

Stimulation of NSF ATPase activity during t-SNARE priming

Lee P. Haynes, Richard J.O. Barnard¹, Alan Morgan, Robert D. Burgoyne*

The Physiological Laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX, UK

Received 20 July 1998

Abstract *N*-Ethylmaleimide-sensitive factor (NSF) plays a key role in vesicular traffic by disassembling and priming SNARE proteins for their function in docking and fusion. We demonstrate that the ATPase activity of NSF is activated by α -soluble NSF attachment protein (α -SNAP) in a complex with syntaxin 1A. In addition, we show that a construct consisting of the H3 domain of syntaxin 1A (GST-synt(195–263), which does not support NSF disassembly in the presence of MgATP gave a larger stimulation. NSF ATPase activation was specific and did not occur using mutant α -SNAPs unable to bind GST-synt or with mutated C-termini. We suggest that activation of NSF ATPase activity in the SNARE complex may be essential to allow SNARE priming.

© 1998 Federation of European Biochemical Societies.

Key words: Syntaxin; *N*-Ethylmaleimide-sensitive factor; ATPase; Vesicular traffic; Soluble NSF attachment protein receptor

1. Introduction

N-Ethylmaleimide-sensitive factor (NSF) is a member of a large family of ATPases with diverse cellular functions known as the AAA ATPases [1]. NSF [2,3] and its yeast homologue Sec18p [4] have been shown to play essential roles in many aspects of the vesicular traffic within cells including transport from the ER to the Golgi [5], intra-Golgi transport [3], neurotransmitter release [6] and endocytic transport [7,8]. NSF exerts its effects following its recruitment, via soluble NSF attachment proteins (SNAPs) [9,10], to a class of membrane proteins collectively termed SNAP receptors (SNAREs) and originally designated as v-(vesicular) or t-(target) SNAREs [11]. ATP hydrolysis by NSF leads to dissociation of itself and SNAPs from the SNAREs and to disassembly of SNARE protein complexes in vitro [12].

It was originally suggested that disassembly of SNARE complexes, and particularly, disruption of v-SNARE/t-SNARE pairing would occur following transport vesicle docking as a late step leading to membrane fusion [12]. This model has been extensively debated [13] and it is now clear that SNAP and NSF can function prior to vesicle docking in various systems [14–19] and that SNARE complex disassembly and disruption of v-SNARE/t-SNARE interaction by SNAP and NSF can occur on isolated transport vesicles [16,17] as a prelude to their docking and fusion [16]. In addition to this

event, another role of SNAP and NSF has been uncovered, in which they also prime t-SNAREs for their subsequent function [16,20]. In vitro, SNAP and NSF action can modify the conformation of the neuronal t-SNARE syntaxin 1A such that it can no longer bind α -SNAP or the v-SNARE VAMP [20] and in yeast vacuole-vacuole fusion Sec18p and Sec17p have a direct and essential priming action on vacuoles containing t- but not v-SNAREs [16]. The priming reaction, which requires ATP hydrolysis by NSF, thus consists of distinct molecular events including SNARE complex disruption and also specific priming of t-SNAREs for their subsequent role in vesicle docking and fusion [16]. The molecular events during ATP hydrolysis and the chaperone-like action of NSF [13] are thus crucial in the steps leading to transport vesicle fusion with target membranes.

It has previously been demonstrated that, while SNAPs cannot efficiently bind NSF in solution, they can do so following their immobilisation on plastic [9]. This has been presumed to be due to generation of a conformational change in SNAPs similar to that occurring on binding to SNAREs [9]. We have previously demonstrated that plastic-immobilised SNAPs not only recruit NSF but also stimulate its ATPase activity [21–23]. Evidence that this activation is physiologically significant includes the finding that α -SNAP stimulates hydrolysis by the D1 but not the D2 ATPase domain of NSF [24] and only the ATPase activity of the D1 domain is absolutely essential for NSF function in vesicular traffic [25]. In addition, mutations near the C-terminus of α -SNAP abolish its ability to stimulate NSF ATPase activity and such mutant proteins can no longer support SNARE complex disassembly or secretory vesicle fusion [26]. We examine here the ability of α -SNAP to stimulate NSF in a complex with the t-SNARE syntaxin 1A. This SNARE protein is the major neuronal SNAP receptor [20], has an essential role in regulated exocytosis [27–29] and syntaxin homologues are required for various other intracellular fusion events [15,16,27]. The data presented indicate that ATP hydrolysis by NSF is activated within an NSF/ α -SNAP/syntaxin 1A complex suggesting that this activation is a key molecular event in the t-SNARE priming reaction.

