An NSF function distinct from ATPase-dependent SNARE disassembly is essential for Golgi membrane fusion

Joyce M. M. Müller*, Catherine Rabouille*, Richard Newman†, James Shorter*, Paul Freemont†, Giampietro Schiavo‡, Graham Warren* and David T. Shima*§

*Cell Biology Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK
†Molecular Structure Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK
‡Molecular Neuropathobiology Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK
§e-mail: shima@europa.lif.icnet.uk

focus on MEMBRANE TRAFFIC

The precise biochemical role of *N*-ethylmaleimide-sensitive factor (NSF) in membrane fusion mediated by SNARE proteins is unclear. To provide further insight into the function of NSF, we have introduced a mutation into mammalian NSF that, in *Drosophila* dNSF-1, leads to temperature-sensitive neuroparalysis. This mutation is like the *comatose* mutation and renders the mammalian NSF temperature sensitive for fusion of postmitotic Golgi vesicles and tubules into intact cisternae. Unexpectedly, at the temperature that is permissive for membrane fusion, this mutant NSF binds to, but cannot disassemble, SNARE complexes and exhibits almost no ATPase activity. A well-charaterized NSF mutant containing an inactivating point mutation in the catalytic site of its ATPase domain is equally active in the Golgi-reassembly assay. These data indicate that the need for NSF during postmitotic Golgi membrane fusion may be distinct from its ATPase-dependent ability to break up SNARE pairs.

NSF was the first member of the AAA family of ATPases (for ATPases associated with diverse cellular activities)[1,2] to be implicated in the process of membrane fusion[3]. It was first purified and characterized as a component of intra-Golgi transport[4,5] and then shown to be involved in many other vesicle-mediated transport steps on the exocytic and endocytic pathways in organisms from yeast to man[6].

NSF is involved in SNARE-mediated membrane fusion. SNAREs (SNAP (soluble NSF-attachment protein) receptors) act as membrane receptors for NSF[7] via SNAP proteins[8] and they comprise a family of coiled-coil membrane proteins[9]. SNAREs participate in membrane-fusion events, which have been best characterized during neurotransmitter release at the presynaptic membrane[10]. In the simplest model, a vesicle-bound (v-) SNARE interacts with a target-membrane-bound (t-SNARE)[6], forming a parallel, four-helix bundle[11] that brings the vesicle and target membranes close enough together to fuse[12], perhaps with the aid of other downstream factors[13,14].

NSF is a barrel-shaped hexamer, with each subunit forming one of the staves of the barrel[15,16]. Each subunit has an amino-terminal (N) domain and two homologous ATP-binding domains, the D1 domain being in the middle of the protein and the D2 domain at the carboxy terminus[17]. The N domain is required for interactions with SNAPs and SNAREs; the D1 domain acts on these SNAREs; and the D2 domain determines the hexameric state of the protein[18,19].

NSF seems to act in two different ways on SNAREs. The first is to break up SNARE complexes that accumulate in the same membrane as a result of SNARE-mediated fusion[20–22]. To do this, NSF binds to a variety of v-SNARE–t-SNARE complexes, through SNAPs, to form a particle that sediments at 20S (ref. 23). In the presence of Mg-ATP this 20S particle breaks down[24], releasing the individual SNARE molecules and thus allowing their recycling for further rounds of fusion. The second action, perhaps related to the first, may be the 'priming' of the released t-SNARE. In the case of vacuole fusion, this priming event is coupled to transfer of Lma1 to the t-SNARE Vam3 (ref. 25). Lma1 is a heterodimer of thioredoxin and proteinase B[26], and has also been implicated in endoplasmic-reticulum-to-Golgi transport[27].

Our interest in NSF arose during studies of the Golgi apparatus during mitosis[28]. As part of the inheritance process, the stacks of Golgi cisternae are converted into clusters of vesicles and tubules, which reassemble a Golgi apparatus in each daughter cell during telophase[29,30]. Mimicking the reassembly of Golgi cisternae *in vitro* led to the identification of two related ATPases, NSF and p97, as candidates for mediating the membrane-fusion event[31].

To understand more precisely the role played by NSF during postmitotic reassembly of Golgi cisternae, we sought to generate a temperature-sensitive NSF mutant by exploiting the *comatose* mutation in *Drosophila melanogaster*. In flies, this mutation, which is found in the *Drosophila* NSF, dNSF-1, leads to neuroparalysis when the temperature is raised from 25 °C to 37 °C (refs 32, 33). At the restrictive temperature, *comatose* flies accumulate synaptic vesicles and v-SNARE–t-SNARE complexes, consistent with a role for dNSF-1 in synaptic transmission[34]. The *comt17* allele carries a single point mutation of glycine 274 to glutamate (G274E) in a highly conserved region of the D1 domain of dNSF-1 (ref. 33).

Here we show that transferring this mutation to mammalian NSF not only renders mitotic Golgi reassembly temperature sensitive, but also abolishes ATPase-dependent break-up of SNARE pairs at the temperature permissive for membrane fusion.

