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Sequential SNARE disassembly and GATE-16–GOS-28 complex assembly mediated by distinct NSF activities drives Golgi membrane fusion

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Characterization of mammalian NSF (G274E) and *Drosophila* NSF (*comatose*) mutants revealed an evolutionarily conserved NSF activity distinct from ATPase-dependent SNARE disassembly that was essential for Golgi membrane fusion. Analysis of mammalian NSF function during cell-free assembly of Golgi cisternae from mitotic Golgi fragments revealed that NSF disassembles Golgi SNAREs during mitotic Golgi fragmentation. A subsequent ATPase-independent NSF activity restricted to the reassembly phase is essential for membrane fusion. NSF/α-SNAP catalyze the binding of GATE-16 to GOS-28, a Golgi v-SNARE, in a manner that requires ATP but not ATP hydrolysis. GATE-16 is essential for NSF-driven Golgi reassembly and precludes GOS-28 from binding to its cognate t-SNARE, syntaxin-5. We suggest that this occurs at the inception of Golgi reassembly to protect the v-SNARE and regulate SNARE function.

Introduction

NSF belongs to the ATPases associated with diverse cellular activities (Patel and Latterich, 1998) (AAA)* family of ATPases and catalyzes multiple exocytic/endocytic membrane fusion events most likely at a predocking stage (Colombo et al., 1996; Mayer et al., 1996). NSF functions via interactions with SNAREs, integral membrane proteins that recruit NSF to membranes via SNAPs (Söllner et al., 1993). NSF binds to SNAP–SNARE complexes, forming a particle that sediments at 20S and is disassembled upon NSF-driven ATP hydrolysis (Wilson et al., 1992; Söllner et al., 1993). SNARE disassembly occurs as a consequence of a conformational switch between the ATP/ADP states of the NSF hexamer that generates a rotational force that transmitted via SNAPs may help unwind the helical SNARE bundle (Hanson et al., 1997; Owen and Schiavo, 1999).

The cyclic assembly and disassembly of SNAREs is essential for sustainable SNARE function during membrane fusion (Whiteheart et al., 1994; Mayer et al., 1996). A unitary v-SNARE on a vesicle interacts specifically with its cognate t-SNARE complex on the target membrane forming a trans-SNARE complex, which leads to the tight association of the vesicle with its target membrane, termed docking (Pfeffer, 1999; McNew et al., 2000). The release of energy induced by trans-SNARE complex formation may promote fusion directly (Chen et al., 1999; McNew et al., 2000) or serve as a transient signal to downstream effectors that induce fusion (Ungermann et al., 1998; Peters et al., 2001). After fusion, highly stable cis-SNARE complexes accumulate in the fused membranes. The ATPase-dependent SNARE-dissociating activity of NSF allows their recycling for future fusion events (Mayer et al., 1996; Xu et al., 1998).

In vacuole fusion, NSF-catalyzed SNARE disassembly is tightly coupled to the transfer of LMA1 from NSF to the t-SNARE Vam3p (Xu et al., 1998). LMA1 is a thioredoxin/proteinase B inhibitor 2 heterodimer and is proposed to maintain Vam3p in an activated form conducive for future trans-SNARE pairing events (Xu et al., 1998). A similar activity is proposed for GATE-16, a component of the intra-Golgi transport machinery, which binds NSF and GOS-28, a

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*Abbreviations used in this paper: AAA, ATPases associated with diverse cellular activities; MGF, mitotic Golgi fragment; RLG, rat liver Golgi membrane; wt, wild type.

Key words: NSF; α-SNAP; GATE-16; GOS-28; Golgi

Golgi v-SNARE (Sagiv et al., 2000). Thus, NSF not only disassembles SNAREs but also facilitates binding of SNARE activation factors, such as LMA1, that maintain SNARE activity for future fusion events.

To better define the role of NSF in membrane fusion, we focused on a biochemical analysis of various NSF mutants and their activity in a cell-free system that imitates the mitotic fragmentation/reassembly of the mammalian Golgi apparatus (Rabouille et al., 1995a,b). These included a mammalian NSF mutant constructed to mimic one of the *comatose* mutants of *D. melanogaster* (dNSF-1 *comt*^{ST17} [Pallanck et al., 1995]). In flies, *comatose* mutations induce a progressive reduction of neurotransmitter release upon raising the temperature from 25 to 37°C (Siddiqi and Benzer, 1976). Neuroparalysis at the restrictive temperature is accompanied by an accumulation of synaptic vesicles and assembled SNARE complexes within synaptic nerve terminals (Kawasaki et al., 1998; Tolar and Pallanck, 1998). However, a biochemical analysis of *comatose* dNSF-1 mutants is still lacking. Analysis of NSF ATPase mutants (in particular D1 G274E and D1 E329Q) revealed that NSF-driven Golgi reassembly can occur without NSF-driven ATP hydrolysis and so is uncoupled from SNARE disassembly (Müller et al., 1999).

By analyzing the state of SNAREs and the need for NSF during the mitotic fragmentation/reassembly of the Golgi apparatus, we now show that NSF has two functionally and biochemically separable, sequential functions. The first corresponds to the ATPase-dependent disassembly of SNAREs and occurs during mitotic Golgi fragmentation. The second is needed during the fusion of mitotic Golgi fragments (MGFs) and occurs in the absence of ATP hydrolysis. Functional and biochemical comparison of the original *comatose* dNSF-1 mutant (*comt*^{ST17}) of *Drosophila* with its mammalian counterpart suggests that the ATPase-independent NSF activity may be evolutionarily conserved.

To define the ATPase-independent function, we focused on the NSF–GATE-16 interaction (Sagiv et al., 2000). We now show that GATE-16 is an essential component of NSF-driven Golgi reassembly. GATE-16 acts at a terminal stage in membrane fusion coincident with GOS-28. Furthermore, NSF/α-SNAP stimulate the recruitment of GATE-16 to unpaired GOS-28 in a manner that requires ATP but not ATP hydrolysis. GATE-16 precludes GOS-28 from binding its cognate t-SNARE syntaxin-5 (Subramaniam et al., 1997). Thus, GATE-16 is a v-SNARE protector (Pfeffer, 1999) that may prevent GOS-28 from assembling into unproductive cis-SNARE complexes.

Results

A role for NSF in Golgi reassembly that is independent of SNARE disassembly

Golgi reassembly can be catalyzed by NSF mutants (D1 E329Q, D2 D604Q, and D1 G274E) defective in ATP hydrolysis (Müller et al., 1999). This appears to contradict data from other membrane fusion assays, such as intra-Golgi transport (Whiteheart et al., 1994), endosome–endosome fusion (Colombo et al., 1996), and regulated exocytosis (Banerjee et al., 1996). These studies purport an obligate role for ATP hydrolysis and SNARE disassembly by NSF. To address the nucleotide requirements necessary for NSF-driven Golgi reassembly, reactions were performed with either NSF (wild type [wt]) or NSF (G274E) in the presence of various adenine nucleotides or nucleotide analogues.

MGFs were treated with NEM to inactivate endogenous NSF and α-SNAP (Wattenberg et al., 1992). NEM was quenched with DTT, and Golgi reassembly was initiated by addition of exogenous NSF, α-SNAP, γ-SNAP, and the vesicle tethering protein p115. Membrane fusion (cisternal regrowth) was monitored by quantitative EM (Rabouille et al., 1995a,b). Both NSF wt and mutant (G274E) catalyzed membrane fusion in the presence of ATP, ATPγS (a slowly hydrolyzable ATP analogue), AMP-PNP, and AMP-PCP (both nonhydrolyzable ATP analogues). In contrast, in the presence of ADPβS or the absence of adenine nucleotide little reassembly occurred (Fig. 1 a). Membrane fusion was also abolished when ATP-degenerating systems, such as glucose/hexokinase or apyrase, were added to convert ATP to ADP or AMP, respectively (unpublished data).

Titration of NSF into the reassembly reaction in the presence of ATP or ATPγS revealed that both the wt and mutant proteins had similar activity profiles (Fig. 1, b and c). This suggests that NSF acts by a common mechanism to promote Golgi membrane fusion in the presence or absence of ATP hydrolysis.