2. Materials and methods

2.1. Buffers

ATPase buffer: 100 mM KCl, 25 mM Tris, 2 mM MgCl₂, 0.6 mM ATP, 0.5 mM DTT, 10% (v/v) glycerol, pH 7.4. Binding buffer: 150 mM NaCl, 20 mM HEPES, 2 mM MgCl₂, 1 mM DTT, 0.5% (v/v) Triton X-100, pH 7.4. GST elution buffer: 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0.

2.2. Plasmid constructs

A plasmid encoding essentially the full length cytoplasmic domain of syntaxin 1A (amino acids 4–266) fused to glutathione-S-transferase (GST) was a gift from Dr R. Scheller (Howard Hughes Medical Institute, Stanford University, CA, USA). A truncated syntaxin consist-

*Corresponding author. Fax: (44) (151) 794-5337.
E-mail: burgoyne@liverpool.ac.uk

¹Present address: Department of Genetics, University of Wisconsin, Madison, Wisconsin, USA.

Abbreviations: GST, glutathione-S-transferase; NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor

ing of the H3 domain (synt 195–263) was amplified by PCR using Taq polymerase. The primers used contained restriction endonuclease sites to permit subcloning into the pGex 4T-2 vector (Pharmacia). Expression from this vector generates GST fusion proteins where the GST moiety is attached, via a thrombin cleavable linker region, to the N-terminus of the cloned protein. Primers used for amplification were as follows: sense: *Eco*RI 5'-GCCCGAATTCAGATCGAGACCAGG-CACAGT-3'; antisense: *Xho*I 5'-CCCCCTCGAGTTACCTGCGT-GCCTTGCTCTGG-3'. The amplified PCR product was ligated into pGex4T-2 and checked by automated sequencing.

2.3. Recombinant protein expression

GST-syntaxin fusion protein constructs were expressed in *E. coli* M15 [pREP4] cells (Qiagen) and protein expression induced using 1 mM isopropyl-1-thio- β -D-galactopyranoside for 4 h. Proteins were purified from cytosolic fractions on glutathione-Sepharose 4B (Pharmacia) and eluted by application of buffer containing 10 mM reduced glutathione. NSF, α -SNAP and α -SNAP mutant constructs [24] were expressed as His₆-tagged proteins and purified using Nickel-NTA agarose (Qiagen) according to published methods [10,26]. GST-VAMP was a gift from Dr Clifford Shone (PHLS, Salisbury, UK).

2.4. NSF ATPase assays

For analysis of the ATPase activity of NSF immobilised on plastic, α -SNAP and α -SNAP mutant proteins were immobilised by a 20-min incubation in 1.5 ml polypropylene Eppendorf tubes at room temperature (5 μ g protein in a final volume of 20 μ l). SNAP solutions were discarded and 50 μ l NSF (20 μ g/ml, 0.044 μ M) in ATPase buffer added to each tube. For every condition, control tubes were incubated with 50 μ l of NEM-inactivated NSF (2 mM NEM for 20 min on ice). All samples were then incubated for 1 h at 37°C and ATPase activity measured using a spectrophotometric method [30,31]. Corrections for contaminating ATPase and endogenous phosphate in protein samples were made by subtracting the NEM-treated control values from the non-NEM-treated incubations.

For analysis of NSF ATPase activity during SNARE priming, syntaxin constructs (at 5 μ M unless otherwise stated) and α -SNAP or α -SNAP mutants (2.5 μ M) were preincubated on ice for 2 h together in glass test tubes. NSF was then added to a final concentration of 50 nM, the concentration based on NSF as a hexameric protein [32], in a final reaction volume of 50 μ l. All samples were then incubated for 1 h at 37°C and ATPase activity determined as described above with NEM-treated NSF samples run as controls for all conditions. All data are shown with NEM-treated control values subtracted. These values were closely similar under all conditions.

