Domains of α -SNAP Required for the Stimulation of Exocytosis and for *N*-ethylmaleimide-sensitive Fusion Protein (NSF) Binding and Activation

Richard J.O. Barnard, Alan Morgan, and Robert D. Burgoyne*

The Physiological Laboratory, University of Liverpool, Liverpool L69 3BX, United Kingdom

Submitted January 22, 1996; Accepted March 1, 1996
Monitoring Editor: Randy W. Schekman

The binding of α -SNAP to the membrane proteins syntaxin, SNAP-25, and synaptobrevin leads to the recruitment of the *N*-ethylmaleimide-sensitive fusion protein (NSF). ATP hydrolysis by NSF has been suggested to drive conformational changes in one or more of these membrane proteins that are essential for regulated exocytosis. Functional evidence for a role of α -SNAP in exocytosis in adrenal chromaffin cells comes from the ability of this protein to stimulate Ca^{2+} -dependent exocytosis in digitonin-permeabilized cells. Here we examine the effect of a series of deletion mutants of α -SNAP on exocytosis, and on the ability of α -SNAP to interact with NSF, to define essential domains involved in protein-protein interactions in exocytosis. Deletion of extreme N- or C-terminal regions of α -SNAP produced proteins unable to bind to syntaxin or to stimulate exocytosis, suggesting that these domains participate in essential interactions. Deletion of C-terminal residues abolished the ability of α -SNAP to bind NSF. In contrast, deletion of up to 120 N-terminal residues did not prevent the binding of NSF to immobilized α -SNAP and such mutants were also able to stimulate the ATPase activity of NSF. These results suggest that the C-terminus, but not the N-terminus, of α -SNAP is crucial for interactions with NSF. The involvement of the C-terminus of α -SNAP, which contains a predicted coiled-coil domain, in the binding of both syntaxin and NSF would place the latter two proteins in proximity in a ternary complex whereupon the energy derived from ATP hydrolysis by NSF could induce a conformational change in syntaxin required for exocytosis to proceed.

INTRODUCTION

Regulated exocytosis in neurons and neuroendocrine cells is inhibited by the clostridial neurotoxins that cleave the proteins synaptobrevin (VAMP), syntaxin, or SNAP-25 (Schiavo *et al.*, 1992; Blasi *et al.*, 1993a,b; Montecucco and Schiavo, 1994; Niemann *et al.*, 1994), indicating an essential role for these proteins in Ca^{2+} -dependent exocytosis. Synaptobrevin was first identified as a synaptic vesicle protein (Baumert *et al.*, 1989; Trimble *et al.*, 1989), whereas syntaxin (Bennett *et al.*, 1992) and SNAP-25 (Oyler *et al.*, 1989) were originally identified as synaptic plasma membrane proteins. These three proteins have been termed SNAREs (SNAP receptors) because, when in a complex in de-

tergent extracts of brain membranes, they were found to bind α -SNAP and in turn the *N*-ethylmaleimide-sensitive fusion protein (NSF) to form a 20S complex (Sollner *et al.*, 1993a). It has been proposed that the SNAREs mediate secretory vesicle docking on the target membrane and that subsequent ATP hydrolysis by NSF leads to disassembly of the SNARE complex as a late event in the steps leading to membrane fusion in exocytosis (Sollner *et al.*, 1993b). NSF as well as α -, β -, and γ -SNAP were initially identified as soluble factors required for intra-Golgi transport (Wilson *et al.*, 1989; Clary *et al.*, 1990; Whiteheart *et al.*, 1993). Homologues of the SNAREs as well as SNAPs and NSF have been implicated in many vesicular transport steps in mammalian cells (Rothman, 1994; Sollner, 1995; Whiteheart and Kubalek, 1995) and evidence from mutations of homologous proteins in yeast (Pryer *et al.*, 1992; Ferro-

* Corresponding author.

Novick and Jahn, 1994) provides convincing proof that these proteins form part of an essential conserved mechanism leading to fusion of transport vesicles with target membranes.

A fuller understanding of vesicular transport mechanisms will require detailed analysis of the molecular interactions between the key participating proteins, some of which have now been identified, and investigation of their exact functional roles. Recent work using purified recombinant proteins has characterized the domains of the SNAREs involved in their interactions with one another (Calakos *et al.*, 1994; Chapman *et al.*, 1994; Hayashi *et al.*, 1994; Pevsner *et al.*, 1994). In addition, it has been demonstrated that α -SNAP binding to syntaxin (Hanson *et al.*, 1995; Hayashi *et al.*, 1995; Kee *et al.*, 1995; McMahon and Sudhof, 1995) and indeed recruitment of NSF and the dissociation of α -SNAP from syntaxin following ATP hydrolysis by NSF (Hanson *et al.*, 1995) can occur in the absence of any other proteins. Synaptobrevin does not bind to α -SNAP, and SNAP-25 binds α -SNAP with 10-fold lower affinity than syntaxin (Hanson *et al.*, 1995; Kee *et al.*, 1995). These findings suggest that syntaxin is the major receptor for α -SNAP. The role of NSF may be to act as a molecular chaperone (Morgan and Burgoyne, 1995a) to modify the conformation of syntaxin (Hanson *et al.*, 1995) and possibly other SNAREs (Pellegrini *et al.*, 1995) and this may be directly regulated by α -SNAP, which is able to stimulate the ATPase activity of NSF (Morgan *et al.*, 1994). α -SNAP contains three predicted coiled-coil domains (Whiteheart *et al.*, 1993) that have been suggested to mediate protein-protein reactions required for α -SNAP's role in neurotransmitter release (DeBello *et al.*, 1995). Direct evidence of a requirement for these domains in the function of α -SNAP is not yet available.