Whether NSF could hydrolyze ATPγS was determined by monitoring the release of [γ-^{35}S] from [γ-^{35}S]ATPγS compared with the release of [γ-^{32}P] from [γ-^{32}P]ATP at 25°C (Fig. 1 d). The ATPase activity of NSF wt (6.5 ± 1.3 μmol P_i per mg NSF per h; in [γ-^{32}P]ATP) was ~30-fold decreased in the presence of [γ-^{35}S]ATPγS. Furthermore, this reduction was observed even if the stimulating factor α-SNAP was added to the reaction. NSF (G274E) had no ATPase activity under any of these conditions (Fig. 1 d). Thus, it is unlikely that a low level of ATPγS hydrolysis by NSF wt is required for NSF-driven Golgi reassembly, since under nonsaturating NSF (wt and mutant) concentrations membrane fusion occurs to ~60% in ATPγS (Fig. 1 c). This finding is reinforced by the fact that NSF (wt or mutant) can induce Golgi membrane fusion in the presence of the nonhydrolyzable ATP analogues AMP-PNP and AMP-PCP (Fig. 1 a). Together, these results imply that ATP hydrolysis, and therefore SNARE disassembly, is not required during NSF-driven Golgi reassembly, yet the presence of ATP or an ATP analogue is essential.

The nature of this distinct role for NSF in Golgi reassembly has raised various issues (Müller et al., 1999; Schwarz, 1999), such as whether it is akin to an NSF activity that induces liposome fusion (Otter-Nilsson et al., 1999). However, this is unlikely, since the NSF activity required for Golgi reassembly is NEM sensitive and requires α-SNAP (Müller et al., 1999), unlike the liposome fusion activity of NSF (Brügger et al., 2000). We have proposed that an alternative to this extreme view would be that NSF performs multiple functions during membrane fusion and that an abundant source of disassembled SNAREs already exists on MGFs. If so, NSF-driven SNARE disassembly may be required before membrane fusion but is an event that is temporally and functionally distinct from the ATPase-independent function of NSF during membrane fusion.

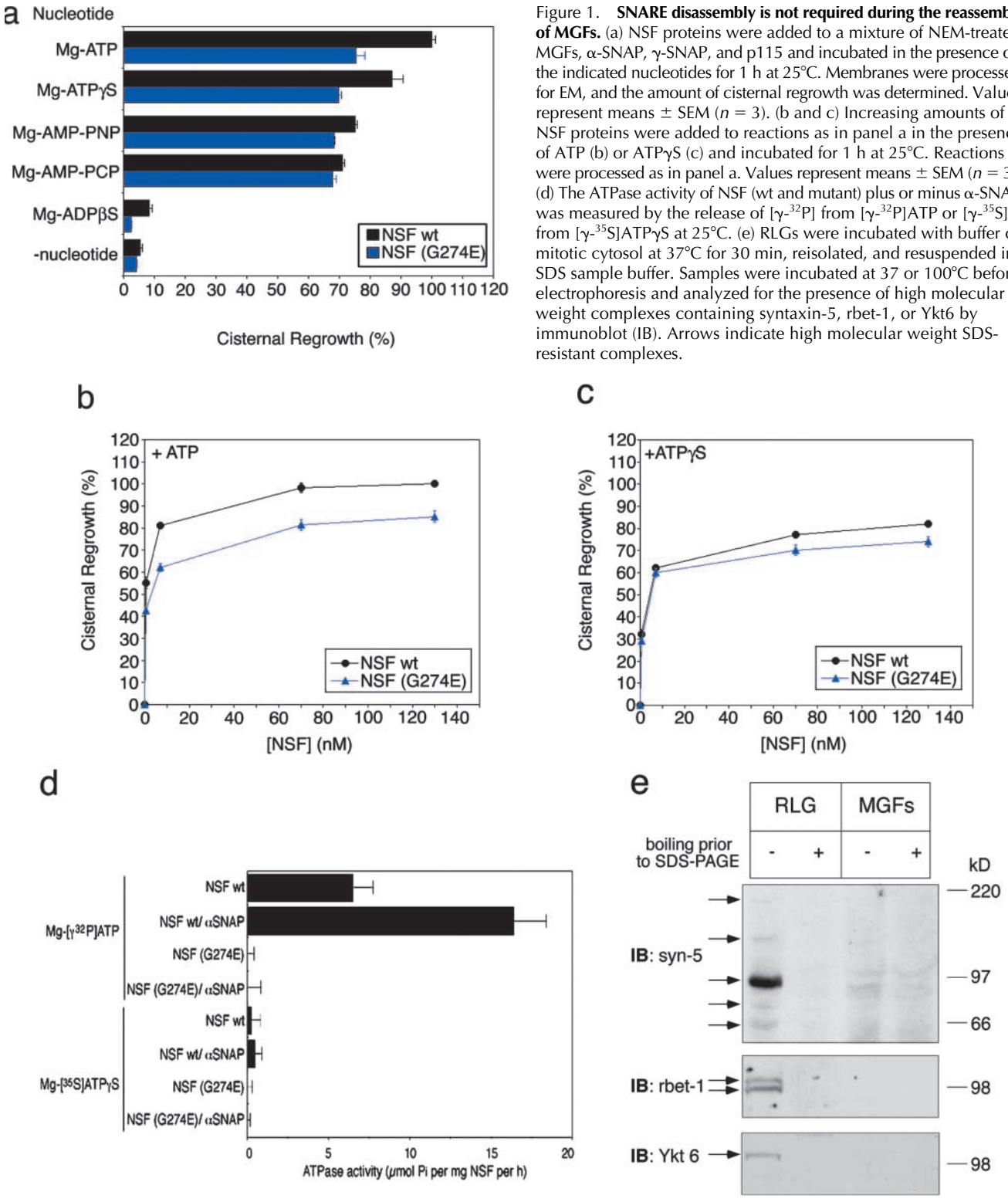

Figure 1. **SNARE disassembly is not required during the reassembly of MGFs.** (a) NSF proteins were added to a mixture of NEM-treated MGFs, α-SNAP, γ-SNAP, and p115 and incubated in the presence of the indicated nucleotides for 1 h at 25°C. Membranes were processed for EM, and the amount of cisternal regrowth was determined. Values represent means ± SEM ($n = 3$). (b and c) Increasing amounts of NSF proteins were added to reactions as in panel a in the presence of ATP (b) or ATPγS (c) and incubated for 1 h at 25°C. Reactions were processed as in panel a. Values represent means ± SEM ($n = 3$). (d) The ATPase activity of NSF (wt and mutant) plus or minus α-SNAP was measured by the release of [γ-^{32}P] from [γ-^{32}P]ATP or [γ-^{35}S] from [γ-^{35}S]ATPγS at 25°C. (e) RLGs were incubated with buffer or mitotic cytosol at 37°C for 30 min, reisolated, and resuspended in SDS sample buffer. Samples were incubated at 37 or 100°C before electrophoresis and analyzed for the presence of high molecular weight complexes containing syntaxin-5, rbet-1, or Ykt6 by immunoblot (IB). Arrows indicate high molecular weight SDS-resistant complexes.

To test this hypothesis, the oligomeric state of Golgi SNAREs was examined on MGFs and rat liver Golgi membranes (RLGs) by taking advantage of the fact that SNARE complexes are SDS resistant at 37°C and can be visualized as high molecular weight complexes by immunoblot (Otto et al., 1997; Shorter et al., 2002). Similar to neuronal SNAREs (Müller et al., 1999), multiple SDS-resistant complexes were observed at ~100 kD for the Golgi SNAREs syntaxin-5, rbet-1, and Ykt6 on RLGs (Fig. 1 e) (Shorter et al., 2002). These complexes disappeared upon boiling the membranes before electrophoresis. In contrast, these high molecular weight complexes were absent on MGFs (Fig. 1 e). This suggests that SNARE complexes preexist on RLGs and are disassembled during mitotic fragmentation. This also provides an explanation for the finding that SNARE disassembly is not required during the