2.5. GST-syntaxin/ α -SNAP/NSF binding and disassembly assay

GST syntaxin 4–266 and GST syntaxin 195–263 (1 μ M) were immobilised onto 40 μ l of a 50% slurry of GST-Sepharose in a 30-min incubation at 4°C. α -SNAP was then added to a final concentration of 5 μ M in a total volume of 100 μ l of binding buffer. Incubations were then continued for a further 2 h at 4°C with periodic agitation. Sepharose bound syntaxin/SNAP complexes were then pelleted by brief centrifugation and supernatants removed. NSF in either binding buffer, or binding buffer with excess EDTA (5 mM) was added to each condition at 100 nM in a total volume of 100 μ l and incubations continued for a further hour at 4°C. GST-Sepharose was again pelleted and washed twice with 1 ml of the appropriate binding buffer (with or without EDTA). Finally beads were removed to fresh centrifuge tubes and bound proteins solubilised by boiling in the presence of 40 μ l SDS dissociation buffer. Bound NSF was analysed by SDS-PAGE on 15% polyacrylamide gels and immunoblotting.

2.6. GST-syntaxin/ α -SNAP binding assay

GST-synt(195–263) at 5 μ M was incubated with 2.5 μ M α -SNAP or α -SNAP mutants in a total volume of 100 μ l of binding buffer for 2 h at 4°C with periodic mixing. 20 μ l of a 50% slurry of GST-Sepharose was then added to each condition and incubation continued for a further 1 h at 4°C. GST-Sepharose was pelleted by centrifugation at 13 000 rpm in a microfuge for 1 min and supernatants discarded. Each

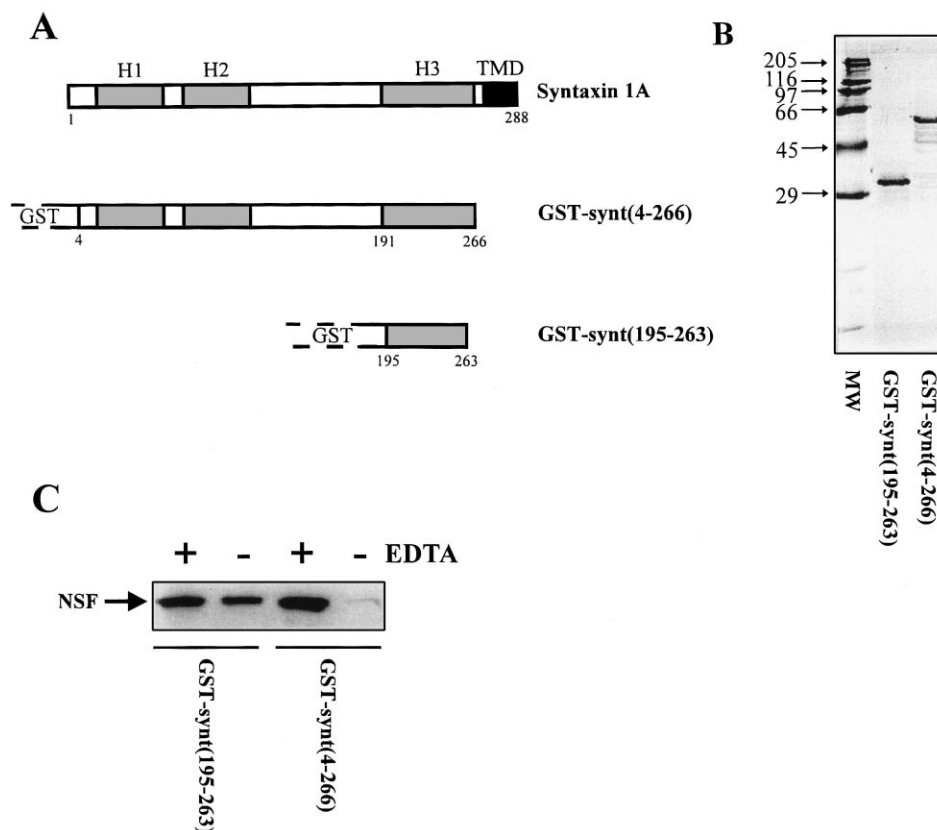


Fig. 1. GST-Syntaxin constructs, analysis of purified proteins and binding of NSF. A: Schematic representation of syntaxin 1A and the two syntaxin GST-fusion proteins used in this study. B: SDS-PAGE analysis of purified GST-synt(195–263) and GST-synt(4–266). C: Association of NSF with α -SNAP and syntaxin GST-fusion proteins in the presence of MgATP or with excess EDTA to prevent ATP hydrolysis.

pellet was washed twice with binding buffer and bound proteins solubilised by boiling in 50 μ l SDS dissociation buffer. Bound His-tagged SNAPs were analysed by SDS-PAGE on 15% polyacrylamide gels and immunoblotting with monoclonal anti-polyhistidine antibody (Sigma).

