Analysis of NSF Mutants Reveals Residues Involved in SNAP Binding and ATPase Stimulation†

William G. C. Horsnell, Gregor J. Steel,‡ and Alan Morgan*

The Physiological Laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX, U.K.

Received December 12, 2001; Revised Manuscript Received February 16, 2002

ABSTRACT: *N*-Ethylmaleimide-sensitive fusion protein (NSF) and its yeast orthologue, Sec18, are cytoplasmic AAA$^+$ ATPases required for most intracellular membrane fusion events. The primary function of NSF is thought to be the disassembly of *cis*-SNARE complexes, thus allowing *trans*-SNARE complex formation and subsequent membrane fusion. The importance of NSF/Sec18 in intracellular membrane traffic in vivo is highlighted by the inhibition of neurotransmission in *Drosophila comatose* (NSF) mutants and of constitutive secretion in yeast *sec18* mutants. However, the underlying biochemical defects in these mutant proteins are largely unknown. Here, we identify the *sec18-1* mutation as a G89D substitution in the N domain of Sec18p. This mutation results in an inhibition of the mutant protein's ability to bind to Sec17p (yeast α-SNAP). In contrast, engineering the *comatose*^{st53} mutation (S483L) into mammalian NSF (S491L) has no effect on α-SNAP binding. Instead, the stimulation of ATPase activity by α-SNAP required for wild-type NSF to disassemble SNARE complexes does not occur in the mutant NSF^{st53} protein. This biochemical phenotype predicts a dominant negative effect, which was confirmed by engineering the *st53* mutation into Sec18 (A505L), resulting in a dominant lethal phenotype in vivo. These findings suggest a biochemical basis for the block in membrane fusion observed in the mutant organisms. Furthermore, the mutants characterized here define key residues involved in two essential, but mechanistically distinct, biochemical functions of NSF: SNAP binding and SNAP-dependent ATPase stimulation.

Intracellular membrane fusion is known to require interactions between SNARE[1] proteins. Although controversy remains over whether such interactions drive bilayer fusion directly (*1*, *2*), it is generally accepted that the formation of a *trans*-SNARE complex between SNAREs on opposing vesicle and target membranes is essential in enabling membrane fusion. For *trans*-SNARE complex formation to proceed, however, *cis*-SNARE complexes residing in the same membrane must first be disassembled. Disassembly is clearly necessary to recycle *cis*-SNARE complexes formed after membrane fusion, but is also required prior to fusion to separate vesicular *cis*-SNARE complexes (*3*). In most cases, the disassembly of cellular SNARE complexes is performed by α-SNAP and the hexameric ATPase NSF (*4*). It is thought that α-SNAP both recruits NSF to the SNARE complex and stimulates ATP hydrolysis by NSF, resulting in dissassembly of the complex (*5*, *6*). This would then release SNARE proteins to engage in *trans* and hence function in further membrane fusion events.

In view of the near ubiquitous requirement for NSF in membrane fusion, much work has gone into structural and functional analysis of NSF and its yeast orthologue, Sec18. Both proteins share a characteristic domain structure, with an N-terminal domain (N) followed by two AAA$^+$ ATPase domains (D1 and D2). The N domain is required for interactions with α-SNAP (Sec17 in yeast), the D1 domain for ATP hydrolysis, and the D2 domain for hexamerization (*4*). These domains are arranged such that the SNARE complex is positioned inside the NSF hexamer, with α-SNAP interacting with both the SNARE complex and the outside face of the N domains (*7*). The crystal structures of the NSF and Sec18 N domains reveal remarkable similarity, with both comprising two β-sheet-rich subdomains, N_A and N_B, in near identical orientations (*8*−*10*). This structural similarity extends to function, as Sec18p can replace mammalian NSF in biochemical and membrane traffic assays (*11*−*13*).

In vivo evidence for a requirement of NSF in membrane fusion comes from the *comatose* mutants in *Drosophila melanogaster* (*14*) and *sec18* mutants in *Saccharomyces cerevisiae* (*15*). These mutants are characterized by temperature-sensitive blocks in presynaptic neurotransmission and membrane traffic through the biosynthetic pathway, respectively. The original *comatose* mutants isolated by Siddiqi and Benzer (*14*) are known to be due to G274E (*st17* allele) and S483L (*st53* allele) substitutions (*16*). Engineering the *st17* mutation into mammalian NSF (G282E) results in an abolition of NSF ATPase activity, consistent with the position of this residue in the Walker A box required for nucleotide

† This work was supported by project grants from The Wellcome Trust to A.M.