In support of a key role of α -SNAP in regulated exocytosis, it has been shown that exogenous α -SNAP stimulates exocytosis following its injection into the squid giant synapse (DeBello *et al.*, 1995) or introduction into permeabilized adrenal chromaffin cells (Morgan and Burgoyne, 1995b) where its effect is on an ATP-dependent priming step that precedes Ca^{2+} -triggered membrane fusion (Chamberlain *et al.*, 1995). All three synaptic SNAREs are expressed in chromaffin cells (Hodel *et al.*, 1994; Roth and Burgoyne, 1994) and it is probable that α -SNAP acts in chromaffin cells by binding to a SNARE or the SNARE complex. Consistent with this is the finding that its action on exocytosis is prevented by treatment of the cells with botulinum neurotoxin A (Morgan and Burgoyne, 1995b), which cleaves SNAP-25 (Blasi *et al.*, 1993a). In addition, it is likely to act in conjunction with NSF but this has not been directly demonstrated. To investigate the protein-protein interactions involving α -SNAP and their relevance to exocytosis in chromaffin cells we have examined the effects of full length α -SNAP and

deletion mutants of the protein in assays for exocytosis in permeabilized chromaffin cells, for NSF binding, and for activation of NSF ATPase activity. It has been possible to define domains of α -SNAP crucial for both exocytosis and NSF binding/activation. We report that both extreme N- and C-terminal domains of α -SNAP are essential for its role in exocytosis and that the C-terminus of α -SNAP is required not only for syntaxin binding but also for NSF binding and activation.

MATERIALS AND METHODS

Materials

Plasmids encoding His₆-NSF and His₆- α -SNAP were gifts from Dr. J.E. Rothman (Sloan-Kettering Memorial Institute, New York, NY) and GST-Syntaxin1A(4–266) was a gift from Dr. R.H. Scheller (Department of Cellular and Molecular Physiology, Stanford, CA). High purity digitonin was obtained from Novabiochem (Nottingham, UK). Fetal calf serum and DMEM with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) were obtained from Life Technologies (Paisley, UK). Nickel-nitriloacetic acid-agarose (Ni-NTA-agarose) and anti-MRGS-His antibody were obtained from Qiagen (Dorking, UK). All other reagents were of analytical grade from Sigma (Poole, UK).

Buffers

Buffers were as follows. 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: DMEM with 25 mM HEPES, 10% fetal calf serum, 8 μM fluorodeoxyuridine, 50 $\mu\text{g}/\text{ml}$ gentamicin, 10 μM cytosine arabinofuranoside, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Permeabilization buffer: 139 mM potassium glutamate, 20 mM piperazine-N,N'-bis(ethanesulfonic acid) (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, 5 mM EGTA, 2 mM ATP, and 2 mM MgCl_2 , pH 6.5. SNAP wash buffer (SWB): 25 mM Tris-HCl, 50 mM KCl, 1 mM dithiothreitol (DTT), and 10 mg/ml bovine serum albumin, pH 7.4. NSF binding buffer (NBB): 20 mM HEPES, 2 mM EDTA, 100 mM KCl, 500 μM ATP, 1 mM DTT, 1% (w/v) Peg₄₀₀₀, and 250 $\mu\text{g}/\text{ml}$ soyabean trypsin inhibitor, pH 7.4. ATPase assay buffer: 25 mM Tris-HCl, 0.5 mM DTT, 2 mM MgCl_2 , 0.6 mM ATP, and 10% (w/v) glycerol, pH 9.0.

Plasmid Constructs

Truncated α -SNAP coding sequences were amplified by the polymerase chain reaction 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 a 6-histidine tag and a 2-amino acid linker sequence fused to the amino terminus. Primers used for amplification were as follows. α -SNAP (1–250): sense, BamHI 5'-CGGGATCCATGGACAACCTCCGGGAAGG-3'; antisense, 5'-TCAAGCTTGGCTTCTAACAGCTTTTGTG-3'. α -SNAP (41–295): sense, BamHI 5'-TAGGATCCGCGCATGTGAAATCTACGCCAG-3'; antisense, HindIII 5'-CTGAAGCTTTAGCGCAGGTCTTCTCTCGT-3'. α -SNAP(81–295): sense, BamHI 5'-AGGGATCCGCGAGCCACCTGCTTCGTGG-3'; antisense, HindIII 5'-CTGAAGCTTTTAGCGCAGGTCTTCTCTCGT-3'. α -SNAP (121–295): sense, BamHI 5'-ACCGGATCCGCGCAAGCACACATCTCCAT-3'; antisense, HindIII 5'-CTGAAGCTTTTAGCGCAGGTCTTCTCTCGT-3'. The plasmid for α -SNAP(1–250) encoded a protein with nine additional C-terminal vector-derived amino acids. The plasmids were confirmed as correct by automated sequencing.