3. Results

Assembly of SNAP and NSF onto a t-SNARE protein, subsequent disassembly of this complex and priming of the t-SNARE have been demonstrated in a minimal system consisting of α -SNAP, NSF and syntaxin 1A [20]. We examined whether SNAP-activation of the NSF ATPase would occur in the minimal complex of these three proteins. In this system, the primed syntaxin 1A cannot subsequently rebinding α -SNAP or NSF [20] suggesting that only short-lived stimulation of NSF ATPase activity would occur under these conditions. In contrast, constructs in which only the H3 domain of syntaxin 1A is fused to GST can bind α -SNAP and NSF but do not support ATP-dependent disassembly and NSF and α -SNAP remain bound in the presence of MgATP [20,33,34]. A complex formed with an H3 domain construct could potentially support continued ATPase activation. We aimed, therefore, to compare NSF ATPase activity in complexes containing the cytoplasmic domain of syntaxin 1A or only the H3 domain as GST fusion proteins (Fig. 1A,B). The two syntaxin 1A constructs were found to bind α -SNAP and the ability of both GST-synt(4–266) and GST-synt(195–263) to recruit α -SNAP and NSF but only GST-synt(4–266) to support ATP-dependent disassembly of NSF was demonstrated with the constructs used here (Fig. 1C).

When α -SNAP and NSF are combined in glass tubes, relatively little activation of NSF ATPase activity occurs as the proteins do not efficiently associate under these conditions [21]. In the present experiments, α -SNAP was added in a large molar excess over NSF to ensure maximal activation of the NSF ATPase. At these molar ratios a low level of activation of NSF due to α -SNAP alone was seen in all experiments.

Addition of increasing amounts of GST-synt(4–266) or the H3 domain construct GST-synt(195–263) to α -SNAP and NSF in glass resulted in a stimulation of NSF ATPase activity over that due to α -SNAP alone (Fig. 2A). For reasons that are unclear, higher concentrations of the GST syntaxin constructs were less effective. The peak stimulation (at 5 μ M syntaxin) was consistently greater for GST-synt(195–263) (4.51 ± 0.52 -fold, compared to control which in this case had both α -SNAP and NSF present, $n=8$ experiments) than for GST-synt(4–266) (1.96 ± 0.15 -fold, $n=7$). A difference in the extent of stimulation cannot be explained by differences in NSF recruitment to the syntaxin constructs (Fig. 1C). It is, however, consistent with the behaviour of the complexes of these proteins in previous assembly/disassembly studies [20,33,34] and as shown in Fig. 1C, and suggests that in the non-disassembling complex with the H3 domain construct continuing futile cycles of ATP hydrolysis occur. The stimulation of the NSF ATPase was specific since no significant stimulation over that due to α -SNAP alone was seen in the presence of GST or of GST-VAMP (Fig. 2B). In this experiment GST-synt(195–263) present with α -SNAP gave a 9-fold stimulation of NSF ATPase activity compared to that of NSF alone without added α -SNAP (Fig. 2B, column 4 vs. column 1).

In order to establish that the stimulation of NSF ATPase activity by syntaxin 1A is a specific α -SNAP mediated process, we used a series of α -SNAP mutants [26]. We first examined the ability of these mutant α -SNAPs to bind to GST-synt(195–263). The α -SNAP (L294A) and α -SNAP(1–285) bound to GST-synt(195–263) to a similar extent as wild-type α -SNAP (Fig. 3). In contrast, the two mutants with large C- or N-terminal deletions, α -SNAP(1–200) and α -SNAP(121–295) gave little or no detectable binding. In the plastic-immobilisation assay, α -SNAP (L294A) and α -SNAP(1–285) can bind NSF but cannot stimulate its ATPase activity [26]. In the present experiments this was confirmed and it was also seen that α -SNAP(1–200) could not stimulate NSF ATPase activity on plastic but the N-terminally deleted α -SNAP(121–