Results

Mitotic Golgi reassembly in the presence of wild-type or mutant NSF. The mammalian *comatose* NSF mutant (G274E) was generated by a site-directed mutagenesis approach, using the complementary DNA encoding Chinese hamster ovary (CHO) NSF containing a Myc tag at the C terminus as a template[19]. Recombinant proteins were expressed in *Escherichia coli* and purified, using their N-terminal His_6 tag, to ~95% purity as assessed by SDS polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie staining (Fig. 1a).

We tested both wild-type and mutant NSFs for their ability to re-form Golgi cisternae from mitotic Golgi fragments (MGFs). MGFs were prepared by incubating rat liver Golgi membranes with mitotic cytosol. After re-isolation, the fragments were treated with *N*-ethylmaleimide (NEM) so that cisternal regrowth depended on

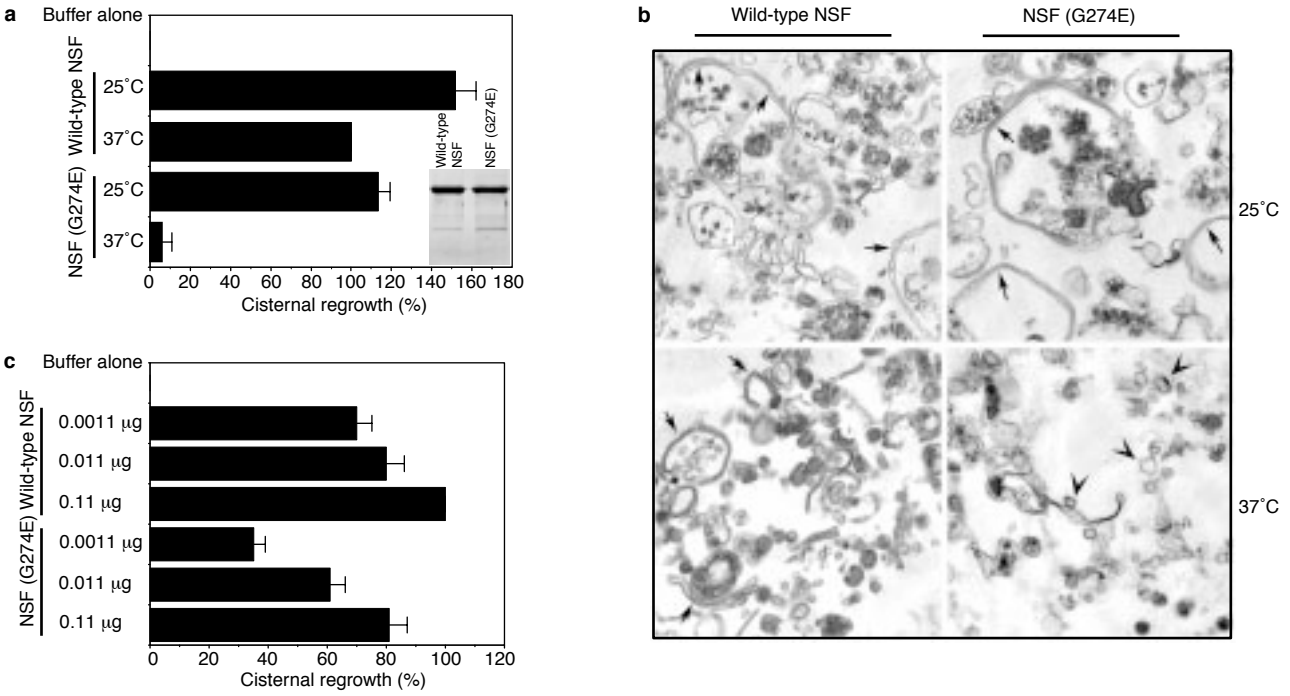

Figure 1 **Fusion of postmitotic Golgi membranes in the presence of wild-type NSF or NSF(G274E). a**, Temperature dependence of cisternal regrowth. NSF proteins were preincubated and added to a mixture of NEM-treated MGFs, α-SNAP, γ-SNAP and p115 at 25 °C or 37 °C for 60 min. Membranes were processed for electron microscopy and the percentage cisternal regrowth was determined. SDS–PAGE analysis of the purified recombinant His_6-tagged NSF proteins is shown in the inset. **b**, Electron micrographs show the typical appearance of membranes following reassembly reactions in the presence of wild-type and mutant NSF proteins at 25 °C or 37 °C. Arrows denote cisternae; arrowheads denote unfused tubulovesicular membranes. **c**, Dose dependence of cisternal regrowth. Various amounts of the NSF proteins were assayed under standard conditions at 25 °C for 60 min. Samples were processed and analysed as above.

the addition of exogenous NSF and its accessory proteins, α- and γ-SNAP, together with the vesicle-docking protein p115 (ref. 35).

Wild-type NSF catalysed cisternal regrowth at both 25 °C and 37 °C (Fig. 1a). The higher activity at 25 °C may reflect the greater stability of the components at this temperature. However, the mutant NSF was active at 25 °C but almost completely inactive at 37 °C. The typical appearance of membranes following the reassembly reactions are shown in electron micrographs (Fig. 1b).

Lowering the amounts of NSF by 10- and 100-fold showed that both the wild-type and the mutant proteins had similar activity profiles at the permissive temperature of 25 °C (Fig. 1c). Together, these data indicate that the temperature-sensitive phenotype of the *comt17* allele was transplanted to CHO NSF, as measured by the Golgi-reassembly assay.