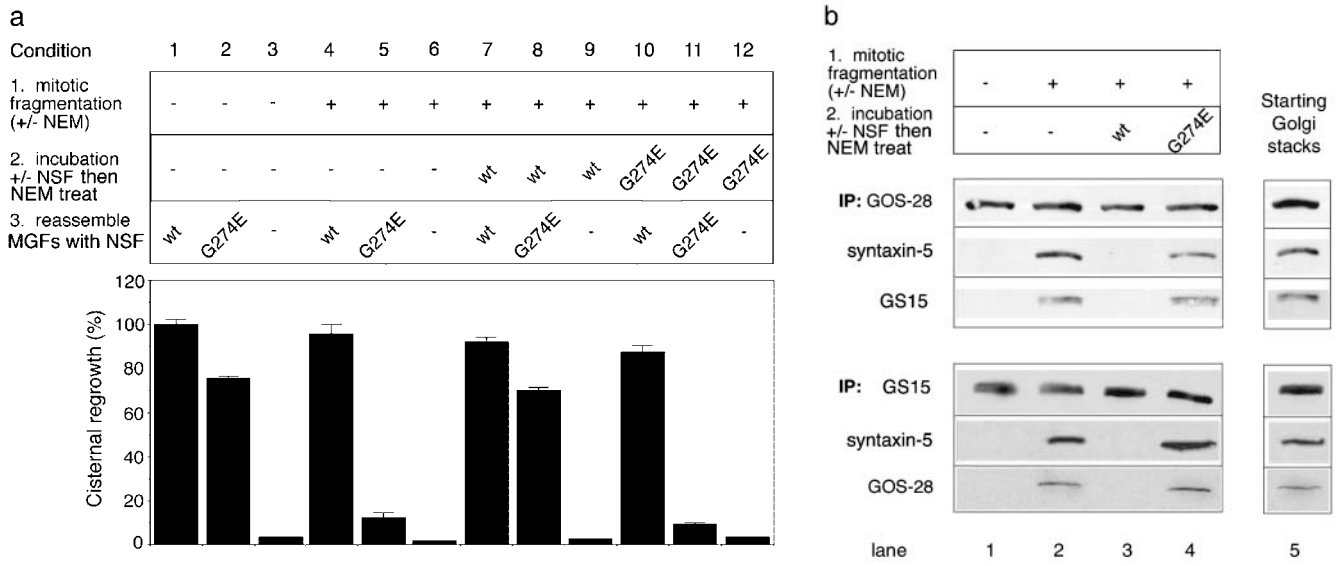

Figure 2. **Two NSF activities are required for the fusion of MGFs.** (a) Fragmentation reactions were pretreated with NEM for 15 min on ice followed by DTT for 15 min on ice (conditions 4–12). NEM prequenched with DTT served as the control (conditions 1–3). NSF (wt or mutant) and α-SNAP (conditions 7–12) or buffer (conditions 1–6) was then added, and reactions were incubated for 10 min at 25°C. NSF was then inactivated with NEM, and the reaction continued for 20 min at 37°C. MGFs were isolated and incubated in standard fusion assays with NSF (wt or mutant) or buffer at 25°C. Reactions were processed for EM, and the extent of cisternal regrowth was determined. Values represent means ± SEM ($n = 3$). (b) Fragmentation reactions were performed as in panel a except that MGFs were solubilized with Triton X-100 buffer. RLGs incubated with buffer instead of mitotic cytosol served as the control (lane 5). GOS-28 or GS15 was then immunoprecipitated. Immunocomplexes were analyzed for the presence of syntaxin-5, GOS-28, and GS15 by immunoblot.

fusion of MGFs. Importantly, these data imply that NSF is required twice during the fragmentation/reassembly cycle of the Golgi apparatus, since MGFs still require NSF in an ATPase-independent role to reform cisternae.

If NSF performs two functions in Golgi reassembly, we might expect that the *comatose* NSF mutant would catalyze fusion only if MGFs had been exposed to functional wt NSF during the fragmentation phase to generate a pool of disassembled SNAREs. To test this hypothesis, RLGs and mitotic cytosol were treated separately with NEM (to inactivate endogenous NSF/α-SNAP) that was then quenched with DTT before mixing for mitotic fragmentation. Mitotic fragmentation is unaffected by this NEM treatment (Rabouille et al., 1995b). NEM-treated RLGs and NEM-treated mitotic cytosol were mixed and incubated with α-SNAP and NSF (wt or mutant) to selectively restore SNARE disassembly. Reactions were then treated with NEM again to inactivate added NSF/α-SNAP, and mitotic fragmentation was allowed to proceed. MGFs were then isolated and reassembled in the presence or absence of either wt or mutant NSF. In the absence of added NSF, no reassembly occurred after any previous fragmentation condition (Fig. 2 a, conditions 3, 6, 9, and 12). Thus, no residual NSF activity from the mitotic fragmentation phase persists into the reassembly phase after NEM treatment. NSF wt catalyzed fusion irrespective of whether the MGFs were generated previously in the absence or presence of NEM pretreatment (Fig. 2 a, conditions 1 and 4). In contrast, mutant NSF only promoted fusion of MGFs that were generated either in the absence of NEM pretreatment (i.e., in the presence of endogenous NSF [Fig. 2 a, condition 2]) or generated in the presence of NEM but treated with wt NSF before fusion (Fig. 2 a, condition 8). This implies that MGFs only reform cisternae if they have been exposed to wt NSF at one stage of the assay. Thus, the SNARE-dissociating function of NSF is required for membrane fusion but can be fulfilled during mitotic fragmentation. These data also suggest that SNARE pairing is inhibited under mitotic conditions, which likely contributes to the mitotic inhibition of intra-Golgi and ER-Golgi transport that induce Golgi fragmentation (Warren, 1985). This may be due to the mitotic release of p115 from Golgi membranes and inhibition of vesicle tethering events that facilitate downstream trans-SNARE complex formation (Nakamura et al., 1997; Shorter et al., 2002).

To substantiate that the role of NSF during Golgi fragmentation is the generation of disassembled SNAREs (Fig. 2 a, conditions 2 and 8), MGFs were prepared and treated with NSF (wt or mutant) as described in Fig. 2 a. Membranes were then solubilized and either GOS-28 or GS15 was immunoprecipitated. A SNARE complex comprising GOS-28, GS15, and syntaxin-5 was isolated from RLG extract (Fig. 2 b, lane 5) (Shorter et al., 2002). Upon incubation of the membranes with mitotic cytosol, this SNARE complex was not detectable (Fig. 2 b, lane 1) yet was preserved upon NEM pretreatment (Fig. 2 b, lane 2). This suggests that endogenous NSF supplied with mitotic cytosol disassembled preexisting SNARE complexes. This is supported by the finding that supplementing reactions with wt NSF (Fig. 2 b, lane 3) but not mutant NSF (Fig. 2 b, lane 4) restored SNARE disassembly. Together, these data imply that SNARE complexes on RLGs are broken up by wt NSF during the mitotic fragmentation of RLGs and that SNARE disassembly is essential for the Golgi fragmentation/reassembly cycle. Moreover, these findings discriminate two independent NSF activities required for Golgi reassembly, since MGFs that are exposed to wt NSF before reassembly and so