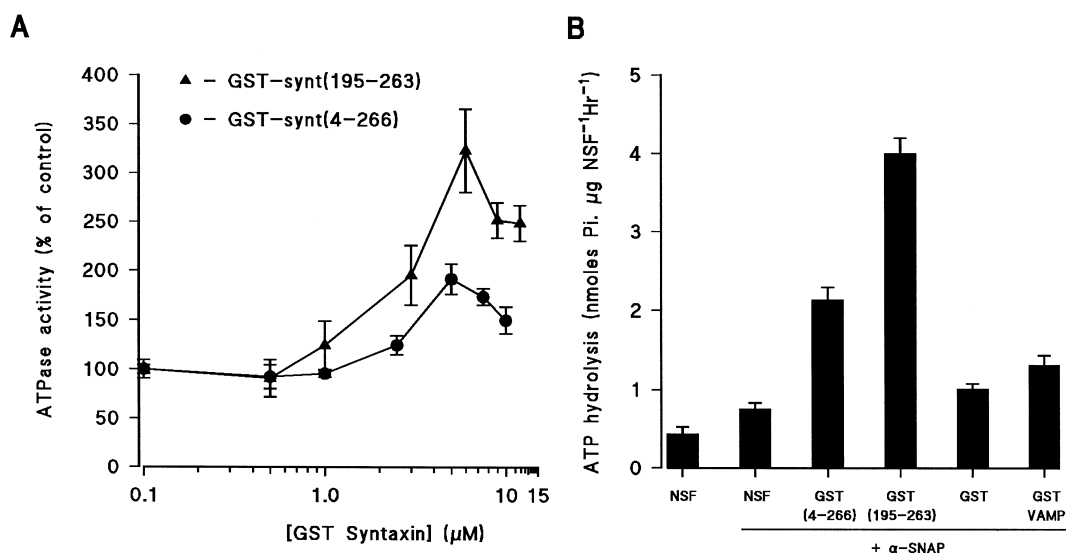


Fig. 2. Effect of GST-fusion proteins on SNAP-activation of the NSF ATPase activity. A: GST-synt(4–266) or GST-synt(195–263) were added at various concentrations to incubations containing 2.5 μ M α -SNAP and 50 nM NSF and ATPase activity was determined. For each condition, control values from NEM-treated NSF incubations were subtracted ($n=4$). B: NSF (50 nM) was incubated alone with 2.5 μ M α -SNAP or with α -SNAP plus GST-synt(4–266), GST-synt(195–263), GST or GST-VAMP, all at 5 μ M. All data are shown with NEM-treated controls subtracted ($n=4$).

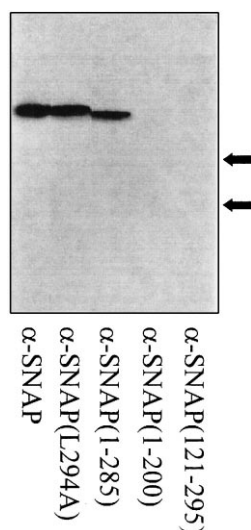


Fig. 3. Analysis of binding of α -SNAP and α -SNAP mutants to GST-synt(195–263). GST-synt(195–263) at 5 μ M was incubated with 2.5 μ M α -SNAP or α -SNAP mutants as indicated. GST-synt(195–263) and any bound α -SNAPs were recovered on glutathione-Sepharose beads and bound α -SNAPs detected using anti-polyhistidine antibody that recognises the His₆-tag in all of the α -SNAPs. The positions of migration of α -SNAP(1–200) and α -SNAP(121–295) which did not bind are indicated by the upper and lower arrows, respectively. No binding of α -SNAP to beads alone was detected.

295) did so (Fig. 4A). In incubations in glass with GST-synt(195–263), activation of NSF ATPase activity only occurred to any significant extent with wild-type α -SNAP (11-fold over that due to NSF alone) and not with any of the α -SNAP mutants (Fig. 4B). The lack of ability of α -SNAP(L294A), α -SNAP(1–285) and α -SNAP(1–200) to stimulate the NSF ATPase is consistent with the plastic-immobilisation assay in which they are inactive. In contrast, α -SNAP(121–295) could stimulate NSF on plastic but not in conjunction with GST-synt(195–263) (Fig. 4B) presumably due to its in-

ability to bind to GST-synt(195–263) (Fig. 3). These data show that the stimulation of NSF ATPase activity requires an α -SNAP that can bind syntaxin. Binding and complex formation is, however, not sufficient and ATPase activation cannot occur with α -SNAP constructs with C-terminal mutations that have previously been shown to be inactive in both NSF ATPase activation on plastic and in SNARE complex disassembly [26].