NSF(G274E) binds to SNARE complexes only at the permissive temperature. As shown by work on *comatose* flies, dNSF-1 operates at the level of synaptic SNARE complexes[34]. At the non-permissive temperature, synaptic SNARE complexes accumulate but it is not clear whether this is caused by a lack of binding of NSF to SNARE complexes or an inability of NSF to break them up. We tested each of these possibilities in turn using the mammalian NSF mutant.

First, we studied binding of NSF(G274E) to synaptic SNAREs at different temperatures. To do so, NSF (wild-type or mutant) was preincubated at 25 °C or 37 °C for 45 min in the presence of Mg-ATP, mixed with α-SNAP and brain membrane extract and incubated at the same temperature in the presence of EDTA to prevent ATP hydrolysis. Anti-syntaxin-1 beads (syntaxin is a t-SNARE) were added to the mixture and incubations continued for 4 h at the same temperature. Immunocomplexes were isolated and analysed for bound α-SNAP, VAMP/synaptobrevin (a v-SNARE) and NSF. Wild-type NSF formed a complex at both 25 °C and 37 °C (Fig. 2a). At 25 °C, mutant NSF bound to the SNAP–SNARE complex, though with slightly less efficiency than wild-type NSF. In marked contrast, at 37 °C binding of the mutant to SNAP–SNARE complexes was completely abolished. To test the reversibility of this effect, we dropped the temperature of one set of samples from 37 °C to 4 °C for the last 2 h of the incubation. Binding activity was not, however, recovered (Fig. 2a). We verified the specificity of NSF binding to the SNARE complex by using different combinations of α-SNAP and membrane extract (Fig. 3a).

As the binding activity of NSF(G274E) was irreversibly lost at the restrictive temperature, we determined whether the amount of soluble mutant NSF was decreased at 37 °C. Incubations with α-SNAP and membrane extract at 25 °C or 37 °C for 2 h showed no difference in the protein half-life (data not shown).

Conformation of NSF(G274E) changes at the restrictive temperature. We next examined the structure of NSF(G274E) at 25 °C and 37 °C in the presence of Mg-ATP by negative staining and electron microscopy. In projection, wild-type NSF has similar structures at 25 °C and 37 °C (Fig. 2b). In marked contrast, the mutant has a structure comparable to that of wild-type NSF at 25 °C but not at 37 °C. At this higher temperature, the individual subunits of the NSF mutant are splayed out in projection.

NSF(G274E) does not break up SNARE complexes. As the mutant NSF bound to SNAP–SNARE complexes at the permissive temperature we next examined whether it could break them up. For this we used a well-characterized assay for NSF-dependent disassembly of SNARE complexes[23,24]. We incubated a brain membrane extract with α-SNAP and NSF in the presence or absence of Mg-ATP at 4 °C, and isolated and analysed anti-syntaxin-1 immunocomplexes. Wild-type NSF was present in a complex together with α-SNAP and VAMP/synaptobrevin that was broken up in the

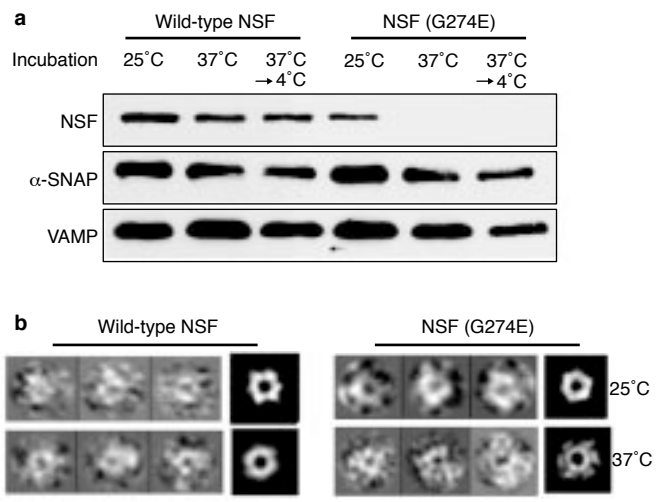

Figure 2 **Stability of NSF complexes and NSF at different temperatures. a**, 20S-complex formation. Incubations were carried out at 25 °C or 37 °C. NSF (wild-type or mutant) was preincubated in the presence of Mg-ATP for 45 min and then added to brain membrane extract and α-SNAP in the presence of EDTA for 30 min. Anti-syntaxin-1 beads were added and incubations continued at the same temperature for 4 h. After 2 h, one set of the 37 °C samples was shifted to 4 °C. Immunocomplexes were analysed by western blotting for the presence of NSF, α-SNAP and VAMP/synaptobrevin. **b**, Negative staining. NSF proteins were incubated at 25 °C or 37 °C in the presence of Mg-ATP for 1 h and samples processed for negative staining. For each condition a set of three end-on views is shown on the left, together with an averaged and 6-fold 'symmetrized' view on the right.