Purification of Fusion Proteins

Recombinant His₆-tagged NSF, α -SNAP, α -SNAP(41–295), α -SNAP(81–295), and α -SNAP(121–295) were purified from the cytosolic fraction of *Escherichia coli* XL-1 blue (Stratagene, Cambridge, UK), or in the case of α -SNAP(121–295) from *E. coli* M15 [pREP4] (Qiagen), on Ni-NTA-agarose based on previously published methods (Whiteheart *et al.*, 1993). GST-Syntaxin1A(4–260) was purified by chromatography on glutathione-agarose (Calakos *et al.* 1994).

Isolation and Culture of Chromaffin Cells and Assay of Catecholamine Secretion

Chromaffin cells were isolated from bovine adrenal medullae by enzymic 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 days before use. For permeabilization and assay of catecholamine secretion each well was washed twice with 1 ml phosphate-buffered saline, and the cultured cells were permeabilized for 45 min with permeabilization buffer. Cells were then stimulated for 30 min with KGEP/MgCl₂ containing 10 μ M calcium and purified proteins, at concentrations indicated in the text. Catecholamine release from the stimulation step 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. Twenty microliters of 100 μ g/ml α -SNAP or α -SNAP deletion mutant protein was incubated for 20 min in 0.5 or 1.5 ml polypropylene microcentrifuge tubes at room temperature. The α -SNAP protein was removed and the tubes incubated with 100 μ l of SWB buffer for 2 min on ice. The SWB was

discarded and 20 μ l of 100 μ g/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 μ l of NBB. Fifty microliters of SDS dissociation buffer was added, each tube was boiled for 5 min, and duplicate 50- μ l samples were then pooled together. The samples were run on 15% polyacrylamide gels and proteins were detected by silver staining or immunoblotting. Immunoblotting was carried out as previously described (Roth and Burgoyne, 1994) using rabbit anti-NSF (Morgan and Burgoyne, 1995b) or an anti-MRGS-His antibody (Qiagen).

NSF ATPase Assay

α -SNAP and α -SNAP deletion mutant proteins were immobilized by incubation in 1.5 ml polypropylene microcentrifuge tubes for 20 min at room temperature (100 μ l containing 10 μ g of protein). The SNAP solution was discarded and 50 μ l of 8 μ g/ml NSF in ATPase assay buffer was added to each tube. 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 (because stored NSF requires ATP for stability) and nonenzymic breakdown of ATP by incubating duplicate samples on ice and subtracting these values from those obtained at 37°C. Absorbance was read at A₆₆₀ and the values were compared with a standard curve using KH₂PO₄ as standard.

RESULTS

A series of mutants of α -SNAP with deletion of the extreme N-terminal or C-terminal domains was generated (Figure 1A) and the proteins were purified as His₆-tagged constructs (Figure 2). α -SNAP was suggested to contain three potential coiled-coil domains, one at the N-terminus and two in the center of the

Figure 1. Schematic diagram of α -SNAP structure and deletion mutants generated in this study. (A) α -SNAP and the N- and C-terminal deletion mutants used in this study. (B) Analysis of potential coiled-coil domains in α -SNAP using the Lupas *et al.* (1991) algorithm. Potential coiled-coil domains are shown and above them the mean scores derived from the analysis.

Figure 2. SDS-PAGE analysis of α -SNAP and deletion mutants. Coomassie blue-stained 15% SDS-polyacrylamide gel of purified His₆-tagged recombinant SNAP proteins generated in this study.

protein (Whiteheart *et al.*, 1993). We reexamined the potential for coiled-coil formation using the algorithm of Lupas *et al.* (1991). This analysis confirmed the three originally proposed domains and revealed an additional possible coiled-coil domain close to the C-terminus of α -SNAP (Figure 1B). The mean scores from the analysis of these domains indicate moderate likelihood (a score of 1.3 or above indicates a strong likelihood) of coiled-coil structures similar to that for proposed coiled-coil structures that mediate SNARE interactions (Chapman *et al.*, 1994). The deletion mutants that have been generated should allow the significance of the predicted coiled-coil domains for α -SNAP function to be assessed.