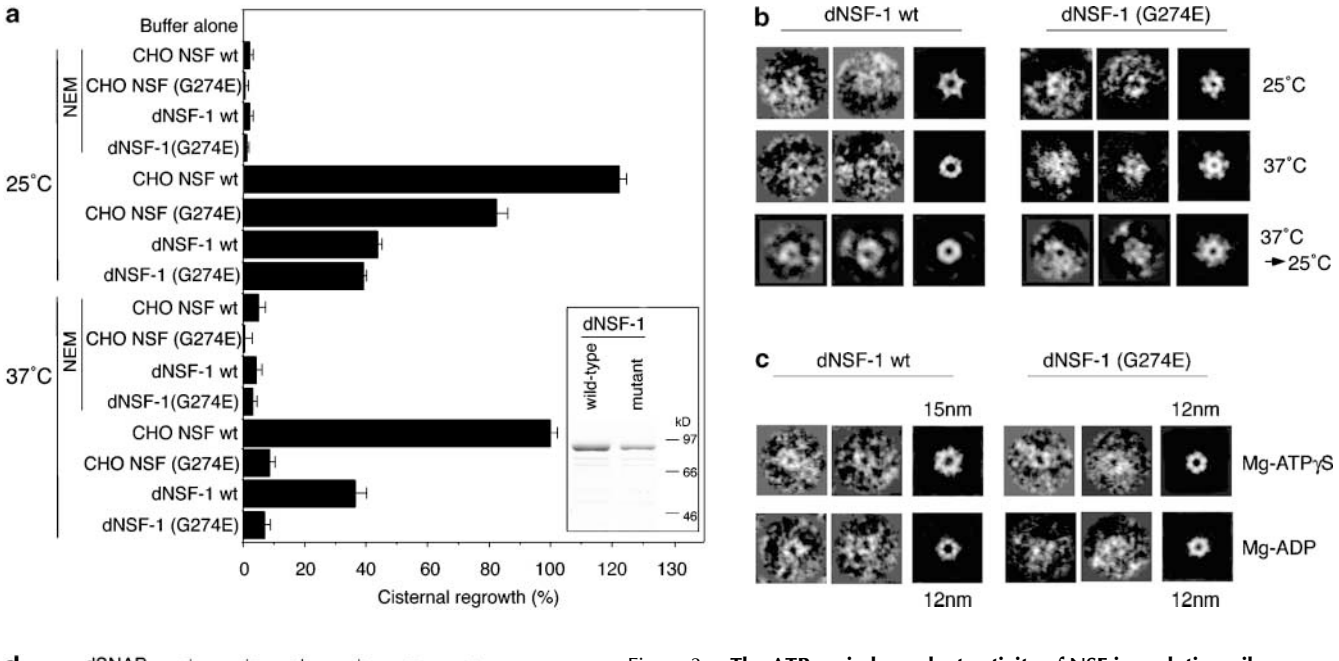

Figure 3. **The ATPase-independent activity of NSF is evolutionarily conserved.** (a) Golgi reassembly was performed with the indicated NSF proteins (*Drosophila* or CHO) at 25 or 37°C. NSF proteins preincubated with NEM served as negative control. Reactions were processed for EM, and the amount of cisternal regrowth was determined. Values represent means ± SEM ($n = 2$). SDS-PAGE analysis of the pure His_6-tagged dNSF proteins is shown in the inset. (b) *Comatose* dNSF-1 irreversibly changes conformation at 37°C. dNSF-1 proteins were incubated in the presence of Mg-ATP for 90 min at 25 or 37°C. After 45 min, one set of the 37°C samples was shifted to 25°C. Samples were processed for negative staining. A representative set of three end-on views is shown on the left, together with an averaged ($n = 300$) and sixfold symmetrized view on the right. (c) dNSF-1 proteins were incubated with Mg-ATP γS or Mg-ADP for 45 min at 4°C. Samples were processed and analyzed as in panel b. Note that Mg-ATPγS increases the diameter of wt dNSF-1 but not mutant dNSF-1 barrel from ~12 to 15 nm. (d) NEM-treated light membranes isolated from fly heads were incubated with dSNAP and dNSF-1 (wt or mutant) in Mg-ATP for 40 min at 25°C. SDS sample buffer was then added, and the presence of high molecular weight complexes containing dsyntaxin-1A was determined by immunoblot.

contain a pool of disassembled SNAREs (Fig. 2 b, lanes 1 and 3) still require NSF (wt or mutant) to fuse and reform cisternae (Fig. 2 a, conditions 2 and 8).

The ATPase-independent activity of NSF is evolutionarily conserved

The identification of a second NSF function during membrane fusion is largely based on the use of the mammalian *comatose* NSF mutant. To elucidate whether this *comatose*-like NSF mutant is a biochemical phenocopy of the $comt^{ST17}$ dNSF-1 allele of *D. melanogaster*, the *Drosophila* NSF-1 proteins were characterized. This would help resolve whether the ATPase-independent activity is an evolutionarily conserved NSF activity. Thus, *Drosophila* NSF-1 wt and mutant proteins were expressed in *E. coli* and purified using their NH_2-terminal His_6-tags (Fig. 3 a, inset). Both proteins were tested for their ability to promote Golgi reassembly at 25 and 37°C, the permissive and restrictive temperature for neuronal function of *comatose* flies. dNSF-1 wt promoted cisternal regrowth at both temperatures with an average fusion activity of ~40% compared with its mammalian counterpart (Fig. 3 a). The decreased activity of dNSF-1 is likely due to an ~50% reduction, relative to mammalian NSF, in its ability to bind to mammalian SNAP–SNARE complexes (unpublished data). In contrast, mutant dNSF-1 catalyzed reassembly to the levels observed with the dNSF-1 wt at 25°C but was inactive at 37°C. Thus, the *comatose* dNSF-1 mutant is temperature sensitive for membrane fusion and therefore functionally mimics its mammalian counterpart.

Structural and biochemical analyses of the dNSF-1 mutant uncovered additional similarities between the mammalian and *Drosophila comatose* mutants. As for the mammalian mutant (Müller et al., 1999), the *Drosophila* mutant (a) irreversibly changes conformation at 37°C (Fig. 3 b), (b) does not change conformation in the presence of different adenine nucleotides (Fig. 3 c), (c) has no detectable ATPase activity at temperatures ranging from 20 to 40°C (although neither did dNSF-1 wt; unpublished data), and (d) is defective in disassembling *Drosophila* SNAP–SNARE complexes (Fig. 3 d). Together, these results suggest that the mammalian NSF mutant and the authentic *comatose* mutant are structural and biochemical phenocopies. Moreover, they in-

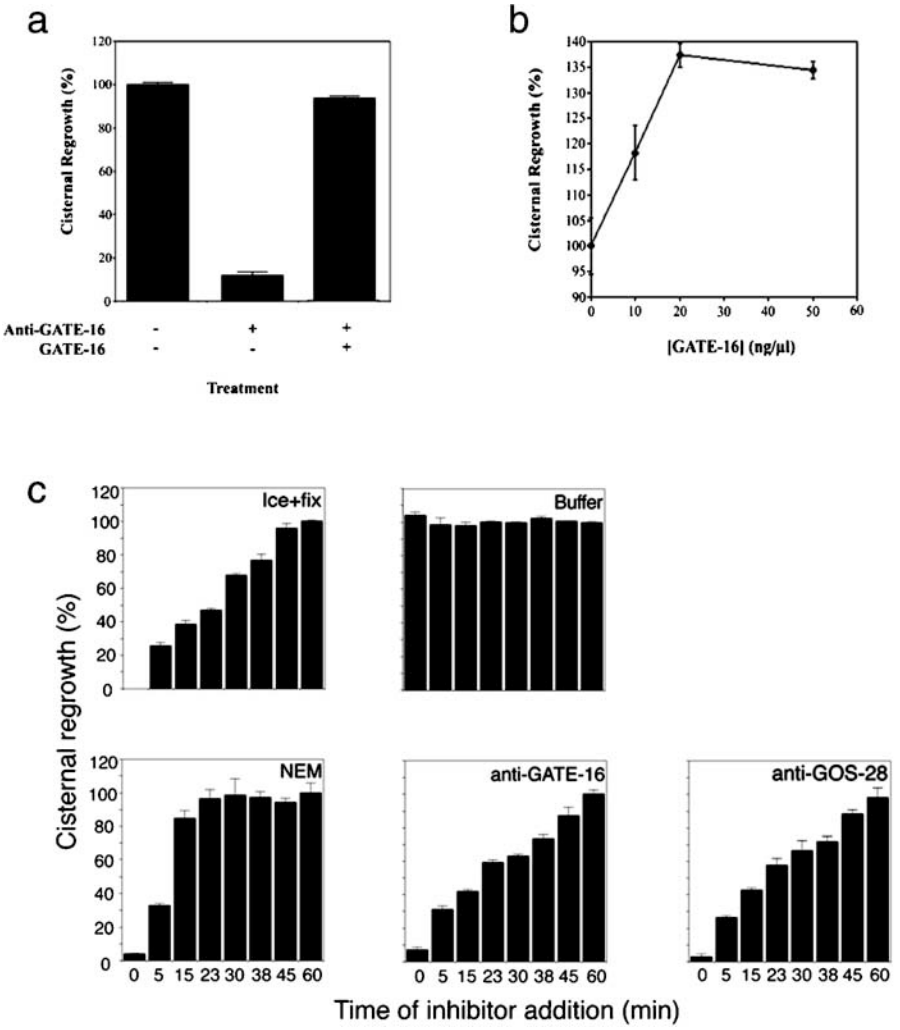

Figure 4. **GATE-16 is involved in Golgi reassembly.** (a) Standard assays were performed with anti–GATE-16 antibodies and His_6–GATE-16 protein as indicated, and the amount of cisternal regrowth was determined. Values represent means ± SEM ($n = 3$). (b) Increasing concentrations of His_6–GATE-16 were added to standard assays. Values represent means ± SEM ($n = 3$). (C) Standard fusion assays were performed except that at the indicated times reactions were either terminated by fixation or treated with either buffer, NEM, anti–GOS-28 antibodies, or anti–GATE-16 antibodies after which the reaction was allowed to proceed for a total time of 1 h. Samples were analyzed as in panel a. Values represent means ± SEM ($n = 4$).

dicate that the ATPase-independent activity of NSF is preserved in the dNSF-1 mutant and reveal an evolutionarily conserved facet of this alternative NSF function.