4. Discussion

We have demonstrated that NSF ATPase activity is stimulated following assembly of α -SNAP and NSF into a complex with the t-SNARE syntaxin 1A in a specific process that requires assembly of the three components since α -SNAP mutants that cannot bind the H3 domain of syntaxin 1A do not stimulate the NSF ATPase. Another SNARE, VAMP, was unable to stimulate NSF ATPase in the presence of α -SNAP consistent with its inability to detectably bind α -SNAP in the absence of other SNARE proteins [20,33,34]. Finally, assembly into a complex is insufficient for NSF ATPase activation as α -SNAPs with C-terminal mutations were unable to stimulate NSF ATPase in the presence of GST-synt(195–263) despite their ability to bind to this protein. This lack of stimulation was consistent with the inability of these C-terminal mutants to stimulate NSF ATPase after immobilisation on plastic. These latter findings support the idea that activation of NSF ATPase activity by immobilised α -SNAP is a faithful representation of a physiologically occurring process normally requiring assembly onto a SNARE protein and emphasises the crucial nature of the C-terminus of α -SNAP for a productive NSF/ α -SNAP/SNARE interaction [26].

It seems likely that activation of the NSF ATPase by α -SNAP within a SNARE complex is a crucial event for subsequent complex disassembly and t-SNARE priming as previously suggested from the inability of C-terminally mutated

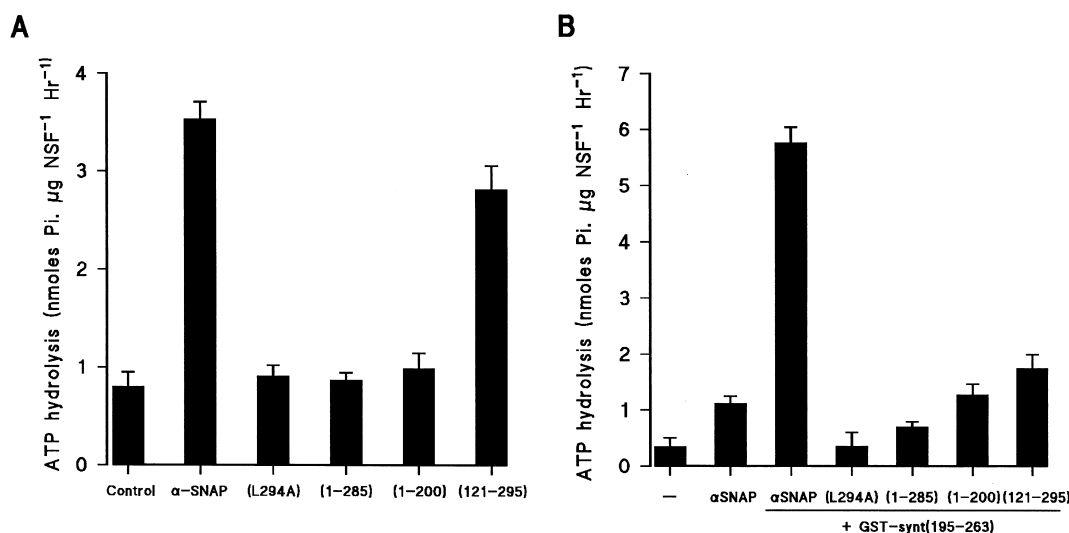


Fig. 4. Stimulation of NSF ATPase activity by α -SNAP and effects of α -SNAP mutants. A: Effect of α -SNAP and indicated mutants in a plastic-immobilisation assay with 5 μ g per tube α -SNAP and 1 μ g NSF showing the inability of α -SNAP(L294A), α -SNAP(1–285) and α -SNAP(1–200) to stimulate NSF ATPase activity whereas wild-type α -SNAP and α -SNAP(121–295) could do so ($n=4$). B: Comparison of the effect of α -SNAP and indicated α -SNAP mutants to activate NSF ATPase in glass in the presence of GST-synt(195–263). The first two incubations contained NSF alone or NSF and α -SNAP. The other incubations contained NSF, α -SNAP or α -SNAP mutants and GST-synt(195–263). Only wild-type α -SNAP was able to significantly stimulate NSF ATPase activity in the presence of GST-synt(195–263) ($n=4$).