The ability of the C-terminal and N-terminal deletion mutants to stimulate exocytosis in digitonin-permeabilized chromaffin cells was compared with that of the full length protein. Chromaffin cells were permeabilized for 45 min to allow extensive protein leakage and then stimulated for 30 min with 10 μ M Ca²⁺ in the absence of (control) or presence of exogenous SNAP proteins. As shown in Figure 3, full length α -SNAP stimulated catecholamine release to around 50% above control levels in this series of experiments and its effect was Ca²⁺ dependent. No effect of α -SNAP(41–295), α -SNAP(81–295), or α -SNAP(1–250) was observed on release in the absence or presence of 10 μ M Ca²⁺. To characterize the effects further, α -SNAP and deletion mutants were tested over a range of concentrations. The stimulatory effect of full

length α -SNAP was half-maximal at 3 μ g/ml (0.1 μ M) and maximal at 10–15 μ g/ml. Proteins with deletions of 40 or 80 N-terminal [α -SNAP(41–295) or α -SNAP(81–295)] or 45 C-terminal [α -SNAP(1–250)] residues had no significant effect on catecholamine release over a range of protein concentrations up to 40 μ g/ml (Figure 4A). An α -SNAP mutant with a 120-amino acid deletion [α -SNAP(121–295)] was also without effect on secretion (Figure 4B). In nine separate experiments, stimulation by full length α -SNAP was seen on each occasion but in no case was a significant effect of any of the deletion mutants observed. These results show that both N-terminal and C-terminal domains of α -SNAP are essential for its ability to stimulate exocytosis in permeabilized chromaffin cells.

The ability of α -SNAP mutants with N-terminal or C-terminal deletions to bind to syntaxin, the major binding partner for α -SNAP, was determined in an assay based on binding to the cytoplasmic domain of syntaxin1A as a GST fusion protein (Calakos *et al.*, 1994) immobilized on glutathione-Sepharose beads. Binding of full-length α -SNAP to syntaxin was detected but deletion of 40 or 80 N-terminal or 45 C-terminal residues abolished syntaxin binding, confirming reports (Hanson *et al.*, 1995; Hayashi *et al.*, 1995) that both the extreme N- and C-terminal domains of α -SNAP are required for tight binding to syntaxin or the SNARE complex. This could explain

Figure 3. Effect of α -SNAP and deletion mutants on Ca²⁺-dependent secretion from permeabilized adrenal chromaffin cells. Chromaffin cells were permeabilized by incubation with digitonin for 45 min, stimulated with 0 or 10 μ M Ca²⁺ for 30 min in the absence or presence of the indicated proteins, and released catecholamine was assayed. All proteins were at 15 μ g/ml except α -SNAP(1–250) at 20 μ g/ml. The data are expressed in relation to the control 10 μ M Ca²⁺ value that was set at 100% and are shown as means $\pm$ SEM (n = 4).

Figure 4. Dose dependency of the effect of α -SNAP on catecholamine release. Chromaffin cells were permeabilized for 45 min, stimulated with 10 μ M Ca^{2+} for 30 min in the presence of the indicated concentration of protein, and released catecholamine was assayed. (A) Comparison of the effects of α -SNAP, α -SNAP(1-250), α -SNAP(41-295), and α -SNAP(81-295). (B) A separate experiment comparing the effect of α -SNAP and α -SNAP(121-295). The data are expressed in relation to the control 10 μ M Ca^{2+} value that was set at 100% and are shown as means $\pm$ SEM ($n = 4$).

the inability of the deletion mutants to stimulate exocytosis.

NSF does not bind to α -SNAP in solution but can bind α -SNAP immobilized on plastic (Clary *et al.*, 1990) and under these conditions the ATPase activity of NSF is stimulated by α -SNAP (Morgan *et al.*, 1994). To assess the interaction of NSF with full length α -SNAP and deletion mutants, the SNAP proteins were first immobilized on the surface of polypropylene tubes and incubated with his₆-NSF to assess their ability to bind NSF. NSF binding was assessed using two different antibody detection methods with anti-NSF or with anti-MRGS-His and also with silver staining. All three methods gave essentially the same results and two of the approaches are illustrated (Figure 5). In this assay the limiting factor is α -SNAP binding to plastic with only 5% of the added α -SNAP becoming immobilized (Morgan *et al.*, 1994). A correspondingly small proportion of added excess NSF becomes bound to α -SNAP and from quantitative densitometry the stoichiometry of bound α -SNAP for the full length proteins was around 0.2 mol NSF monomer:1 mole α -SNAP. In Figure 5A it is clear that similar levels of α -SNAP, α -SNAP (1-250), α -SNAP(41-295), and α -SNAP(81-295) became immobilized on the tubes. In the absence of α -SNAP, NSF did not bind to the tubes but NSF binding could be detected in the presence of full length α -SNAP (Figure 5A). The N-terminally truncated mutants, α -SNAP(41-295) and α -SNAP(81-295), were able to efficiently bind NSF. In contrast, no binding of NSF was detected to the C-terminally truncated mutant α -SNAP(1-250). In a separate experiment, using detection with anti-MRGS-His, immobilized α -SNAP, α -SNAP(1-250), and the additionally N-terminally truncated α -SNAP(121-295) were detected (Figure 5B). α -SNAP(121-295) appeared to bind less well to plastic than the full length α -SNAP (Figure 5B) and quantitative densitometry indicated that the level was around 50% of that of the full length

protein. Additional larger polypeptides detected at low levels are likely to be dimers as they corresponded in size to dimers of α -SNAP or the mutants and they were recognized not only by the anti-MRGS-His monoclonal but also by anti- α -SNAP polyclonal antisera. NSF binding to α -SNAP and to α -SNAP(121-295) was observed but again no NSF binding to the C-terminally truncated α -SNAP(1-250) was seen (Figure 5B).