NSF-driven Golgi reassembly requires GATE-16

To examine the precise nature of the ATPase-independent role of NSF in Golgi reassembly, we searched for NSF binding partners implicated in Golgi membrane fusion. GATE-16, a cytosolic and Golgi peripheral membrane protein required for intra-Golgi transport, was identified as a candidate, since it binds NSF and GOS-28 (Sagiv et al., 2000), a Golgi v-SNARE involved in the NSF-driven Golgi reassembly (Shorter et al., 2002).

If GATE-16 were involved in the ATPase-independent function of NSF, it would also be required for NSF-driven Golgi reassembly. This possibility was tested using affinity purified neutralizing antisera raised to GATE-16. Preincubation of MGFs with these antibodies inhibited membrane fusion by ~90% (Fig. 4 a). This inhibition was reversed by preadsorption of the antibodies with His_6–GATE-16. Furthermore, titration of His_6–GATE-16 into reassembly reactions elevated cisternal regrowth by a maximum of ~37% at 20 ng/µl His_6–GATE-16 (Fig. 4 b).

Next, the sequence of NSF, GATE-16, and GOS-28 action during Golgi reassembly was established using well-defined inhibitors in a kinetic analysis. Thus, Golgi reassembly reactions were terminated at different time points by fixation (negative control), treated with buffer alone (positive control) or with different inhibitors, after which the reaction was allowed to proceed for a total time of 1 h. Termination of the reaction by fixation revealed that cisternal regrowth proceeds with approximately linear kinetics for the first 45 min (Fig. 4 c). Addition of buffer at any time point during the reaction had no effect on membrane fusion (Fig. 4 c). All inhibitors tested abolished fusion almost completely when added at the onset of the incubation. Inactivation of NSF and α-SNAP by addition of NEM (Wattenberg et al., 1992) inhibited the reaction within the initial 15 min of the incubation (Fig. 4 c) and could be rescued by addition of fresh NSF and α-SNAP, suggesting that no additional NEM-sensitive factors had been inactivated (unpublished data). This inhibition was much greater at 5 min than at 15 min, suggesting that NSF completes much of its function during the 5–15-min interim. At later time points, the reaction was resistant to NEM, indicating that NSF and α-SNAP were only required at this early stage (Fig. 4 c). In contrast, addition of anti–GOS-28 or anti–GATE-16 antibodies inhibited cisternal regrowth throughout the reaction, indicating that both proteins were required at a terminal stage of fusion (Fig. 4 c). Together, this suggests that the ATPase-indepen-

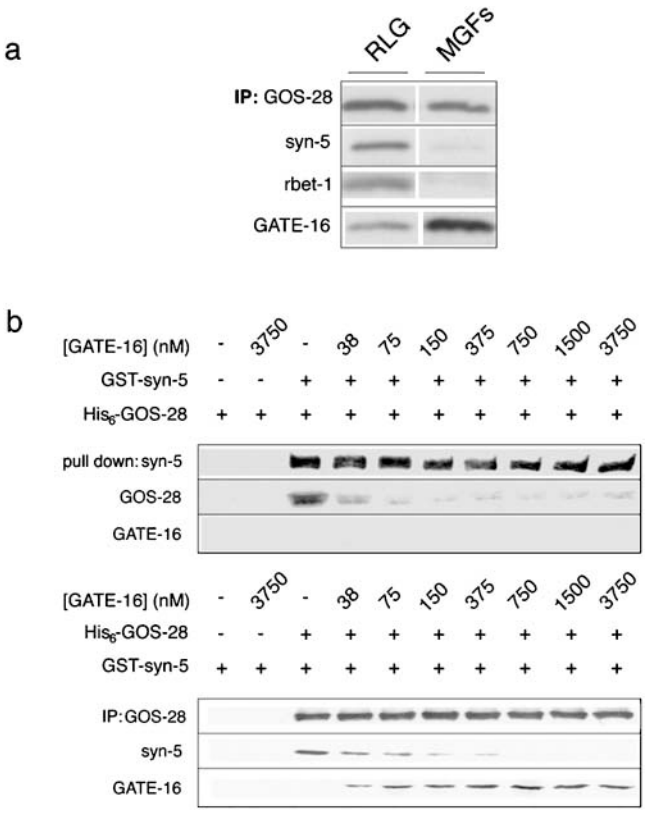

Figure 5. **GATE-16 regulates GOS-28–syntaxin-5 binding.** (a) GATE-16 was mixed with RLG or MGF extract followed by the addition of anti–GOS-28 beads (IP). Immunocomplexes were isolated and analyzed for the presence of GOS-28, syntaxin-5, rbet1, and GATE-16 by immunoblot. (b) His_6–GOS-28 (75 nM) was incubated for 1 h on ice with GST–syntaxin-5 (75 nM) and increasing concentrations of His_6–GATE-16 (0–3,750 nM). GST–syntaxin-5 was retrieved with glutathione sepharose (top), or His_6–GOS-28 was immunoprecipitated (bottom). Reactions were then processed for immunoblot.

dent function of NSF occurs upstream of the obligate functions of GATE-16 and GOS-28.

GATE-16 regulates the ability of GOS-28 to assemble into a SNARE complex

What is the role of GATE-16 in NSF-driven Golgi reassembly? One possibility is that it preserves the dissociated form of GOS-28 in a fusion-competent state, perhaps similar to the activation of the t-SNARE Vam3p by LMA1 in vacuole fusion (Xu et al., 1998). If so, GATE-16 should preferentially bind unpaired GOS-28. Thus, His_6–GATE-16 was mixed with RLG or MGF detergent extract, a source for paired and unpaired SNAREs, respectively. GOS-28 was then immunoprecipitated and the extent of syntaxin-5, rbet-1, and GATE-16 coprecipitation was analyzed by immunoblot. GOS-28 was present in a SNARE complex together with syntaxin-5 and rbet-1 on RLGs but not on MGFs (Fig. 5 a). GATE-16 binding to GOS-28 was ~5-fold more efficient when MGFs were used instead of RLGs, suggesting that only unpaired GOS-28 can bind GATE-16.

Next, we tested the effect of GATE-16 on GOS-28–syntaxin-5 pairing in pure protein binding assays. His_6–GOS-28 was mixed at equimolar concentration with GST–syntaxin-5 in the presence of increasing amounts of His_6–GATE-16. GST–syntaxin-5 was retrieved with glutathione beads, or His_6–GOS-28 was immunoprecipitated. GATE-16 was an extremely potent inhibitor of GOS-28–syntaxin-5 binding, since even when present at equimolar levels with the SNAREs GATE-16 almost completely inhibited GOS-28–syntaxin-5 binding (Fig. 5 b). These data imply that GOS-28–syntaxin-5 and GOS-28–GATE-16 form mutually exclusive complexes.

ATPase-independent stimulation of GATE-16–GOS-28 complex formation by NSF/α-SNAP

Based on the requirements and kinetics of NSF and GATE-16 function, it may be that the ATPase-independent function of NSF in Golgi reassembly is to enhance GATE-16–GOS-28 complex formation. For such an activity to accurately reflect the ATPase-independent role of NSF in Golgi reassembly, it should be NEM sensitive and temperature sensitive for the NSF mutant.

The effect of NSF/α-SNAP on GOS-28–GATE-16 binding was tested by mixing His_6–GATE-16 with different combinations of MGF extract, α-SNAP and NSF (wt or mutant). GATE-16 was immunoprecipitated, and the extent of GOS-28, syntaxin-5, α-SNAP, and NSF coprecipitation was determined by immunoblot (Fig. 6 a). NSF wt coprecipitated with GATE-16 when preincubated at 25 (Fig. 6 a, lane 3) and 37°C (Fig. 6 a, lane 5). In contrast, mutant NSF was coprecipitated when preincubated at 25°C (Fig. 6 a, lane 6) but not if preincubated at 37°C (Fig. 6 a, lane 8), most likely because the mutant is irreversibly denatured at 37°C. Further, GOS-28 bound to GATE-16 (Fig. 6 a, lane 1) and was stimulated ~9-fold by addition of α-SNAP/NSFSNAP/NSF. NSF wt enhanced GOS-28 binding when preincubated at both 25 and 37°C (Fig. 6 a, lanes 9 and 11). In contrast, mutant NSF stimulated GOS-28 binding when preincubated at 25°C (Fig. 6 a, lane 12) but not if preincubated at 37°C (Fig. 6 a, lane 14). Stimulation was NEM sensitive, indicating that functional NSF was required for this reaction (Fig. 6 a, lanes 10 and 13). Although required for enhanced GATE-16–GOS-28 binding, α-SNAP was not present in GATE-16 immunocomplexes. This suggests that α-SNAP triggers GATE-16–GOS-28 complex formation, perhaps by recruiting NSF/GATE-16 to unpaired GOS-28, consistent with the observed binding of α-SNAP to unpaired GOS-28 (Subramaniam et al., 1997). Further, syntaxin-5 was not coprecipitated, suggesting that GOS-28 only interacts with GATE-16 when dissociated from syntaxin-5.