* To whom correspondence should be addressed. Telephone: +44 (0)1517945333. Fax: +44(0)1517945337. E-mail: amorgan@liverpool.ac.uk.

‡Present address: School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, U.K.

[1] Abbreviations: NEM, *N*-ethylmaleimide; NSF, NEM-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; AAA$^+$ ATPases, ATPases associated with a variety of cellular activities; DTT, dithiothreitol; BSA, bovine serum albumin; SEM, standard error of the mean.

10.1021/bi0160359 CCC: $22.00
Published on Web 03/29/2002

hydrolysis (*17*). However, no such biochemical analysis has been performed on the *st53* mutation, which maps to a region of unknown function at the interface of the D1 and D2 domains. Of the original *sec18* mutants isolated by Novick et al. (*15*), all that is known is that the widely used *sec18-1* allele maps to a 120-amino acid region in the N domain (*18*). Here, we report the identification of the *sec18-1* mutation as a G89D substitution and the biochemical characterization of this and the comatose *st53* allele using recombinant proteins. Our results suggest a mechanistic basis for the block in membrane fusion seen in the mutant organisms and also provide novel information about key residues that are important for SNAP binding and ATPase stimulation by SNAP.

EXPERIMENTAL PROCEDURES

Materials. Affinity-purified anti-Sec18p antiserum was a generous gift from A. Haas (University of Wurzburg, Wurzburg, Germany). The *sec18-1* yeast strain RSY271 (MAT**a**; *his4-619*; *ura3-52*; *sec18-1*) and its isogenic wild-type parent, RSY249 (MAT**a**; *his4-619*; *ura3-52*), were generously provided by R. Schekman (University of California, Berkeley, CA) via A. Haas. Monoclonal anti-NSF antibody was a generous gift from M. Tagaya (Kyoto University, Kyoto, Japan). The monoclonal anti-VAMP2 antibody was a generous gift from M. Takahashi (Mitsubishi Kasei Institute of Life Science, Tokyo, Japan). The pSEY8 plasmid was a gift from S. Emr (University of California, San Diego, CA). Plasmids encoding His_6-tagged NSF and α-SNAP were gifts from J. Rothman (Memorial Sloan Kettering Cancer Center, New York, NY). Monoclonal anti-α/*β*-SNAP antibody was obtained from Synaptic Systems (Göttingen, Germany). Yeast transformation kits were purchased from Invitrogen (Groningen, The Netherlands); QIAAMP genomic DNA isolation kits, pQE vectors, and nitrilotriacetic acid (NTA) agarose were purchased from Qiagen (Dorking, U.K.). Unless otherwise specified, all other reagents were analytical grade and obtained from Sigma (Poole, U.K.).

Identification and Construction of Mutants. Genomic DNA was isolated from RSY271 and the open reading frame of the *sec18-1* allele amplified by PCR as previously described for *SEC18* (*13*). Automated sequencing (Oswell, Southampton, U.K.) of the first 800 nucleotides of the PCR product revealed the only difference from *SEC18* to be a G → A substitution at position 266. Mutagenic primers (sense, 5′-GCGTACCTGGGACGGTTGGTCCC-3′; and antisense, 5′-GGGACCAACCGTCCCAGGTACGC-3′) were then used to introduce this mutation into the bacterial expression vector, pQE30-*SEC18* (*13*), and the yeast expression vector, pSEY8-*SEC18* (*18*), creating the pQE30-*sec18-1* and pSEY8-*sec18-1* plasmids. The *comatose st53* mutation was introduced into pQE9-NSF (*19*) using mutagenic primers (sense, 5′-GACTTCCTGGCTCTTTTGGAGAATGAT-3′; and antisense, 5′-ATCATTCTCCAAAAGAGCCAGGAAGTC-3′) to create the pQE9-NSF^{st53} plasmid. The analogous mutation was introduced into pSEY8-*SEC18* using the following mutagenic primers to create the pSEY8-$sec18^{st53}$ plasmid: sense, 5′-GACTTTTTAAATTTACTCAACGATGTTACTCC-3′; and antisense, 5′-CTGAAAAATTTAAATGAGTTGCTACAATGAGG-3′. All mutations were introduced using the "Quickchange" site-directed mutagenesis kit (Stratagene, La Jolla, CA) and constructs checked by DNA sequencing (University of Durham, Durham, U.K.).

Purification of Recombinant Proteins. Recombinant His_6-tagged proteins were purified from *Escherichia coli* extracts using Ni^{2+}−NTA chromatography (*13*). To minimize potentially irreversible changes caused by incubation at 37 °C, expression of mutant constructs was induced at 21−22 °C. All chromatography was performed at 21−22 °C using a Pharmacia FPLC system.