To determine whether deletion mutants that bound NSF could also activate its ATPase activity, α -SNAPs were immobilized on the tubes, incubated with NSF, and then the ATPase activity of NSF was assayed. NSF alone had a low ATPase activity, and full length α -SNAP stimulated the NSF ATPase activity approximately threefold (Figure 6A) in agreement with previous results (Morgan *et al.*, 1994). The C-terminal deletion mutant α -SNAP(1-250) did not increase the ATPase activity of NSF. In contrast, α -SNAP(41-295) and α -SNAP(81-295) were both able to stimulate NSF ATPase activity but to a lesser extent than the full length protein (Figure 5A). Similar results were obtained in three separate experiments and the data shown is pooled from all three assays. Analysis of the effect of the further N-terminally truncated mutant, α -SNAP(121-295), showed that this was also able to effectively stimulate the ATPase activity of NSF (Figure 6B). These results indicate that the N-terminal 120 residues of α -SNAP are not absolutely essential for NSF binding and activation whereas the C-terminal deletion mutant is unable to bind and activate NSF.

DISCUSSION

As previously demonstrated (Chamberlain *et al.*, 1995; Morgan and Burgoyne, 1995b) recombinant α -SNAP stimulates Ca^{2+} -dependent exocytosis leading to catecholamine release from permeabilized chromaffin cells. The ability of exogenous α -SNAP to stimulate

Figure 5. Binding of NSF to immobilized α -SNAPs. α -SNAPs (2 μ g per tube) were immobilized by binding to the surface of polypropylene tubes for 20 min. After washing, the tubes were incubated with buffer containing bovine serum albumin followed by incubation with NSF (2 μ g per tube). The tubes were washed, and bound proteins were solubilized in SDS-buffer and analyzed by SDS-polyacrylamide gel electrophoresis. (A) Detection of bound α -SNAPs by silver staining and bound NSF by immunoblotting with anti-NSF. NSF was detected bound to α -SNAP, α -SNAP(41-295), and α -SNAP(81-295) but not α -SNAP(1-250). (B) Detection of bound α -SNAP and NSF using anti-MRGS-His. NSF was detected bound to α -SNAP and α -SNAP(121-295) but not α -SNAP(1-250). The additional band detected in the α -SNAP tracks is of the expected size of an α -SNAP dimer.

exocytosis in the absence of added NSF is consistent with recent findings suggesting that NSF can be bound to the synaptic vesicle protein synaptotagmin via β -SNAP and then subsequently bind α -SNAP and SNARE complex (Schiavo *et al.*, 1995). It is possible, therefore, that exogenous α -SNAP could interact with pre-bound NSF in the permeabilized cells following interaction with a membrane SNARE.

The half-maximal concentration of α -SNAP for stimulation (0.1 μ M) is close to the estimated cellular concentration in intact cells (0.2 μ M) based on immunoblotting in comparison with recombinant protein standards (our unpublished observations), suggesting

that α -SNAP levels are limiting for exocytosis. A similar conclusion was reached based on stimulation of neurotransmitter release from the squid giant synapse after microinjection of α -SNAP (DeBello *et al.*, 1995). The concentration of α -SNAP required for half-maximal stimulation of exocytosis is lower than that for half-maximal syntaxin binding (0.5–1 μ M; Hanson *et al.*, 1995; Hayashi *et al.*, 1995; Kee *et al.*, 1995; McMahon and Sudhof, 1995) but close to the calculated half-maximal concentration (0.07 μ M) for α -SNAP-mediated disassembly of the SNARE complex in vitro (Hayashi *et al.*, 1995). SNAP-25 binds α -SNAP with a 10-fold lower affinity than syntaxin (Hanson *et al.*, 1995; Kee *et al.*, 1995), suggesting that syntaxin may be the major receptor for α -SNAP. The stimulation of exocytosis in chromaffin cells was previously shown to be prevented by treatment of cells with botulinum neurotoxin A, which cleaves SNAP-25 (Morgan and Burgoyne, 1995b). In the present study it was found that deletion of either the extreme N-terminal or the C-terminal residues of α -SNAP abolished its ability to stimulate exocytosis. These mutants were unable to bind to syntaxin 1A-GST consistent with other recent findings (Hanson *et al.*, 1995; Hayashi *et al.*, 1995), showing that syntaxin-binding to α -SNAP requires both the N- and C-terminal domains of α -SNAP. Overall, these data support a stimulatory action of α -SNAP on exocytosis in chromaffin cells through a syntaxin- and SNAP-25-dependent mechanism. α -SNAP was originally suggested to contain three predicted coiled-coil domains that may be involved in protein-protein interactions (Whiteheart *et al.*, 1993), although in our analysis an additional domain close to the C-terminus was detected. This possible role of the N-terminal coiled-coil domain was tested for regulated exocytosis (DeBello *et al.*, 1995) using synthetic peptides in attempts to inhibit exocytosis in the squid giant synapse. N-terminal α -SNAP peptides encompassing the squid equivalent of residues 1–41 of mammalian α -SNAP were inhibitory when injected into the synapse. No direct evidence that this was due to disruption of α -SNAP function was provided but it was concluded that the N-terminus of α -SNAP is important in exocytosis. Our data now provides direct evidence for this contention because deletion of the N-terminal 40 residues abolished the ability of α -SNAP to stimulate exocytosis, suggesting that the predicted N-terminal coiled-coil domain of α -SNAP does indeed participate in key protein-protein interactions (most probably with syntaxin) in exocytosis. The deletion mutants of α -SNAP do not stimulate exocytosis nor, alternatively, do they have any inhibitory effects. This is not surprising because none of them bind syntaxin. Although the N-terminal deletion mutants bind NSF in the plastic binding assay they would not be expected to bind NSF in the cell because such binding would require the ability of the α -SNAP to first interact with syntaxin