α -SNAPs to support NSF-dependent disassembly of VAMP from SNARE complexes immunoprecipitated from brain membranes [26]. These previous findings suggested that the intrinsic ATPase activity of NSF is insufficient for the disruption of v-SNARE/t-SNARE interaction [26] and that the increase in ATPase activity due to α -SNAP stimulation of the D1 domain of NSF [24] is essential. It is not known for certain whether simultaneous ATP hydrolysis by all NSF subunits within the hexamer is required for the action of NSF in SNARE complex disruption but this is likely as NSF containing only a single non-enzymatically active subunit is non-functional in Golgi transport assays [25]. The maximal level of stimulation of the NSF ATPase seen here in the presence of α -SNAP and GST-synt(195–263) was considerably greater (around 10-fold increase over NSF alone) than that previously observed [21–23,26] in the plastic-immobilisation assay and indicates that a substantial increase in NSF ATPase activity occurs within a SNARE complex. By analogy with well characterised molecular chaperones it could be envisaged that the chaperone-like action of NSF would require one or more cycles of concerted ATP hydrolysis to allow correct re-folding of the t-SNARE to occur. It is possible that activated NSF functions as a conformational quality control check by continuing to hydrolyse ATP in the complex until the correctly printed t-SNARE conformation is achieved.

The correct interaction of SNARE, SNAP and NSF to allow NSF ATPase activation may be an important molecular switch that, when tripped, initiates SNARE complex disassembly and t-SNARE priming. If so, cycles of nucleotide binding and hydrolysis by NSF would determine SNARE conformation. NSF in the ATP-bound state would interact with SNAREs via SNAPs [32,35]. Formation of the SNARE/SNAP/NSF complex would activate the NSF ATPase resulting in t-SNARE priming and conversion of NSF into the ADP bound state which is unable to interact with SNAPs/SNAREs [32,35] and so release NSF from the complex. Exchange of ADP for ATP would then complete the cycle and allow NSF to catalyse another round of priming. This hypothetical model is very similar to that proposed for the rab GTPases, in which only rabs in the GTP-bound state are able to interact with their membrane effectors, and where GTPase activity results in conversion to the GDP-bound form which allows release of rab into the cytosol. Exchange of GDP for GTP then completes the cycle. Although there are differences between the two proposed cycles (e.g. nucleotide hydrolysis by NSF enables subsequent membrane fusion, whereas the time spent by rabs in the GTP-bound state acts as a fusogenic window of opportunity [36]), the similarities are intriguing in view of the requirement for both proteins in membrane traffic. In particular, since rab GTP/GDP exchange and GTP hydrolysis are precisely regulated by various proteins, it may be that NSF nucleotide binding and hydrolysis are also under tight control by protein factors other than SNAPs and SNAREs during the complex membrane fusion process.

Acknowledgements: We are grateful to Drs. J. Rothman and R. Scheller for the gift of plasmids, Dr. Greg Steel for NSF, Dr. Clifford Shone for GST-VAMP protein and Dr. Albert Haas for comments on this paper. This work was supported by grants from the Wellcome Trust to R.D.B. and A.M. This work was supported by grants from the MRC and the Wellcome Trust. L.P.H. was in receipt of a Wellcome Trust Prize Studentship.