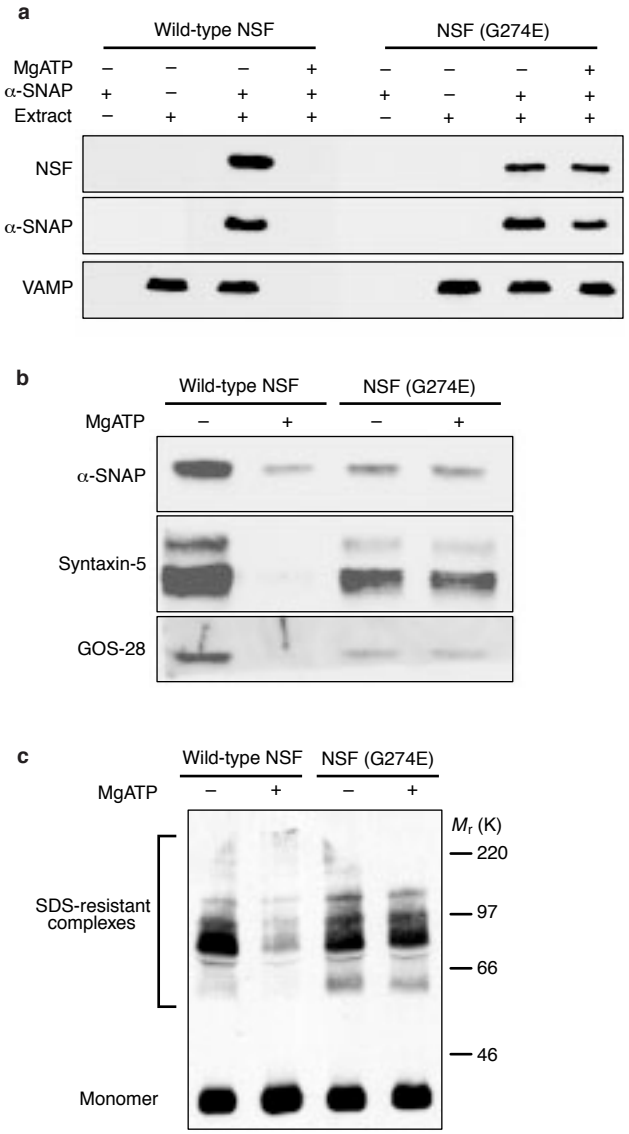

Figure 3 **Disassembly of 20S complexes containing synaptic or Golgi SNAREs. a**, Synaptic SNARE complex. Combinations of brain membrane extract and α-SNAP were incubated with wild-type or mutant NSF in the presence or absence of Mg-ATP at 4 °C, followed by the addition of anti-syntaxin-1 beads. Immunocomplexes were analysed for the presence of the indicated proteins by western blotting. **b**, Golgi SNARE complex. NEM-treated Golgi extract, α-SNAP and NSF (wild-type or mutant) were incubated in the presence or absence of Mg-ATP at 4 °C, followed by the addition of anti-Myc-tag beads to isolate NSF complexes. Immunocomplexes were analysed for the presence of α-SNAP, syntaxin-5 and GOS-28 by western blotting. Note that there are two forms of syntaxin-5, with M_r values of 35K and 42K, respectively[48]. **c**, Disassembly of SDS-resistant complexes containing syntaxin-1. NEM-treated synaptic vesicles were incubated with α-SNAP and wild-type or mutant NSF in the presence or absence of Mg-ATP at 25 °C, followed by the addition of SDS sample buffer and analysis for the presence of high-molecular-mass complexes containing syntaxin-1 by western blotting.

presence of Mg-ATP (Fig. 3a). In contrast, the complex containing the NSF mutant was stable in the presence of Mg-ATP. The same result was obtained when the experiment was done at 25 °C, the permissive temperature for membrane fusion (data not shown).

Next, we carried out similar experiments using Golgi membranes. We used anti-Myc-tag beads to immuno-isolate complexes containing the recombinant Myc–NSF proteins from NEM-treated Golgi extracts. The blots were probed for syntaxin-5 and GOS-28, the two Golgi SNAREs implicated in the reassembly process[36], as well as for α-SNAP. Disassembly occurred only when using wild-type, and not mutant, NSF (Fig. 3b).

Lastly, we studied the ability of wild-type and mutant NSF to disassemble SNARE complexes in intact synaptic vesicle membranes, using an assay that exploits the ability of the detergent SDS to preserve existing SNARE complexes while also preventing *de novo* formation of complexes[20,37]. We observed several distinct SDS-resistant synaptic-vesicle SNARE complexes containing syntaxin-1 (Fig. 3c, lanes 1, 3, 4) and VAMP-1 (data not shown) at 25 °C, with a major protein complex being observed at relative molecular mass 85,000–100,000 (M_r ~85K–100K), as previously described[20]. SDS-resistant SNARE complexes were broken up following an incubation at 100 °C before electrophoresis[37] (data not shown) or upon incubation of the intact vesicles with wild-type NSF in the presence of Mg-ATP at 25 °C before membrane solubilization (Fig. 3c, compare lanes 1, 2). The NSF mutant, however, was not able to disassemble the SNARE complexes under these conditions (Fig. 3c, compare lanes 3, 4). Together these results show that, at the temperature permissive for membrane fusion, NSF(G274E) is defective in the disassembly of native or detergent-solubilized SNARE complexes.