Figure 6. Comparison of the effects of α -SNAPs on the ATPase activity of NSF. α -SNAPs were immobilized by incubation in polypropylene tubes, the solution was removed, and then the tubes were incubated with NSF (0.4 μ g per tube) for 1 h at 37°C. The ATPase activity of NSF was assayed by measurement of released inorganic phosphate. (A) Comparison of the effect of α -SNAP, α -SNAP(1–250), α -SNAP(41–295), and α -SNAP(81–295) with $n = 12$ in each case. Stimulation by α -SNAP was 2.7-fold and that by α -SNAP(41–295) and α -SNAP(81–295) was 1.3- and 1.6-fold, respectively, after correction for molar concentration to that of α -SNAP. (B) Comparison of the effect of α -SNAP and α -SNAP(21–295)

at two different concentrations of each added to the tubes ($n = 4$). Stimulation by α -SNAP was 3.5- and 4.2-fold at 10 and 40 μ g/ml and 1.7- and 3.7-fold for α -SNAP(121–295) after correction for molar concentration to that of α -SNAP.

(Sollner *et al.*, 1993a; Hanson *et al.*, 1995). The deletion mutants would, therefore, be unable to disrupt the function of endogenous NSF.

Deletions of up to 120 N-terminal residues of α -SNAP had no effect on the ability of the deletion mutants to become immobilized on plastic and to bind NSF. In contrast, α -SNAP(1–250) bound to plastic but did not bind NSF. The C-terminus of α -SNAP, therefore, appears to be important to allow NSF-binding, and α -SNAP(1–250) was unable to activate the ATPase activity of NSF. The N-terminally deleted α -SNAP mutants not only bound NSF but were also able to stimulate the ATPase activity of NSF, indicating that these mutants were able to make a significant interaction with NSF. α -SNAP(121–295) apparently bound to plastic only half as well as the full-length protein in experiments in which similar amounts of α -SNAP proteins were added and yet resulted in a similar fold stimulation of NSF ATPase activity. The reduced level of α -SNAP bound would be compensated by the smaller molecular mass and thus increased molar concentration of α -SNAP(121–295). Therefore on a molar basis (Figure 6 legend) the N-terminally deleted α -SNAP(121–295) had similar activity to the full length α -SNAP in NSF ATPase activation. The N-terminal 120 amino acids of α -SNAP are clearly not essential for NSF binding and activation.

The α -SNAP(1–250) C-terminal deletion construct was unable to bind or activate NSF. This does not necessarily mean that the C-terminal 45 amino acids constitute the NSF binding site. Their removal may have resulted in changes in the folding of the C-terminus of the mutant. In addition, the C-terminus deletion construct contained nine vector-derived

amino acids that could have interfered with binding. Nevertheless, the finding that disruption of the C-terminal predicted coiled-coil domain prevented NSF ATPase activation would be consistent with the role of such domains in protein-protein interactions. Further, mapping of this region will be necessary to define the essential and sufficient residues for NSF binding. Our data suggest, however, that the NSF-binding site of α -SNAP is very close to the syntaxin-binding domain. In a ternary complex of these three proteins (or in the 20S complex also containing synaptobrevin and SNAP-25), it would therefore be expected that NSF and syntaxin would be in close proximity. This could facilitate a mechanism whereby the putative chaperone-like activity of NSF (Hanson *et al.*, 1995; Morgan and Burgoyne, 1995a) could use the energy of ATP hydrolysis to induce a transient conformational change in syntaxin, rendering it temporarily incapable of binding synaptobrevin (Sollner *et al.*, 1993b; Hanson *et al.*, 1995) and other components of the SNARE complex. Considerable information is now available on the interactions between α -SNAP and syntaxin and other SNARE proteins. The binding site on syntaxin for α -SNAP resides in the predicted coiled-coil domain that is also required for binding of SNAP-25 and synaptobrevin to syntaxin (Chapman *et al.*, 1994; Hayashi *et al.*, 1994, 1995; Kee *et al.*, 1995). It is now clear that both N- and C-terminal domains of α -SNAP are required for its binding to syntaxin (Hanson *et al.*, 1995; Hayashi *et al.*, 1995; this study). It appears that syntaxin plays the major role in transmitting the conformational change leading to disassembly of the 20S complex following ATP hydrolysis by NSF because NSF cannot dissociate α -SNAP from SNAP-25 (Ha-