To define these interactions more precisely, binding assays were performed with pure proteins. Thus, biotinylated His_6–GATE-16 (bio–GATE-16) was mixed with His_6–GOS-28 at equimolar concentration in the presence of Mg-ATP and incubated with various combinations of α-SNAP and NSF (wt or G274E) that had been left untreated, incubated at 37°C, or inactivated with NEM. bio–GATE-16 was then retrieved with monomeric avidin beads, and bound proteins were eluted and fractionated by SDS-PAGE. The extent of His_6–GOS-28, α-SNAP, and NSF coprecipitation was determined by Coomassie brilliant blue staining. In the absence of NSF and α-SNAP, there was some interaction between bio–GATE-16 and His_6–GOS-28 (Fig. 6 b). However, this interaction was stimulated ~5-fold by the inclusion of α-SNAP and NSF (wt

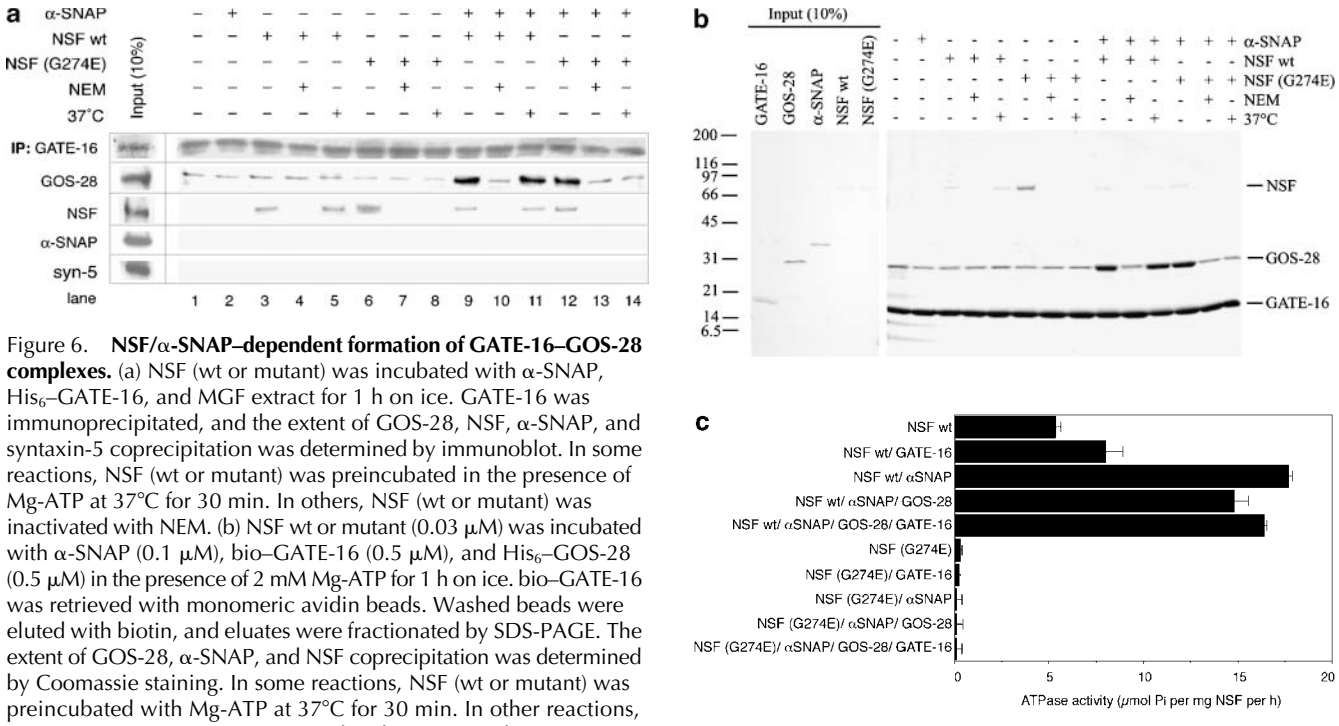

Figure 6. **NSF/α-SNAP–dependent formation of GATE-16–GOS-28 complexes.** (a) NSF (wt or mutant) was incubated with α-SNAP, His_6–GATE-16, and MGF extract for 1 h on ice. GATE-16 was immunoprecipitated, and the extent of GOS-28, NSF, α-SNAP, and syntaxin-5 coprecipitation was determined by immunoblot. In some reactions, NSF (wt or mutant) was preincubated in the presence of Mg-ATP at 37°C for 30 min. In others, NSF (wt or mutant) was inactivated with NEM. (b) NSF wt or mutant (0.03 μM) was incubated with α-SNAP (0.1 μM), bio–GATE-16 (0.5 μM), and His_6–GOS-28 (0.5 μM) in the presence of 2 mM Mg-ATP for 1 h on ice. bio–GATE-16 was retrieved with monomeric avidin beads. Washed beads were eluted with biotin, and eluates were fractionated by SDS-PAGE. The extent of GOS-28, α-SNAP, and NSF coprecipitation was determined by Coomassie staining. In some reactions, NSF (wt or mutant) was preincubated with Mg-ATP at 37°C for 30 min. In other reactions, NSF (wt or mutant) was inactivated with NEM. (c) The ATPase activity of NSF (wt and mutant) was measured by the release of [γ-^{32}P] from [γ-^{32}P]ATP plus or minus different combinations of GATE-16, α-SNAP, and GOS-28 at 25°C.

or G274E) (Fig. 6 b). Neither α-SNAP nor NSF alone enhanced the interaction, although NSF but not α-SNAP bound to bio–GATE-16 (Fig. 6 b). Again, the α-SNAP/NSF stimulation of GOS-28–GATE-16 binding was abolished by inactivation of NSF (wt and G274E) with NEM. Further, wt NSF but not mutant NSF stimulated GOS-28–GATE-16 binding if preincubated at 37°C (Fig. 6 b).

Next, we asked whether GATE-16, α-SNAP, and GOS-28 affect the ATPase activity of NSF (wt or mutant). Thus, the ATPase activity of NSF (wt and mutant) was determined by measuring the release of [γ-^{32}P] from [γ-^{32}P]ATP in the presence of various combinations of GATE-16, α-SNAP, and GOS-28 at 25°C. NSF wt hydrolyzed ATP (5.4 ± 0.3 μmol P_i per mg NSF per h), and this activity was enhanced ~1.5-fold by addition of GATE-16 and ~3.3-fold by α-SNAP (Fig. 6 c). Addition of GOS-28 slightly reduced the α-SNAP–stimulated activity of NSF wt; however, this reduction was reversed by addition of GATE-16 (Fig. 6 c). In vivid contrast, NSF (G274E) had no ATPase activity and could not be rescued by any combination of α-SNAP, GATE-16, and GOS-28 (Fig. 6 c; unpublished data).

In total, these data strongly suggest that NSF/α-SNAP stimulated GATE-16–GOS-28 complex formation represents part of the ATPase-independent function of NSF required for Golgi reassembly, since it is accomplished by the ATPase-defective *comatose* NSF mutant in an NEM- and temperature-sensitive manner.

