mutation may therefore be a specific tool to probe the physiological significance of NSF ATPase activation by α-SNAP.

In our study of the effect of COOH-terminal mutations on 20S complex disassembly, some NSF dissociation occurred with the mutants as well as with the wild-type protein. Since the mutants did not impair the intrinsic ATPase activity of NSF, this may be the explanation for this common action. This could also explain the partial dissociation of the α-SNAP mutants themselves. Alternatively, this may be due to the pool of nonexchangeable endogenous α-SNAP present in the extracts. These data also suggest that SNARE complex disassembly may not be a simple process, but rather consisting of various subreactions. Some 20S complex disassembly, measured as VAMP dissociation, occurred with α-SNAP (1–285) and α-SNAP (L294A), but only to the same extent as in controls with no added SNAP. Levels of endogenous α-SNAP sufficent to recruit NSF were detected, which are likely to be the cause of this dissociation. It appears, therefore, that the intrinsic ATPase activity of NSF is not sufficient for full 20S complex disassembly. In contrast, α-SNAP activation of NSF ATPase activity correlates well with SNARE disassembly, implying that NSF activation is essential for this process.

Unlike wild-type α-SNAP, α-SNAP (L294A) and α-SNAP (1–285) were unable to stimulate Ca^{2+}-dependent exocytosis in chromaffin cells. These results suggest that the stimulation of NSF ATPase activity is essential for the steps leading to exocytosis. This is likely to be due to the inability of α-SNAP (L294A) or α-SNAP (1–285) to support ATP-dependent disassembly of the 20S SNARE complex, which may itself be an essential step for Ca^{2+}-triggered exocytosis to occur (as originally suggested by Söllner et al., 1993*b*). Recent data has suggested that α-SNAP/NSF may act before vesicle docking (Mayer et al., 1996; Morgan and Burgoyne, 1995*b*), but the discovery of most of the components of the 20S complex on the synaptic vesicle and chromaffin granule (Hong et al., 1994; Tagaya et al., 1995; Walch-Solimena et al., 1995), including SNAP and NSF (Burgoyne and Williams, 1997), suggests that 20S complex assembly/disassembly may be related to priming events on the vesicle rather than late steps before fusion. As well as being unable to stimulate exocytosis, both of these mutants inhibited the stimulatory effect of wild-type α-SNAP when the cells were preincubated with the mutants. Chromaffin cells possess few docked granules (<1% of total), and so the effect of the mutants cannot be due to interaction with SNAREs at the site of docking but must be on undocked granules or syntaxin on the plasma membrane (Hanson et al., 1995). Neither mutant inhibited endogenous exocytosis even when added at high concentrations. Since α-SNAP acts to prime the exocytotic mechanism (Chamberlain et al., 1995), it is possible that endogenous exocytosis comprises those granules already primed by α-SNAP/NSF (Morgan and Burgoyne, 1995*b*; Haas et al., 1996). Alternatively, since 30–50% of the α-SNAP and NSF remain cell-associated after permeabilization (Morgan and Burgoyne, 1995*a*), it is possible that endogenous exocytosis is mediated by α-SNAP and NSF already associated with SNAREs. This component would not be inhibited by the COOH-terminal α-SNAP mutants because of insufficient time for exchange in our incubation protocols, which are limited by the time-dependent run down of exocytosis. It is possible that α-SNAP (L294A) and α-SNAP (1–285) may be more effective as dominant negative inhibitors under conditions where their presence for longer periods could allow their exchange with bound endogenous α-SNAP or, alternatively, in membrane traffic assays comprising vesicle formation as well as consumption.

NSF contains two ATPase domains, and the activity of both is required for efficient vesicular transport in intra-Golgi transport assays (Sumida et al., 1994; Whiteheart et al., 1994; Nagiec et al., 1995). Activity of the D1 domain but not the second D2 domain appears to be absolutely essential (Nagiec et al., 1995). Since the stimulation of NSF ATPase activity is consistent with an increase in the affinity of only one site (Morgan et al., 1994), we would predict that this would be due to activation of the D1 ATPase site of NSF. The stimulation of NSF ATPase activity by α-SNAP that we have found is around 2–2.5-fold in the assay. Only immobilized α-SNAP is able to activate NSF ATPase activity (Morgan et al., 1994). Since only ~10% of the added NSF becomes bound to the immobilized α-SNAP (Sudlow et al., 1996), the activation of the bound component of NSF must be around 10-fold higher than the measured activation. The activation of bound NSF ATPase activity may, therefore, be more than 20-fold and thus represent a significant activation.

In conclusion, this work has demonstrated an essential requirement for leucine 294 of α-SNAP in the activation of NSF ATPase in an in vitro assay. Use of this mutant protein has suggested that this amino acid is important for 20S SNARE complex disassembly and may be physiologically essential for α-SNAP stimulation of exocytosis. As α-SNAP (L294A) is unable to either stimulate exocytosis or support disassembly of the 20S complex, this mutant will allow further dissection of the role of SNARE disassembly in vesicular transport.

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