yashi *et al.*, 1995) and the N-terminal domain of syntaxin is essential both for NSF-driven dissociation of α -SNAP from syntaxin (Hayashi *et al.*, 1995) and also for disassembly of the in vitro-assembled 20S complex (Kee *et al.*, 1995). The N-terminal domain of syntaxin is capable of binding to the C-terminal domain of syntaxin and inhibiting synaptobrevin binding (Calakos *et al.*, 1994) and this intramolecular interaction may control the binding of syntaxin to other proteins. The energy generated during ATP hydrolysis by NSF could be transmitted via α -SNAP to the C-terminal coiled-coil domain of syntaxin to induce a nonbinding conformation or, alternatively, as suggested by the close proximity of NSF to the syntaxin-binding site, NSF could transmit conformational change via the essential N-terminal disassembly domain of syntaxin. In either case, syntaxin would be crucially important in the regulation of SNARE complex assembly and disassembly during vesicle docking/fusion. Such a scheme would involve a key role for α -SNAP in the activation of the ATPase activity of NSF following its recruitment to its syntaxin substrate in a similar manner to other well characterized chaperone/co-chaperone interactions (Hartl and Martin, 1995).

ACKNOWLEDGMENTS

We are grateful to Dr. J.E. Rothman and for the generous gifts of plasmids encoding His₆- α -SNAP and His₆-NSF and to Dr. R.H. Scheller for GST-syntaxin 1A (4–266). We thank Geoff Williams for assistance with chromaffin cell cultures. This work was supported by a grant from the Wellcome Trust to R.D.B. R.J.O.B. was supported by an Medical Research Council Research Studentship. We thank one of the reviewers of this paper who suggested the reanalysis of coiled-coil domains in α -SNAP.

REFERENCES

- Baumert, M., Maycox, P.R., Navone, F., De Camilli, P., and Jahn, R. (1989). Synaptobrevin: an integral membrane protein of 18 000 daltons in small synaptic vesicles of rat brain. *EMBO J.* 8, 379–384.
- Bennett, M.K., Calakos, N., and Scheller, R.H. (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicle at presynaptic active zones. *Science* 257, 255–259.
- Blasi, J., Chapman, E.R., Link, E., Binz, T., Yamasaki, S., De Camille, P., Sudhof, T.C., Niemann, H., and Jahn, R. (1993a). Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature* 365, 160–163.
- Blasi, J., Chapman, E.R., Yamasaki, S., Binz, T., Niemann, H., and Jahn, R. (1993b). Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. *EMBO J.* 12, 4821–4828.
- Burgoyne, R.D. (1992). Investigation of the intracellular regulators and components of the exocytotic pathway. In: *Neuromethods—Intracellular Messengers*, vol. 20, ed. A. Boulton, G. Baker, and C. Taylor, Totowa, NJ: Humana Press, 433–470.
- Calakos, N., Bennett, M.K., Peterson, K.E., and Scheller, R.H. (1994). Protein-protein interactions contributing to the specificity of intracellular vesicular trafficking. *Science* 263, 1146–1149.
- Chamberlain, L.H., Roth, D., Morgan, A., and Burgoyne, R.D. (1995). Distinct effects of α -SNAP, 14–3-3 proteins and calmodulin on priming and triggering of regulated exocytosis. *J. Cell Biol.* 130, 1063–1071.
- Chapman, E.R., An, S., Barton, N., and Jahn, R. (1994). SNAP-25, a t-SNARE which binds to both syntaxin and synaptobrevin via domains that may form coiled-coils. *J. Biol. Chem.* 269, 27427–27432.
- Clary, D.O., Griff, I.C., and Rothman, J.E. (1990). SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell* 61, 709–721.
- DeBello, W.M., O'Connor, V., Dresbach, T., Whiteheart, S.W., Wang, S.S.-H., Schweizer, F.E., Betz, H., Rothman, J.E., and Augustine, G.J. (1995). SNAP-mediated protein-protein interactions essential for neurotransmitter release. *Nature* 373, 626–630.
- Ferro-Novick, S., and Jahn, R. (1994). Vesicle fusion from yeast to man. *Nature* 370, 191–193.
- Hanson, P.L., Otto, H., Barton, N., and Jahn, R. (1995). The N-ethylmaleimide-sensitive fusion protein and α -SNAP induce a conformational change in syntaxin. *J. Biol. Chem.* 270, 16955–16961.
- Hartl, F.U., and Martin, J. (1995) Molecular chaperones in cellular protein folding. *Curr. Opin. Struct. Biol.* 5, 92–102.
- Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Sudhof, T.C., and Niemann, H. (1994). Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. *EMBO J.* 13, 5051–5061.
- Hayashi, T., Yamasaki, S., Nauenburg, S., Binz, T., and Niemann, H. (1995). Disassembly of the reconstituted synaptic vesicle membrane complex in vitro. *EMBO J.* 14, 2317–2325.
- Hodel, A., Schafer, T., Gerosa, D., and Burger, M.M. (1994). In chromaffin cells, the mammalian Sec1p homologue is a syntaxin 1A-binding protein associated with chromaffin granules. *J. Biol. Chem.* 269, 8623–8626.
- Kee, Y., Lin, R.C., Hsu, S.-C., and Scheller, R.H. (1995). Distinct domains of syntaxin are required for synaptic vesicle fusion complex formation and dissociation. *Neuron* 14, 991–998.
- Lanzetta, P.A., Alvarez, L.J., Reinach, P.S., and Candia, O.A. (1979). An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.* 100, 95–97.
- Lill, R., Dowhan, W., and Wickner, W. (1990) The ATPase activity of secA is regulated by acidic phospholipids, secY and the leader and mature domains of precursor proteins. *Cell* 60, 271–280.
- Lupas, A., VanDyke, M., and Stock, J. (1991). Predicting coiled-coils from protein sequences. *Science* 252, 1162–1164.
- McMahon, H.T., and Sudhof, T.C. (1995). Synaptic core complex of synaptobrevin, syntaxin and SNAP25 forms a high affinity α -SNAP binding site. *J. Biol. Chem.* 270, 2213–2217.
- Montecucco, C., and Schiavo, G. (1994). Molecular mechanisms of action of bacterial protein toxins. *Mol. Microbiol.* 13, 1–8.
- Morgan, A., and Burgoyne, R.D. (1995a). Is NSF a fusion protein? *Trends Cell Biol.* 5, 335–339.
- Morgan, A., and Burgoyne, R.D. (1995b). A role for soluble NSF attachment proteins (SNAPs) in regulated exocytosis in adrenal chromaffin cells. *EMBO J.* 14, 232–239.
- Morgan, A., Dimaline, R., and Burgoyne, R.D. (1994). The ATPase activity of N-ethylmaleimide-sensitive fusion protein NSF is regulated by soluble NSF attachment proteins. *J. Biol. Chem.* 269, 29347–29350.