NSF/α-SNAP–stimulated GOS-28–GATE-16 complex formation is nucleotide dependent

Next, the nucleotide dependence of the NSF/α-SNAP stimulation of GOS-28–GATE-16 binding was tested. Thus, bio–GATE-16 was incubated with MGF detergent extract and α-SNAP/NSF (wt or mutant) in the presence of either Mg-ATP, Mg-ATPγS, or Mg-ADPβS. bio–GATE-16 was then retrieved with monomeric avidin beads, and the extent of GOS-28 and NSF coprecipitation was determined by immunoblot. In the absence of bio–GATE-16, no GOS-28 or NSF was retrieved (Fig. 7 a). Upon addition of bio–GATE-16, low levels of GOS-28 were retrieved in the presence of Mg-ATP and Mg-ATPγS but not in the presence of Mg-ADPβS (Fig. 7 a). Addition of α-SNAP/NSF (wt or mutant) caused NSF to coprecipitate with GATE-16, and the amount of GOS-28 retrieved increased ~10 fold in the presence of Mg-ATP and Mg-ATPγS (Fig. 7 a). However, this stimulation was greatly reduced in the presence of Mg-ADPβS, and NSF no longer coprecipitated (Fig. 7 a). This suggests that like NSF-driven Golgi reassembly the NSF/α-SNAP stimulation of GOS-28–GATE-16 binding requires ATP but not ATP hydrolysis and does not occur in the presence of ADP.

These results were confirmed in the pure protein binding assay (Fig. 7, b and c). Thus, α-SNAP/NSF (wt [Fig. 7 b] or mutant [Fig. 7 c]) stimulated the formation of a GOS-28–GATE-16 complex in the presence of Mg-ATP and in the presence of the slowly hydrolyzable Mg-ATPγS and the nonhydrolyzable Mg-AMP-PNP and Mg-AMP-PCP (Fig. 7, b and c). In contrast, ~5-fold less complex was formed in the absence of nucleotide or in the presence of Mg-ADP or Mg-ADPβS (Fig. 7, b and c). Thus, the nucleotide dependence of NSF-stimulated GOS-28–GATE-16 complex formation is identical to that of NSF-driven Golgi membrane fusion.

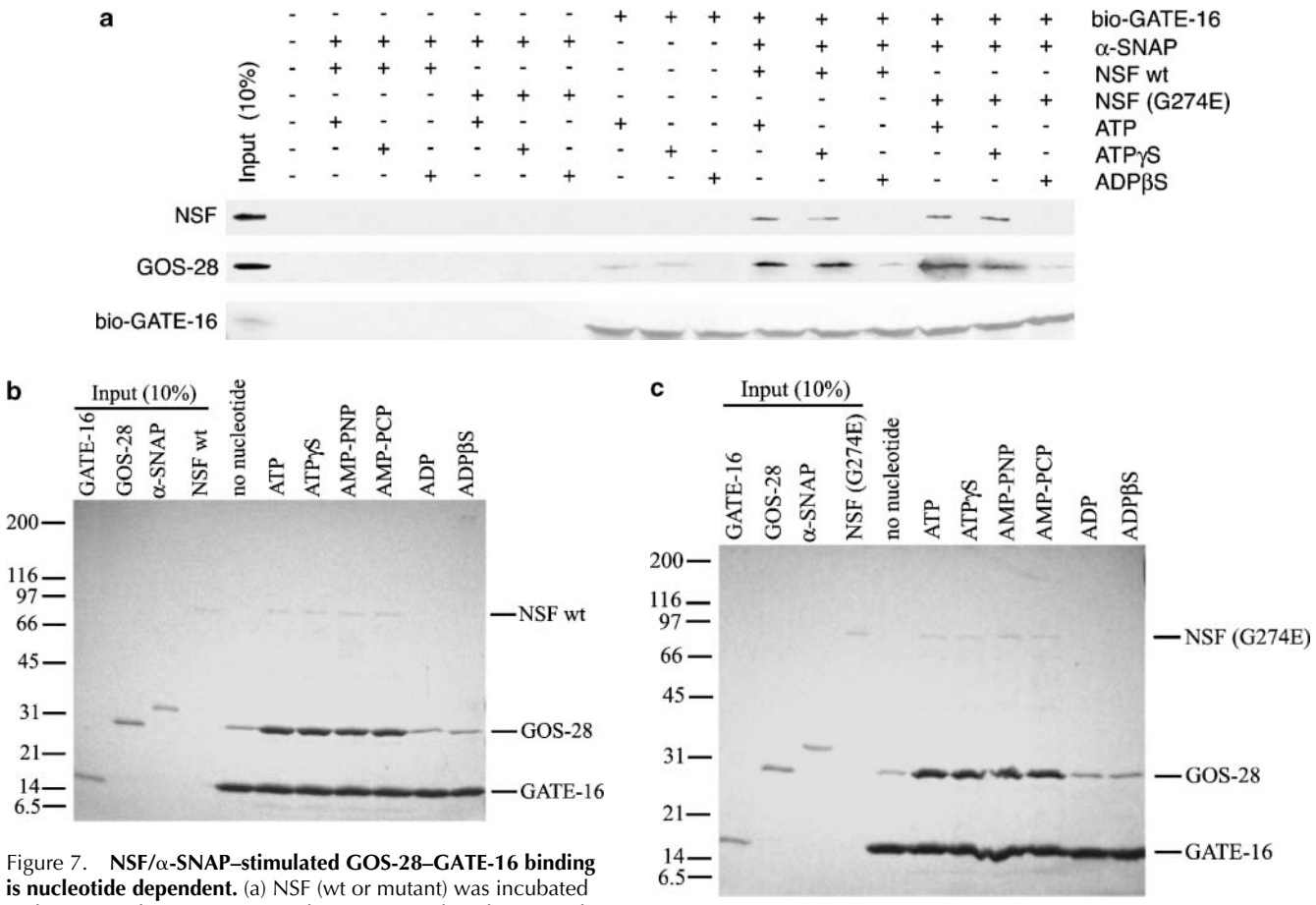

Figure 7. **NSF/α-SNAP–stimulated GOS-28–GATE-16 binding is nucleotide dependent.** (a) NSF (wt or mutant) was incubated with α-SNAP, bio–GATE-16, and MGF extract for 1 h on ice plus either Mg-ATP, Mg-ATPγS, or Mg-ADPβS. bio–GATE-16 was retrieved with monomeric avidin beads, and the extent of GOS-28 and NSF coprecipitation was determined by immunoblot. (b and c) NSF wt (b) or mutant (c) (0.03 μM) was incubated for 1 h on ice with α-SNAP (0.1 μM), bio–GATE-16 (0.5 μM), and His_6–GOS-28 (0.5 μM) plus either 2 mM Mg-ATP, Mg-ATPγS, Mg-AMP-PNP, Mg-AMP-PCP, Mg-ADP, Mg-ADPβS, or no nucleotide. bio–GATE-16 was retrieved with monomeric avidin beads. Washed beads were eluted with biotin and eluates fractionated by SDS-PAGE. The extent of GOS-28, α-SNAP, and NSF coprecipitation was determined by Coomassie staining.

NSF catalyzes the formation of the GOS-28–GATE-16 complex

Next, the effect of substoichiometric levels of NSF (wt and mutant) on the rate of GOS-28–GATE-16 complex formation was tested in the pure protein binding assay. Thus, bio–GATE-16 was immobilized on monomeric avidin beads and incubated with GOS-28 α-SNAP in the presence or absence of substoichiometric (100-fold less) levels of NSF (wt or mutant). These reactions were conducted in the presence of either Mg-ATP or Mg-AMP-PCP. bio–GATE-16 was retrieved at various times (2 min–2 h), and the extent of GOS-28 coprecipitation was determined by Coomassie brilliant blue staining. In the presence of Mg-ATP (Fig. 8, a and b) or Mg-AMP-PCP (Fig. 8, c and d), substoichiometric levels of NSF wt (Fig. 8, a and c) and NSF (G274E) (Fig. 8, b and d) enhanced the rate of GOS-28–GATE-16 complex formation. Coprecipitation of NSF was undetectable by Coomassie staining in these reactions (Fig. 8). The amount of GOS-28–GATE-16 complex formed was at least 20-fold more than the total amount of NSF present. This suggests that NSF catalyzes GOS-28–GATE-16 binding and that ATP but not ATP hydrolysis is required for efficient catalysis.

Discussion

Cell-free analysis of mitotic Golgi fragmentation/reassembly has provided a powerful tool for dissecting the molecular events surrounding membrane fusion. Detailed study of this process revealed a role for NSF in the fusion event (Rabouille et al., 1995a) but one that was later found to be distinct from the accepted role of NSF in SNARE disassembly (Müller et al., 1999). Membrane fusion was supported by NSF mutants defective in ATP hydrolysis (Müller et al., 1999) and in the presence of the slowly hydrolyzable ATP analogue ATPγS or the nonhydrolyzable ATP analogues AMP-PNP and AMP-PCP.