α-SNAP/Sec17p Binding Assay. The α-SNAP binding assay was performed as previously described (*13*). For the Sec17p binding assay, 20 μL of 200 μg/mL Sec17p or control buffer was incubated for 20 min in a 1.5 mL polypropylene microcentrifuge tube. These solutions were then removed, and the tubes were incubated with 100 μL of wash buffer [50 mM KCl, 1 mM DTT, and 25 mM Tris-HCl (pH 7.4)] containing 10 mg/mL BSA for 2 min. This was then removed, and the tubes were further incubated in wash buffer containing 1 mg/mL BSA for 2 min. After removal of this buffer, 20 μL of 10−100 μg/mL Sec18p or Sec18-1p was added in binding buffer [100 mM KCl, 2 mM EDTA, 0.5 mM ATP, 1 mM DTT, 1% (w/v) PEG_{4000}, 250 μg/mL soybean trypsin inhibitor, and 20 mM Hepes (pH 7.4)], and tubes were incubated for 10 min. Supernatants were then removed and all tubes washed with 100 μL of binding buffer. All the above steps were performed on ice. SDS sample buffer (50 μL) was then added, and the contents of the tubes were boiled for 5 min before separation on 10% polyacrylamide gels. Sec17p and Sec18p were detected using a monoclonal anti-His tag antibody (Sigma) and developed using enhanced chemiluminescence.

SNARE Complex Disassembly Assay. Triton X-100-extracted rat brain membrane proteins (200 μL), prepared as previously described (*20*), were incubated with 10 μg of α-SNAP and 10 μg of NSF/NSF $comt^{st53}$ with or without 2 mM $MgCl_2$ and 0.5 mM ATP or ATPγS in a final volume of 1 mL of Complete buffer [20 mM Hepes, 100 mM KCl, 0.7% (w/v) Triton X-100, 1 mM DTT, 1 mM PMSF (pH 7.0), 1% (w/v) PEG 4000, and 1% (w/v) glycerol, with or without 2 mM $MgCl_2$] for 30 min at 4 °C, with head-over-head rotation. Samples were then incubated with 100 μg of HPC-1 anti-syntaxin monoclonal antibody for 2 h at 4 °C, with head-over-head rotation followed by addition of 30 μL of a 50% protein G−Sepharose slurry and incubation for a further 1 h. Samples were then washed four times with wash buffer [20 mM Hepes, 100 mM KCl, 0.7% (w/v) Triton X-100, 1 mM DTT, and 1 mM PMSF (pH 7.0)] with or without $MgCl_2$ and ATP or 2 mM EDTA and ATPγS. Samples were spun down, and the supernatant was removed, followed by addition of 60 μL of 100 mM glycine (pH 2.7) and incubation for 5 min followed by centrifugation at 13000*g* for 2 min. The supernatant was transferred to a fresh tube and neutralized with 2 μL of 1 M Tris followed by addition of 60 μL of SDS sample buffer. Samples were boiled for 5 min and separated on 15% polyacrylamide gels, and blotted with antisera to syntaxin and VAMP, or Coomassie stained (to identify NSF and α-SNAP). Blots were visualized using enhanced chemiluminescence and quantified using ^{125}I-labeled secondary antibodies.

Sec18/NSF ATPase Assay. ATPase assays were performed in flat-bottomed 96-well microtiter plates as described previously (*13*). Values were corrected by running duplicate