NSF(G274E) has no detectable ATPase activity. The surprising observation that NSF(G274E) was able to promote Golgi membrane fusion but unable to disassemble 20S complexes containing either synaptic or Golgi SNAREs led us think that the mutant was defective in its ATPase activity.

We tested this possibility by measuring the release of [^{32}P] from [γ-^{32}P]ATP in the presence of wild-type or mutant NSF. ATPase activity of the wild-type NSF was time (Fig. 4a) and temperature (Fig. 4b) dependent, with a peak activity (~13 μmol mg^{-1} h^{-1} at 37 °C) comparable to published data[17]. In contrast, the mutant had virtually no time-dependent ATPase activity in the *in vitro* assay, at tempera-

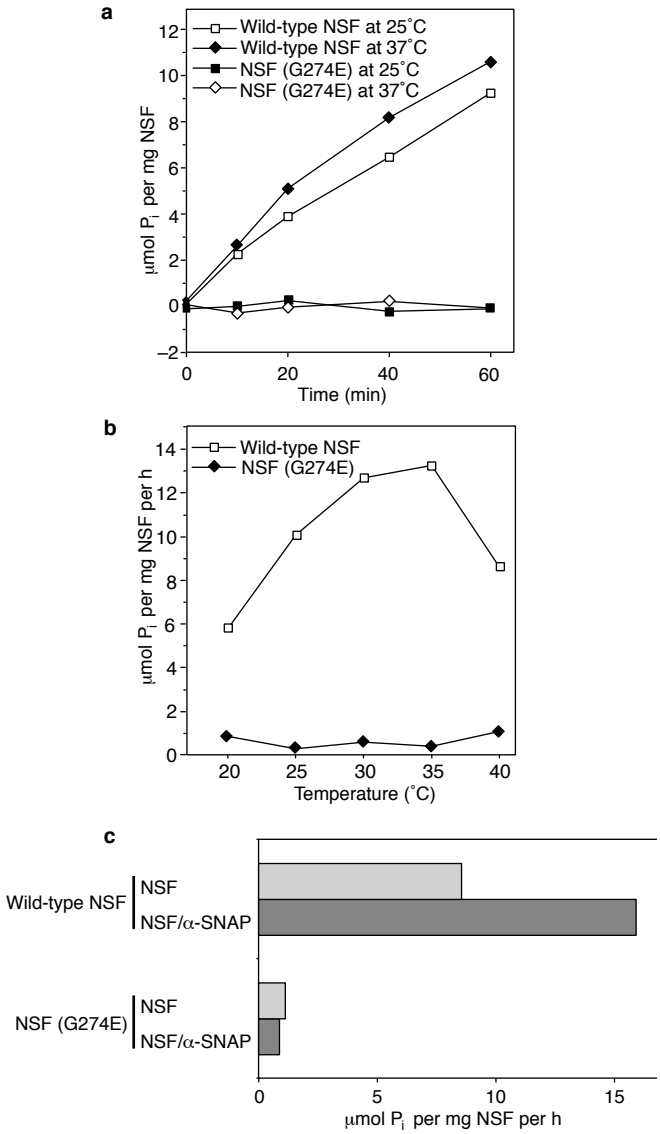

Figure 4 **ATPase activity of the NSF proteins.** The ATPase activity was measured by the release of [^{32}P] from [γ-^{32}P]ATP. Plotted values represent NEM-sensitive activities. **a**, Time course of [^{32}P] release at 25 °C and 37 °C. **b**, Temperature dependence of the ATPase activity at 45 min of incubation. **c**, ATPase activity in the presence of α-SNAP after 45 min of incubation at 25 °C. Experiments shown are representative of four independent assays.

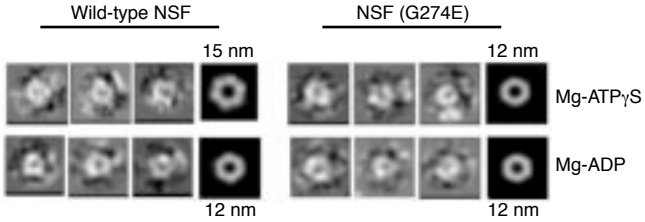

Figure 5 **Negative staining of wild-type and mutant NSF in the presence of different nucleotides.** NSF proteins were incubated in the presence of 2 mM Mg-ATP-γS or Mg-ADP at 4 °C for 1 h, and samples were processed for negative staining. For each condition, a set of three end-on views are shown on the left, together with a averaged and 6-fold 'symmetrized' view on the right. Note that Mg-ATP-γS increases the diameter of the wild-type but not the mutant NSF barrel from ~12 nm to ~15 nm.