- Niemann, H., Blasi, J., and Jahn, R. (1994). Clostridial neurotoxins: new tools for dissecting exocytosis. *Trends Cell Biol.* 4, 179–185.
- Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E., and Wilson, M.C. (1989). The identification of a novel synaptosomal-associated protein SNAP-25, differentially expressed by neuronal subpopulations. *J. Cell Biol.* 109, 3039–3052.
- Pellegrini, L.L., O'Connor, V., Lottspeich, F., and Betz, H. (1995). Clostridial neurotoxins compromise the stability of a low energy SNARE complex mediating NSF activation of synaptic vesicle fusion. *EMBO J.* 14, 4705–4713.
- Pevsner, J., Hsu, S.-C., Braun, J.E.A., Calakos, N., Ting, A.E., Bennett, M.K., and Scheller, R.H. (1994). Specificity and regulation of a synaptic vesicle docking complex. *Neuron* 13, 353–361.
- Pryer, N.K., Wuestehube, L.J., and Sheckman, R. (1992). Vesicle-mediated protein sorting. *Annu. Rev. Biochem.* 61, 471–516.
- Roth, D., and Burgoyne, R.D. (1994). SNAP-25 is present in a SNARE complex in adrenal chromaffin cells. *FEBS Lett.* 351, 207–210.
- Rothman, J.E. (1994). Mechanisms of intracellular protein transport. *Nature* 372, 55–62.
- Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R., and Montecucco, C. (1992). Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature* 359, 832–835.
- Schiavo, G., Gmachl, M.J.S., Stenbeck, G., Sollner, T., and Rothman, J. (1995). A possible docking and fusion particle for synaptic transmission. *Nature* 378, 733–736.
- Sollner, T. (1995). SNAREs and targeted membrane fusion. *FEBS Lett.* 369, 80–83.
- Sollner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R., and Rothman, J. (1993a). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* 75, 409–418.
- Sollner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993b). SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362, 318–324.
- Trimble, W.S., Cowan, D.M., and Scheller, R.H. (1988). VAMP-1: a synaptic vesicle-associated integral membrane protein. *Proc. Natl. Acad. Sci. USA* 85, 4538–4542.
- Whiteheart, S.W., Griff, I.C., Brunner, M., Clary, D.O., Mayer, T., Buhrow, S.A., and Rothman, J.E. (1993). SNAP family of NSF attachment proteins includes a brain-specific isoform. *Nature* 362, 353–355.
- Whiteheart, S.W., and Kubalek, E.W. (1995). SNAPs and NSF general members of the fusion apparatus. *Trends Cell Biol.* 5, 64–68.
- Wilson, D.W., Wilcox, C.A., Flynn, G.C., Chen, E., Kuang, W.J., Henzel, W.J., Block, M.R., Ullrich, A., and Rothman, J.E. (1989). A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature* 339, 355–359.