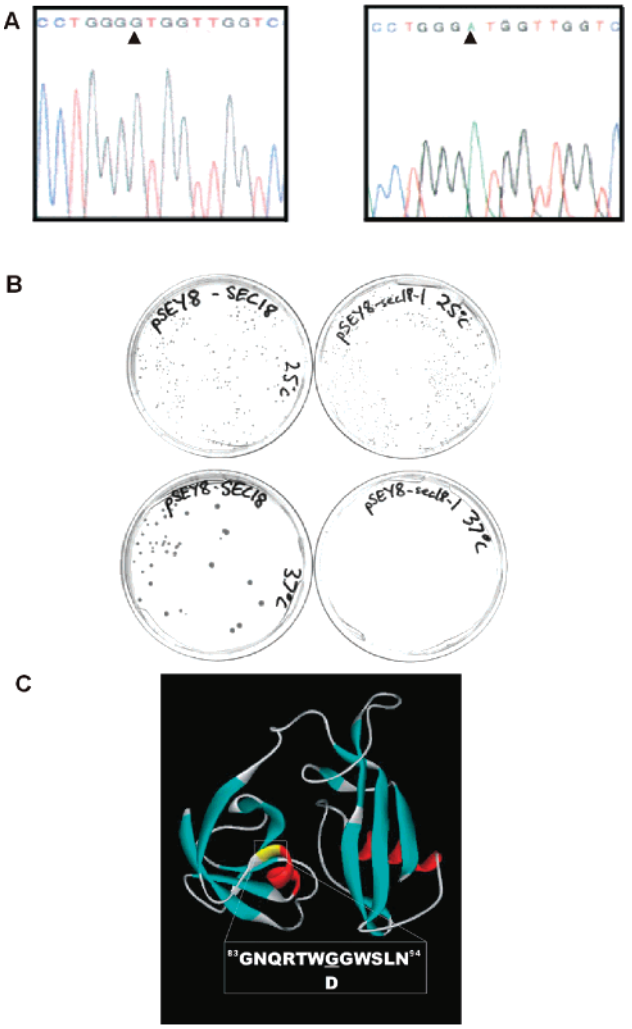

FIGURE 1: The *sec18-1* mutation causes a single amino acid substitution (G89D). (A) The Sec18 open reading frame was amplified from a *sec18-1* strain (RSY271) and its sequence compared to that amplified from a wild-type *SEC18* strain (RSY249). The position of the G266A substitution is indicated. (B) RSY271 cells were transformed with pSEY8-*SEC18* (encoding wild-type Sec18p) or pSEY8-*sec18-1* (encoding mutant Sec18-1p) and inoculated onto minimal plates. Following 3 days growth at 25 or 37 °C, the plates were photographed to assess viability. (C) The *sec18-1* mutation causes a predicted glycine 89 to aspartate substitution. The yellow band indicates the position of glycine 89 in the crystal structure of the Sec18p N domain.

assays either without added proteins (for Sec18) or in the presence of 5 mM *N*-ethylmaleimide (for NSF).

RESULTS

Most studies of the cellular function of Sec18 have been based on analysis of the *sec18-1* strain. Surprisingly, however, all that is known of the *sec18-1* allele itself is that the mutation lies within a 351-base pair *Cla*I restriction fragment (*18*). To identify this mutation, we amplified the *sec18* open reading frame from the *sec18-1* strain and sequenced through the *Cla*I fragment. The only aberration from the wild-type sequence was a single G → A nucleotide substitution at position 266 (Figure 1A). Introduction of this mutation into a Sec18 yeast expression plasmid [pSEY8 (*18*); here termed pSEY8-*SEC18*] abolished its ability to complement the RSY271 *sec18-1* yeast strain at 37 °C (Figure 1B), thus confirming the sequencing information. The result of

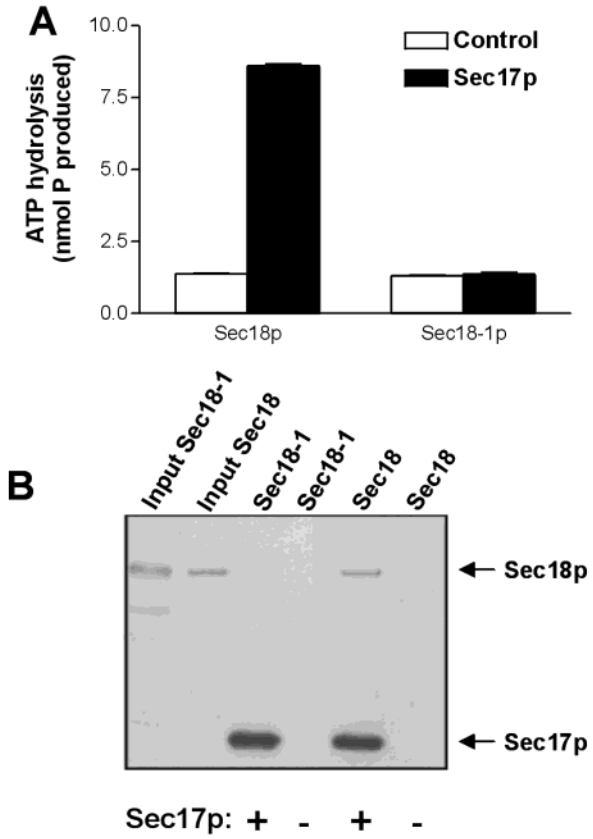

FIGURE 2: Sec17p binding is defective in Sec18-1p. (A) ATPase assay. Plates were preincubated with either 400 μg/mL Sec17p (black bars) or control buffer (white bars) for 30 min. This was then removed and replaced with 10 μg/mL Sec18p or 1 μg/mL Sec18-1p for 120 min before assaying released phosphate spectrophotometrically. Data are expressed as means ± SEM ($n = 4$). (B) Sec17p binding assay. Tubes were preincubated with either 200 μg/mL Sec17p or control buffer for 20 min, washed, and then incubated with 100 μg/mL Sec18p or Sec18-1p for 10 min. Bound protein remaining after washing was solubilized, run on polyacrylamide gels, and detected using an anti-His tag antibody.