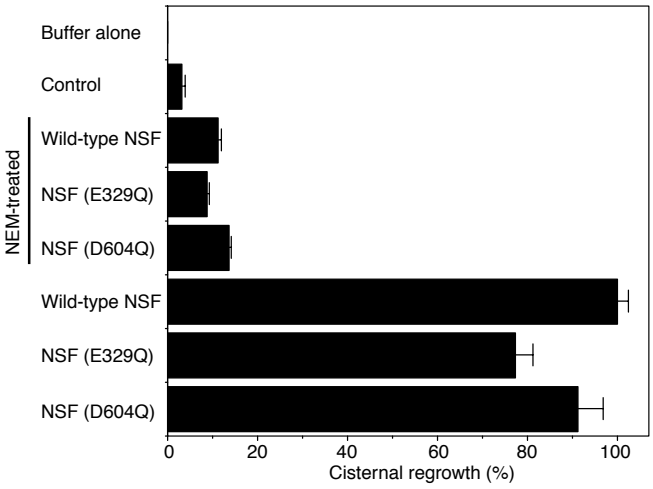

Figure 6 **Cisternal regrowth in the presence of ATPase catalytic-site mutants of NSF.** NSF proteins were mixed with NEM-treated MGFs, α-SNAP, γ-SNAP and p115 and incubated at 37 °C for 60 min. Samples were processed for electron microscopy and the percentage cisternal regrowth was determined. In some experiments, NSF proteins were pretreated with NEM.

tures ranging from 20 °C to 40 °C.

As α-SNAP stimulates NSF's ATPase activity[38], we next determined whether it could rescue the ATPase activity of mutant NSF by adding α-SNAP into standard assays. The ATPase activity of the wild-type NSF was stimulated roughly twofold by α-SNAP (Fig. 4c). In contrast, the ATPase activity of NSF(G274E) remained at background levels in the presence of α-SNAP.

The conformation of NSF(G274E) is insensitive to added nucleotide. We studied the structure of the NSF proteins in the presence of Mg-ADP and Mg-ATP-γS at 4 °C by negative staining and electron microscopy. We used ATP-γS to keep the proteins locked in their ATP-bound state[23]. In projection, the diameter of the wild-type-NSF barrel increased from ~12 nm to ~15 nm, and the central hole from ~3.5 nm to ~4.5 nm, when Mg-ATP-γS was used instead of Mg-ADP (Fig. 5). There was no equivalent change in the NSF mutant even when the protein was incubated in the absence of any nucleotide, conditions under which wild-type NSF is completely unstable (data not shown). Together, these data indicate that the conformation of NSF(G274E) is probably unaffected by the presence of exogenous nucleotides.

Mitotic Golgi reassembly in the presence of mutant NSF proteins. The ability of NSF(G274E) to promote cisternal regrowth despite defects in SNARE disassembly and ATPase activity led us to study the activity of two other NSF mutants in postmitotic Golgi membrane fusion. One mutant, NSF(E329Q), contains a mutation in the catalytic site of the D1 ATPase domain[19] that renders the protein defective in ATPase activity and SNARE disassembly[18], features shared by NSF(G274E) at the permissive temperature. This mutant does not support intra-Golgi transport[19] and endosome fusion[39]. Another mutant, NSF(D604Q), contains the corresponding mutation in the structurally related, but functionally dispensable, D2 ATPase site[19]. This mutation does not affect SNARE-complex disassembly and significant intra-Golgi transport activity is retained[19]. We observed cisternal regrowth from MGFs with both the D1 and the D2 ATPase mutants (Fig. 6), with the D1 ATPase mutant sup-

porting a similar level of activity (~80%) as that observed for NSF(G274E) (Fig. 1) and the D2 ATPase mutant's activity almost reaching that of wild-type NSF.

Discussion

We have introduced the *comatose* mutation of *D. melanogaster* into the mammalian NSF to provide further insights into NSF's role in SNARE-mediated membrane fusion. Projection of the structurally related D1 domain onto the crystal structure of NSF's D2 domain[40] reveals that the *comatose* mutation (G274E) probably results in the addition of a charged residue into a hydrophobic pocket that resides next to the catalytic Walker-A-box motif in the ATPase site. In mammals, this mutation confers a temperature-sensitive phenotype to Golgi reassembly. The mutant mammalian NSF is irreversibly modified at the higher temperature. It can no longer bind to SNAP–SNARE complexes and it undergoes a dramatic change in conformation, the individual subunits splaying out in projection as visualized by electron microscopy. This irreversibility could help to explain the temperature-sensitive phenotype seen in *comatose* flies. The flies are paralysed within a minute at the restrictive temperature but take about 30 min to recover at the permissive temperature[32]. This could represent the time taken for sufficient protein refolding or for new NSF protein to be synthesized.

The mutant NSF bound to SNAP–SNARE complexes at the permissive temperature but could no longer break up these complexes in the presence of Mg-ATP. This was true for the synaptic SNAREs that operate at the plasma membrane as well as for Golgi SNAREs. This defect correlated with a nearly complete lack of time-dependent ATPase activity at temperatures ranging from 20°C to 40°C, and this activity could not be rescued by the presence of α-SNAP. Nevertheless, this mutant was able to catalyse Golgi reassembly at the permissive temperature of 25°C almost as well as the wild-type NSF.