References

- [1] Confalonieri, F. and Duguet, M. (1995) *BioEssays* 17, 639–651.
- [2] Block, M.R., Glick, B.S., Wilcox, C.A., Wieland, F.T. and Rothman, J.E. (1988) *Proc. Natl. Acad. Sci. USA* 85, 7852–7856.
- [3] 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) *Nature* 339, 355–359.
- [4] Pryer, N.K., Wuestehube, L.J. and Schekman, R. (1992) *Annu. Rev. Biochem.* 61, 471–516.
- [5] Beckers, C.J.M., Block, M.R., Glick, B.S., Rothman, J.E. and Balch, W.E. (1989) *Nature* 339, 397–398.
- [6] Pallanck, L., Ordway, R.W. and Ganetzky, B. (1995) *Nature* 376, 25.
- [7] Diaz, R., Mayorga, L.S., Weidman, P.J., Rothman, J.E. and Stahl, P.D. (1989) *Nature* 339, 398–400.
- [8] Rodriguez, L., Stirling, C.J. and Woodman, P.G. (1994) *Mol. Biol. Cell* 5, 773–783.
- [9] Clary, D.O., Griff, I.C. and Rothman, J.E. (1990) *Cell* 61, 709–721.
- [10] Whiteheart, S.W., Griff, I.C., Brunner, M., Clary, D.O., Mayer, T., Buhrow, S.A. and Rothman, J.E. (1993) *Nature* 362, 353–355.
- [11] Sollner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J.E. (1993) *Nature* 362, 318–324.
- [12] Sollner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R. and Rothman, J. (1993) *Cell* 75, 409–418.
- [13] Morgan, A. and Burgoyne, R.D. (1995) *Trends Cell Biol.* 5, 335–339.
- [14] Mayer, A., Wickner, W. and Haas, A. (1996) *Cell* 85, 83–94.
- [15] Nichols, B.J., Ungermann, C., Pelham, H.R.B., Wickner, W.T. and Haas, A. (1997) *Nature* 387, 199–202.
- [16] Ungermann, C., Nichols, B.J., Pelham, H.R.B. and Wickner, W. (1998) *J. Cell Biol.* 140, 61–69.
- [17] Otto, H., Hanson, P.I. and Jahn, R. (1997) *Proc. Natl. Acad. Sci. USA* 94, 6197–6201.
- [18] Chamberlain, L.H., Roth, D., Morgan, A. and Burgoyne, R.D. (1995) *J. Cell Biol.* 130, 1063–1071.
- [19] Rowe, T., Dascher, C., Bannykh, S., Pluttner, H. and Balch, W.E. (1998) *Science* 279, 696–700.
- [20] Hanson, P.I., Otto, H., Barton, N. and Jahn, R. (1995) *J. Biol. Chem.* 270, 16955–16961.
- [21] Morgan, A., Dimaline, R. and Burgoyne, R.D. (1994) *J. Biol. Chem.* 269, 29347–29350.
- [22] Sudlow, A.W., McFerran, B.W., Bodill, H., Barnard, R.J.O., Morgan, A. and Burgoyne, R.D. (1996) *FEBS Lett.* 393, 185–188.
- [23] Barnard, R.J.O., Morgan, A. and Burgoyne, R.D. (1996) *Mol. Biol. Cell* 7, 693–701.
- [24] Steel, G.J. and Morgan, A. (1998) *FEBS Lett.* 423, 113–116.
- [25] Whiteheart, S.W., Rossmagel, K., Buhrow, S.A., Brunner, M., Jaenicke, R. and Rothman, J.E. (1994) *J. Cell Biol.* 126, 945–954.
- [26] Barnard, R.J.O., Morgan, A. and Burgoyne, R.D. (1997) *J. Cell Biol.* 139, 875–883.
- [27] Hay, J.C. and Scheller, R.H. (1997) *Curr. Opin. Cell Biol.* 9, 505–512.
- [28] Broadie, K., Prokop, A., Bellen, H.J., O’Kane, C.J., Schulze, K.L. and Sweeney, S.T. (1995) *Neuron* 15, 663–673.
- [29] Ogawa, H., Harada, S.-I., Sassa, T., Yamamoto, H. and Hosono, R. (1998) *J. Biol. Chem.* 273, 2192–2198.
- [30] Lanzetta, P.A., Alvarez, L.J., Reinach, P.S. and Candia, O.A. (1979) *Anal. Biochem.* 100, 95–97.
- [31] Lill, R., Dowham, W. and Wickner, W. (1990) *Cell* 60, 271–280.
- [32] Hanson, P.I., Roth, R., Morisaki, H., Jahn, R. and Heuser, J.E. (1997) *Cell* 90, 523–535.
- [33] Kee, Y., Lin, R.C., Hsu, S.-C. and Scheller, R.H. (1995) *Neuron* 14, 991–998.
- [34] Hayashi, T., Yamasaki, S., Nauenburg, S., Binz, T. and Niemann, H. (1995) *EMBO J.* 14, 2317–2325.
- [35] Matveeva, E.A., He, P. and Whiteheart, S.W. (1997) *J. Biol. Chem.* 272, 26413–26418.
- [36] Rybin, V., Ullrich, O., Rubino, M., Alexandrov, K., Simon, I., Seabra, M.C., Goody, R. and Zerial, M. (1996) *Nature* 383, 266–269.