this G266A mutation in the predicted *sec18-1* protein would be a single amino acid substitution, changing glycine 89 into an aspartate residue. The position of glycine 89, at the end of an α-helix (α2) in the N_A subdomain of Sec18 (*9*), is shown in Figure 1C.

To determine the functional effect of the *sec18-1* mutation, we introduced the G89D substitution into a Sec18 bacterial expression plasmid and biochemically characterized the resulting purified mutant protein. Sec18-1p exhibited dose-dependent intrinsic ATPase activity at both 25 and 37 °C (data not shown), indicating that this enzymic activity is not grossly inhibited in the mutant. Indeed, although variation was observed between batches of recombinant protein, Sec18-1p generally displayed higher intrinsic ATPase activity than wild-type Sec18p. Controlling for this, by comparing wild-type and mutant proteins at equivalent levels of intrinsic ATPase activity, we find it is clear that whereas ATPase activity in wild-type Sec18p was greatly stimulated by purified Sec17p, no such stimulation was observed for the mutant Sec18-1p (Figure 2A). Similar assays were performed using a range of concentrations of Sec18-1p, but Sec17p stimulation of ATPase activity was never observed (data not shown). To determine if this was a consequence of a reduced level of Sec17p binding in the mutant, in vitro binding assays

Table 1: The st53 Mutation Acts Dominantly When Introduced into Sec18[a]

A

Species	Sequence
D. melanogaster (dNSF1)	VNRDDFLHSLEHDIKPAFG
C. cricetulus (CHO NSF)	VTRGDFLASLENDIKPAFG
S. cerevisiae (Sec18)	VTREDFLNAL-NDVTPAFG

B

Construct	Number of colonies
Empty vector	420
SEC18	369
sec18-1	246
sec18^{st53}	0
sec18-E350Q	0

[a] (A) Comparison of the sequence surrounding the *st53* mutation site (shaded) in fly, mammalian, and yeast NSF. The shaded residues in NSF and Sec18 were changed to leucine in the NSF^{st53} and *sec18*^{st53} constructs. (B) Effect of overexpressing various Sec18 constructs in wild-type cells. RSY249 cells were transformed with YEpURA3 (empty vector), pSEY8-*SEC18*, pSEY8-*sec18-1*, pSEY8-*sec18*^{st53}, and pSEY8-*E350Q* and inoculated onto minimal plates. Following growth for 3 days at 25 °C, the viability of yeast strains was established by counting the number of colonies grown since inoculation.

were performed. Co-incubation of wild-type Sec18p with Sec17p resulted in readily detectable Sec17p-dependent binding of Sec18p, but no such binding was apparent from incubation of Sec18-1p with Sec17p, despite using several-fold higher amounts of the mutant (Figure 2B). Thus, the primary defect in Sec18-1p is an inhibition of Sec17p binding.

The *Drosophila comt*^{st53} mutation is known to cause a single amino acid substitution (S483L) at the interface of the D1 and D2 domains of dNSF1 (*16*), a region of unknown function. To shed light on the biochemical role of this region, we generated a substitution in mammalian CHO cell NSF (S491L) analogous to the *st53* mutation, creating NSF^{st53} (see Table 1A). To determine if this mutation, like that in *sec18-1*, would inhibit SNAP binding, in vitro binding assays were performed. Co-incubation of NSF or NSF^{st53} with α-SNAP resulted in similar SNAP-dependent binding by both proteins (Figure 3A). Assays of intrinsic ATPase activity were also similar in wild-type and mutant NSF (Figure 3B). However, the stimulation of intrinsic NSF ATPase activity by α-SNAP seen in wild-type NSF was not observed in NSF^{st53} (Figure 3B). Furthermore, this lack of SNAP stimulation could not be alleviated by increasing the concentration of NSF^{st53} (data not shown). Taken together, these data suggest that NSF^{st53} has normal intrinsic ATPase activity and SNAP binding ability, but that its ATPase activity is poorly stimulated by α-SNAP.