Results obtained with the comatose-like mutant were substantiated by using a distinct mutant, NSF(E329Q), that is defective in ATPase activity and SNARE disassembly. This mutation has been previously shown to result in a ~75% reduction in ATPase activity, which completely abolished its ability to stimulate fusion in an intra-Golgi transport assay[19]. These results indicated a positive correlation between the membrane-fusion-promoting function of NSF and its ATPase activity. In contrast, our present data show that the ATPase-defective NSF (E329Q), like NSF(G274E), is capable of promoting cisternal regrowth to ~80% of the level of wild-type NSF. Together, data obtained using the mutant NSF proteins indicate that NSF's ATPase activity may not be directly linked to post-mitotic Golgi membrane fusion.

As NSF(G274E) and NSF(E329Q) both lack the ability to break up SNARE complexes, NSF-dependent SNARE disassembly seems to be uncoupled from membrane fusion of postmitotic Golgi fragments. The break-up of SNARE complexes is thought to be essential for the recycling of these proteins for further rounds of fusion; thus, these data indicate that recycling may not be needed for Golgi reassembly in the cell-free assay. On the basis of current models, we therefore predict that there is an abundant source of disassembled SNAREs on MGFs before reassembly. If true, this could help to explain the discrepancy in the requirement for NSF's ATPase activity in other published membrane-fusion assays but not during mitotic Golgi reassembly.

However, this leaves open the nature of the distinct role for NSF during the membrane-fusion process. Interestingly, we found that assembly of synaptic 20S complexes was temperature sensitive in the presence of the NSF mutant, which indicates that the presence of NSF in a SNARE complex might be critical. One possibility is that NSF is needed to prime (for example, by folding or assisting in accessory-factor recruitment) the SNAREs on MGFs in preparation for fusion. Although priming of vacuole and, perhaps, Golgi SNAREs correlates with the presence of Mg-ATP (refs 25, 41), it is not clear that ATP hydrolysis is needed for this process. Another possibility is that a checkpoint exists to ensure that NSF is recruited to the fusion site in preparation for its later function in breaking up SNARE complexes. This would certainly explain why NSF has been found on synaptic and clathrin-coated vesicles that still have to dock and fuse[42,43]. Such recruitment would target NSF to the site at which its action will be needed. A final possibility is that NSF takes part in the actual fusion process itself, as originally proposed[24]. It is, therefore, interesting that NSF–SNAPs can directly fuse liposomes together in an ATP-dependent manner[44]. Furthermore, this happens most efficiently when the NSF–SNAP complex has the lowest ATPase activity[44], the key feature of our NSF mutant.

In conclusion, our studies of the *Drosophila comatose* analogue in mammalian NSF provide clear evidence that NSF has a role in membrane fusion that is divorced from its ability to break up SNARE complexes. The likelihood is that NSF has multiple roles and further structure/function studies should provide the means for their dissection. □

Methods

Plasmid construction.

A polymerase chain reaction (PCR)-based, site-directed mutagenesis approach (Quikchange site-directed mutagenesis kit, Stratagene) was used to introduce a G274E mutation into the cDNA encoding CHO NSF containing a Myc tag at its C terminus[19]. The sense primer used was 5′-GCTCGACAGATT-GAAAAGATGCTGAATGCG, and the antisense primer was 5′-CGCATTCAGCATCTTTTCAATCTGT-CGAGC. cDNAs of NSF and NSF(G274E) were subcloned into pTrc-expression vectors (Invitrogen) to introduce a His_6 tag at the N terminus.

Purification of recombinant proteins.

cDNAs were expressed in *E. coli* at 25°C. Cell homogenates were prepared by freeze/thaw in 100 mM HEPES-KOH, pH 7.4, 500 mM KCl, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 5 mM ATP and protease inhibitors (0.25 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM pepstatin, 2 mM benzamidine and 10 μg ml^{-1} leupeptin) and precleared by centrifugation at 200,000*g* for 1 h at 4°C. Proteins were purified in NSF buffer (20 mM HEPES-KOH, pH 7.4, 200 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 2 mM β-mercaptoethanol and 1 mM ATP) using Ni-NTA–agarose (Qiagen).

Golgi-reassembly assay.

NEM-treated MGFs were prepared as described[35]. If not indicated otherwise, 0.1 μg NSF was mixed on ice with α-SNAP (0.5 μg), γ-SNAP (0.5 μg), p115 (0.15 μg) and MGFs (10–20 μg) in 25 mM HEPES-KOH, pH 7.3, 25 mM magnesium acetate, 25 mM KCl, 2 mM ATP, 1 mM GTP and 1 mM glutathione in a total volume of 20 μl. Reassembly assays were done in the presence of an ATP-regenerating system[35] at 25°C or 37°C for 60 min. In some experiments, NSF was preincubated for 20 min at the temperature of the assay without significantly affecting the results. Samples were fixed and processed for electron microscopy and the percentage of cisternal regrowth was quantified[35]. Control experiments were carried out in the absence of NSF or in the presence of NEM-treated NSF at 37°C.

Assembly of synaptic 20S complexes at different temperatures.