An α-SNAP mutant (L294A) has been described that is able to bind NSF but is unable to stimulate its ATPase activity, consequently preventing SNARE complex disassembly (*21*). As the phenotypes of α-SNAP(L294A) and NSF^{st53} were so similar with respect to SNAP binding and ATPase activation, we tested the prediction that NSF^{st53} would be unable to disassemble SNARE complexes. Solubilized rat brain membranes were incubated with recombinant α-SNAP and NSF or NSF^{st53} before immunoprecipitating SNARE complexes via a syntaxin antibody (*20*). Incubation with wild-type NSF caused an ATP hydrolysis-dependent

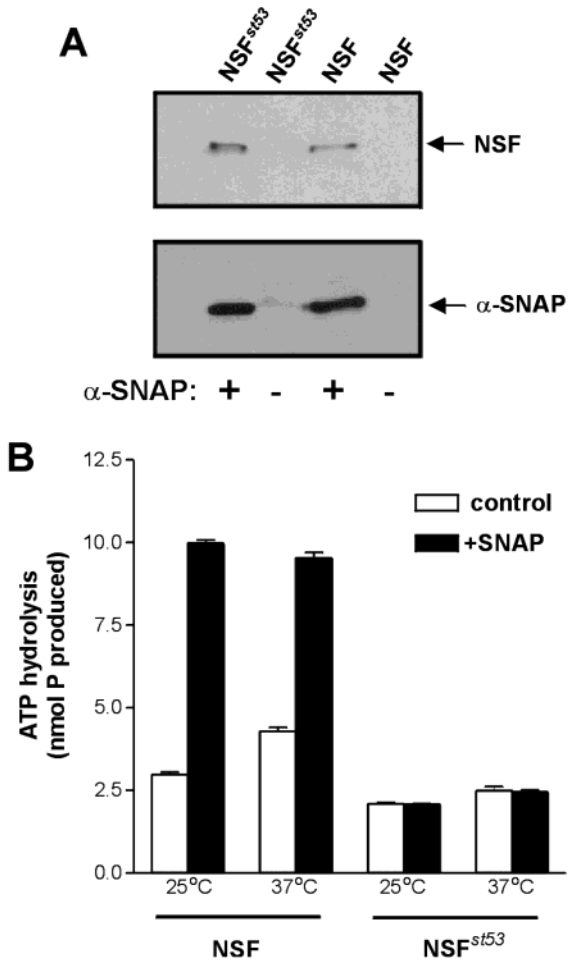

FIGURE 3: SNAP-dependent ATPase stimulation is defective in NSF^{st53}. (A) SNAP binding assay. Tubes were preincubated with either 100 μg/mL α-SNAP or control buffer for 20 min, washed, and then incubated with 100 μg/mL NSF or NSF^{st53} for 10 min. Bound protein remaining after washing was solubilized, run on polyacrylamide gels, and detected by Coomassie blue staining. (B) ATPase assay. Plates were preincubated with either 400 μg/mL α-SNAP (black bars) or control buffer (white bars) for 30 min. This was then removed and replaced with 20 μg/mL NSF or NSF^{st53} for 150 min before assaying released phosphate spectrophotometrically. Data are expressed as means ± SEM (n = 4).

reduction in the amount of VAMP bound to syntaxin, indicating SNARE complex disassembly. In contrast, no significant release of VAMP from syntaxin was observed with mutant NSF^{st53} in the presence of hydrolyzable ATP (Figure 4). NSF^{st53} is therefore defective in SNARE complex disassembly, presumably due to its lack of SNAP-stimulated ATPase activation.

As a consequence of its inability to disassemble SNARE complexes, α-SNAP(L294A) acts as a dominant inhibitor of membrane fusion (*22*). We reasoned that, as NSF^{st53} shared this defect in SNARE complex disassembly, it might also act as a dominant negative mutant. To address this issue in vivo, we generated a substitution in Sec18 (A505L) analogous to the *st53* mutation (see Table 1A). As *SEC18* is an essential gene, any putative dominant mutation should be lethal. The wild-type *S. cerevisiae* strain, RSY249, was then transfected with high-copy plasmids containing wild-type *SEC18*, *sec18*^{st53}, *sec18-1*, *sec18-E350Q*, or an empty vector control (Table 1B). As expected, no colonies were observed in cells transformed with *sec18-E350Q*, as this allele encodes

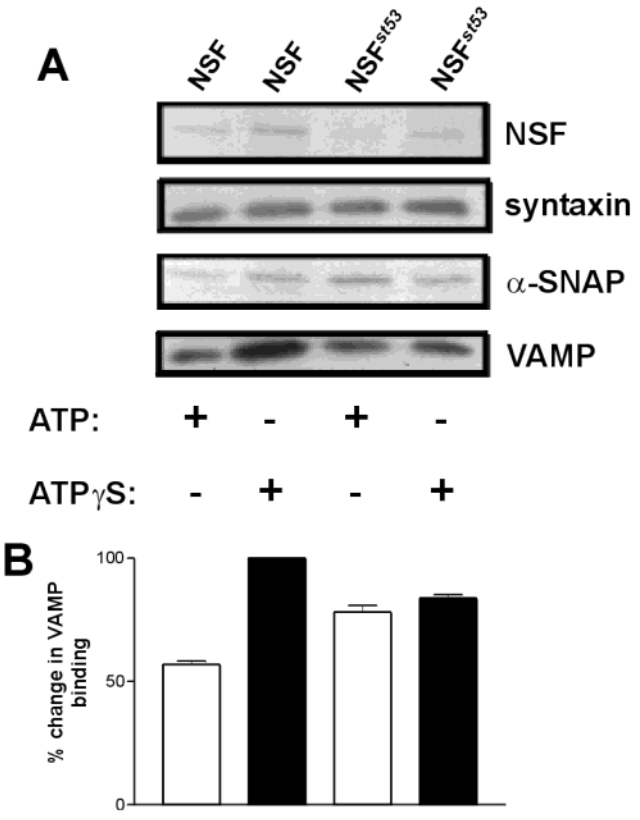

FIGURE 4: NSF^{st53} is defective in SNARE complex disassembly. (A) Solubilized rat brain membrane (200 μL), α-SNAP (10 μg), and NSF (10 μg) were incubated with either ATP or ATPγS at 4 °C for 30 min. Complexes were immunoprecipitated with anti-syntaxin (HPC-1) antibody and protein G−Sepharose beads. Bound proteins were run on polyacrylamide gels and detected by either Coomassie blue staining (NSF and α-SNAP) or immunoblotting (VAMP and syntaxin). (B) The amount of co-immunoprecipitated VAMP relative to syntaxin was determined using ^{125}I-labeled secondary antibodies and quantitative densitometry. Data shown are means ± the standard deviation pooled from three separate experiments.

an intrinsic ATPase-defective mutant that has previously been shown to be dominant lethal (*23*). Transformation with *sec18^{st53}* also resulted in no colonies, indicating a similar dominant lethal phenotype of this mutant. In contrast, large numbers of colonies were observed in cells overexpressing *SEC18* and *sec18-1*. We therefore conclude that the dominant negative phenotype predicted by our biochemical analysis of the *st53* mutation is manifest in vivo.

DISCUSSION

Since the initial isolation of the *sec18-1* strain more than 20 years ago (*15*), this mutant has been the basis of most studies of the cellular function of Sec18. Although the primary phenotype of the *sec18-1* mutant is a temperature-sensitive block in endoplasmic reticulum (ER) to Golgi transport, most other membrane traffic steps are similarly affected (*24*). This indicates that the *sec18-1* mutation interferes with a fundamental function of Sec18 required for its participation in diverse membrane fusion processes. We have now identified the *sec18-1* mutation and demonstrated that this inhibits the mutant protein's ability to bind to Sec17p (yeast SNAP). Clearly, recruitment of Sec18/NSF to SNARE complexes by SNAPs is an essential, early step in the NSF cycle. Therefore, *sec18-1* cells would be predicted to accumulate SNARE complexes containing Sec17p but lacking Sec18-1p. Indeed, it has been demonstrated that incubation of the RSY271 *sec18-1* strain at the restrictive temperature results in the accumulation of the Sed5p/Bos1p/Sec22p/Bet1p SNARE complex involved in ER to Golgi transport (*25*). Moreover, although Sec17p co-immunoprecipitated with this complex, no observable Sec18-1p was associated (*25*), consistent with our biochemical analysis.

The original *Drosophila* comatose mutants, *st17* and *st53*, were isolated 25 years ago (*14*), but only recently were these recognized as NSF1 mutants (*16*). Since then, these mutants have been used to functionally dissect the stage(s) in the synaptic vesicle cycle at which NSF acts. Although opposite interpretations have been reached on this issue (compare refs *26* and *27* and refs *28* and *29*), it is clear that synaptic SNARE complexes accumulate in the mutants at the restrictive temperature. Nevertheless, the biochemical defect causing this phenotype in the *st53* mutant has not been addressed. We found that introducing the analogous mutation into mammalian NSF had no effect on the protein's intrinsic ATPase activity or its ability to bind SNAP, but specifically inhibited the SNAP stimulation of ATP hydrolysis. As this stage in the NSF cycle is essential for SNARE complex disassembly (*21*), this would be predicted to result in the accumulation of SNARE complexes, consistent with the *comatose* phenotype.