NSF proteins were preincubated at 25°C or 37°C for 45 min in NSF buffer. NSF (wild-type or mutant; 7 μg) was mixed with α-SNAP (3.5 μg) and rat brain membrane extract (100 μg)[7] in buffer A (20 mM HEPES-KOH, pH 7.4, 100 mM KCl, 2 mM EDTA, 0.5% Triton-X100, 2 mM dithiothreitol (DTT), 0.5 mM ATP and protease inhibitors (0.25 mM PMSF, 2 mM benzamidine and 10 μg ml^{-1} leupeptin)) in the presence of an ATP-regenerating system in a total volume of 150 μl. Samples were incubated at 25°C or 37°C for 30 min and protein-G–Sepharose beads (Pharmacia) crosslinked to anti-syntaxin-1 IgGs[45] were added. Incubations were continued at 25°C or 37°C for 4 h. For one set of 37°C samples, the temperature was shifted to 4°C after 2 h. Beads were washed, eluted with 0.1 M glycine, pH 2.7, and precipitated with 10% *v/v* trichloroacetic acid (TCA). Samples were analysed by western blotting for the presence of Myc–NSF (monoclonal antibody 9E10), His_6–α-SNAP (RGS antibody, Qiagen) and VAMP/synaptobrevin[46].

Disassembly of 20S complexes.

To assess disassembly of synaptic SNARE complexes, NSF or NSF(G274E) (7 μg), rat brain membrane extract or BSA (100 μg) and/or α-SNAP (3.5 μg) were incubated in a total volume of 150 μl in buffer A in the presence of an ATP-regenerating system at 4°C for 30 min. $MgCl_2$ was present at 8 mM where indicated to generate Mg-ATP. Anti-syntaxin-1 beads were added and incubations continued for 4 h. Immunocomplexes were washed, eluted and precipitated with TCA. Samples were analysed for the indicated proteins by western blotting. To assess disassembly of Golgi SNARE complexes, rat liver Golgi membranes (20 μg)[47] were incubated on ice with 2.5 mM NEM for 15 min in buffer A lacking DTT, quenched with 5 mM DTT for 30 min and mixed with α-SNAP (5 μg) and NSF (wild-type or mutant; 7 μg) in a total volume of 150 μl for 45 min at 4°C in the presence or absence of Mg-ATP. Anti-Myc-tag antibodies (9E10) crosslinked to protein-G–Sepharose beads (Pharmacia) were added and incubations continued for 4 h. Beads were washed, eluted and TCA-precipitated. Samples were analysed by western blotting for the presence of His_6–α-SNAP (Qiagen), syntaxin-5 (ref. 48) and GOS-28 (ref. 49).

Disassembly of SNARE complexes on intact membranes.

NEM-treated synaptic vesicles[50] (10 μg) were incubated with NSF (wild-type or mutant; 3 μg) and α-SNAP (3 μg) in 20 mM HEPES, pH 7.8, 100 mM KCl, 2 mM EDTA, protease inhibitors (0.25 mM PMSF, 2 mM benzamidine and 10 μg ml^{-1} leupeptin), 1 mM DTT, 0.5 mM ATP in the presence of an ATP-

regenerating system in a total volume of 25µl for 40 min at 25°C. $MgCl_2$ was added to 8 mM where indicated. 2× SDS sample buffer (62.5 mM Tris, pH 6.8, 4% SDS, 10% sucrose, 5% β-mercaptoethanol, 0.01% bromphenol blue) was added and incubations continued for 10 min at room temperature. Samples were analysed by western blotting for the presence of monomeric and high-molecular-mass SDS-resistant complexes containing syntaxin-1 (antibody HPC-1, Sigma) and VAMP-1 (data not shown).

Negative staining.

To assess temperature dependency, we incubated NSF proteins at 25°C or 37°C in EM buffer (30 mM HEPES-KOH, pH 7.4, 70 mM KCl, 5 mM $MgCl_2$ and 3 mM EDTA) supplemented with 1 mM ATP for 1 h. Samples were adsorbed onto carbon-coated formvar grids and negatively stained with 1% uranyl acetate before air-drying. Grids were viewed using a Philips CM10 electron microscope and selected images were processed[36]. To assess nucleotide dependency, we incubated NSF (wild-type or mutant) in EM buffer supplemented with 2 mM Mg-ATP-γS or Mg-ADP at 4°C for 1 h. NSF was then negatively stained and viewed as above.

ATPase-activity assay.

NSF proteins (2–4µg) were incubated in a total volume of 50µl in 20 mM Tris-HCl, pH 9.0, 17 mM NaCl, 83 mM KCl, 10 mM $MgCl_2$ and 2 mM ATP supplemented with [γ-^{32}P]ATP at the indicated temperatures for varying times. In one experiment, His_6-tagged α-SNAP was added to a 25°C reaction at a molar ratio of NSF:α-SNAP of 1:2. Nucleotides were bound to 250µl ice-cold, activated charcoal (0.8% *w/v* in 0.2 M HCl, 1 mM NaH_2PO_4) and samples were centrifuged at 12,000*g* for 2 min at 4°C. The resulting supernatants containing released [$^{32}P_i$] were filtered (0.22µm, Millipore) and quantified by liquid scintillation counting. To determine NEM-insensitive activities, we pretreated the NSF proteins on ice with 2.5 mM NEM for 15 min followed by 5 mM DTT for 30 min. About 90% of the wild-type NSF activity was sensitive to NEM compared with about 10% of the mutant NSF activity.

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Correspondence and requests for materials should be addressed to D.T.S.