As pointed out above, the biochemical defects in recombinant Sec18-1p and NSF^{st53} are consistent with the phenotypes of the mutant organisms at restrictive temperatures. However, whereas both yeast *sec18-1* and *Drosophila comt^{st53}* mutants are temperature-sensitive, neither the recombinant protein nor the *sec18^{st53}* allele displayed temperature dependency. Consistent with these findings, cytosol prepared from *sec18-1* cells is unable to function in in vitro membrane traffic assays even at permissive temperatures (*11*), although this may be due to low levels of soluble Sec18-1p (unpublished observations). Nevertheless, it has been pointed out that temperature-sensitive mutants most commonly result "from a constitutive defect that, at higher temperature, is unable to keep up with the increased rate of function required" (*30*). For example, although the yeast *sec9-4* mutant is temperature-sensitive, recombinant Sec9-4p exhibits a temperature-independent defect in SNARE complex assembly (*30*). Furthermore, temperature-sensitive phenotypes can even result from null mutations, as evidenced by the temperature-induced paralysis in *Drosophila csp* null mutants (*31*). A simple explanation of our results is that the *sec18-1* and *st53* mutants are greatly disabled in SNAP binding and ATPase activation, respectively, but retain some vestigial activity that is difficult to detect biochemically, yet sufficient to enable function at low rates. For example, a more transient interaction of Sec18-1p with Sec17p might be missed under stringent binding assay conditions, but could potentially allow the minimal level of SNARE disassembly required in the cell.

Independent of organismal mutant phenotypes, our findings reveal novel residues involved in two key aspects of NSF biochemistry: SNAP binding and SNAP-dependent ATPase activation. Previous analysis of deletion−rearrangement mutants of NSF indicated that the N domain is required for interactions with the SNAP−SNARE complex, the D1 domain for ATP hydrolysis (and hence complex disas-

sembly), and the D2 domain for hexamerization (*19*, *32*). However, most point mutants of NSF and Sec18p described so far have targeted residues in the Walker A and B boxes required for ATP binding or hydrolysis (*13*, *19*, *32*−*36*). The remainder, the *sec18-109* and NSF^{st17} alleles, similarly block intrinsic D1 ATPase activity (*17*, *23*). Thus, our findings provide the first biochemical analysis of loss-of-function point mutants of NSF/Sec18 possessing intrinsic ATPase activity. The glycine residue mutated in *sec18-1* is located at the end of the α2 helix lining the interface of the N_A and N_B subdomains in Sec18 (Figure 1C) (*9*), and the corresponding alanine in NSF is similarly positioned (*8*, *10*). Replacement of glycine 89 with an acidic aspartate residue would be predicted to disrupt the packing of this α2 helix (*10*). It has been suggested that helices α1 and α2 from N_A and strand β10 from N_B together form a basic groove at the interdomain interface which may be the site of interaction with the acidic C-terminus of α-SNAP (*8*). The reduced ability of Sec18-1p to interact with Sec17p observed here provides direct experimental support for this notion. Further mutational studies will be required to identify other residues comprising the reciprocal minimal binding regions on NSF and SNAPs.

The observation that NSF^{st53} is specifically defective in SNAP-stimulated ATPase stimulation suggests a function in this process for the D1−D2 domain interface where the mutation occurs. One possibility is that this region comprises a second SNAP interaction site on NSF, as our results clearly show that the processes of SNAP binding and SNAP stimulation of ATPase activity are mechanistically separate (Figure 3). Nevertheless, the similarity in phenotypes between NSF^{st53} and α-SNAP(L294A) provides independent support for the idea that ATPase stimulation by SNAPs (and not merely intrinsic ATPase activity alone) is essential for SNARE complex disassembly. This notion is supported by the dominant lethal effect of *sec18*^{st53}, as a mutant efficient at SNARE binding but inefficient at disassembly should lock SNAREs into dead-end 20S complexes inaccessible to the wild-type Sec18 protein. In contrast, a mutant poorly able to bind SNAPs should cause little interference with the function of wild-type Sec18p, consistent with the relative lack of an effect of *sec18-1* overexpression. We envisage that similar mutational studies will provide further new information about key residues required for NSF function.

ACKNOWLEDGMENT

We gratefully acknowledge Albert Haas, Randy Schekman, Mitsuo Tagaya, Masami Takahashi, Scott Emr, and James Rothman for generously providing materials. We also thank Richard Barnard and Bob Burgoyne for critical reading of the manuscript.

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BI0160359