Here, we have demonstrated that NSF harbors at least two distinct functional and biochemical activities that can be studied in isolation within the context of the mitotic Golgi inheritance cycle. The first NSF function is the classical ATPase-dependent disassembly of SNARE complexes that preexist on Golgi membranes. This is performed during mitotic Golgi fragmentation and leads to the generation of MGFs containing a pool of dissociated SNAREs. The second NSF function is essential for the fusion of MGFs, is ATPase-independent, requires α-SNAP, and involves the formation of a GATE-16–GOS-28 complex.

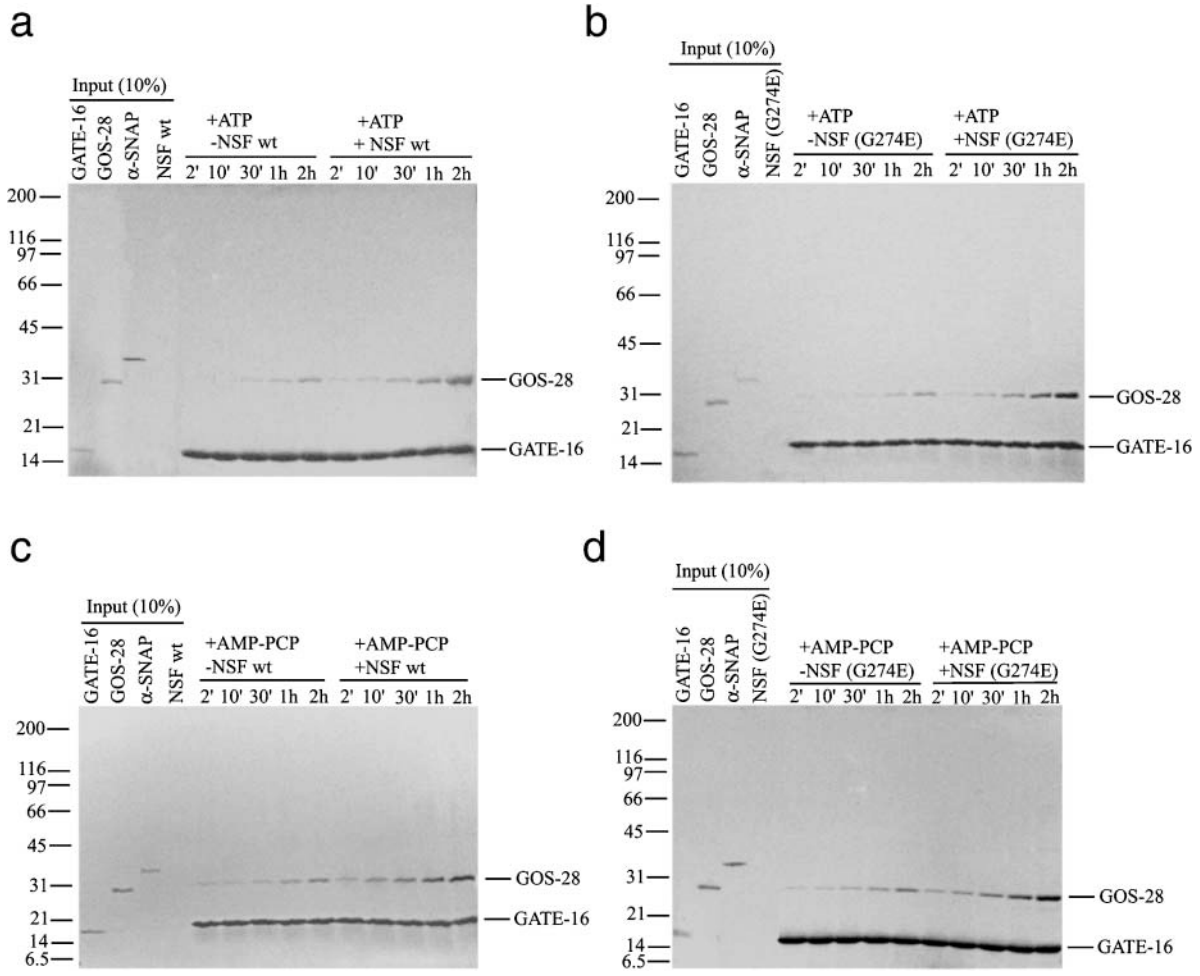

Figure 8. **NSF/α-SNAP catalyze the formation of GATE-16–GOS-28 complexes in the absence of ATP hydrolysis.** (a–d) NSF wt (5 nM; a and c) or NSF G274E (5 nM; b and d) was incubated with α-SNAP (0.1 μM), His_6–GOS-28 (0.5 μM), and bio–GATE-16 avidin beads in the presence of 2 mM Mg-ATP (a and b) or 2 mM Mg-AMP-PCP (c and d) for 2 min–2 h on ice. At the indicated time points, beads were recovered. Washed beads were eluted, and eluates were fractionated by SDS-PAGE. The extent of GOS-28, α-SNAP, and NSF coprecipitation was determined by Coomassie staining.

GATE-16 is one of three mammalian orthologues of the yeast protein Apg8p/Aut7p and is implicated in intra-Golgi transport (Sagiv et al., 2000). In yeast, Apg8p is required for autophagy, the cytoplasm to vacuole targeting pathway (Ichimura et al., 2000; Kirisako et al., 2000), and possibly ER to Golgi transport (Legesse-Miller et al., 2000). We now show that GATE-16 functions in the assembly of Golgi cisternae from MGFs and acts at a terminal phase of membrane fusion coincident with GOS-28. GATE-16 binds to NSF and GOS-28, and formation of a GATE-16–GOS-28 complex is stimulated by α-SNAP/NSF. GATE-16–GOS-28 complex formation on MGFs is also mediated by the *comatose* NSF mutant, indicating that ATP hydrolysis is not required. The interaction between the *comatose* NSF mutant and GATE-16 and the stimulation of GATE-16–GOS-28 binding was temperature sensitive. Preincubation of the *comatose* NSF mutant at 37°C abolished its capacity to stimulate GOS-28–GATE-16 binding, most likely because the NSF mutant denatures at this temperature (Müller et al., 1999). Furthermore, the nucleotide dependence of NSF/α-SNAP-stimulated GOS-28–GATE-16 complex formation was identical to that of NSF-driven Golgi reassembly in that ATP but not ATP hydrolysis was required and ADP and the absence of nucleotide were ineffective.

These findings substantiate that NSF-dependent catalysis of GATE-16–GOS-28 binding represents part of the ATPase-independent step required for Golgi membrane fusion (Müller et al., 1999). The catalytic role of NSF may involve the folding of GATE-16, possibly to liberate the GOS-28 binding domain in GATE-16. Alternatively, NSF/α-SNAP may enable the correct folding of GOS-28 to allow GATE-16 to bind. Consistent with this NSF/α-SNAP binds to unpaired GOS-28 and can induce conformational changes on Q-SNAREs in isolation (Hanson et al., 1995; Subramaniam et al., 1997).

This leaves open the precise role of GATE-16 in Golgi reassembly. GATE-16 preferentially binds to the unpaired form of GOS-28 and interferes with the binding of GOS-28 to its cognate t-SNARE syntaxin-5 in detergent solution. These effects are reminiscent of the inhibition of syntaxin–VAMP binding by Munc18 (Pevsner et al., 1994) and of Sed5p/Bet1p binding by Sly1p (Lupashin and Waters, 1997), suggesting that GATE-16 may be a v-SNARE protector (Pfeffer, 1999). Thus, GATE-16 may prevent GOS-

28 from assembling into unproductive cis-SNARE complexes. Conversely, GATE-16 may promote the regulated assembly of trans-SNARE complexes at appropriate times. It may be that GATE-16 by analogy with LMA1 (Xu et al., 1998) maintains labile GOS-28 in a fusion-competent state. Unpaired GOS-28 may adopt a suboptimal conformation for regulated trans-SNARE complex assembly, and this may be corrected by GATE-16.

GATE-16 is required at a terminal stage of membrane fusion coincident with GOS-28. Presumably, GATE-16 must be displaced from GOS-28 for trans-SNARE complex formation to occur, which may require a Rab GTPase as with the displacement of Sly1p from syntaxin-5 (Lupashin and Waters, 1997). Munc18 has also been implicated in a terminal phase of exocytosis coincident with SNARE function and may aid in fusion pore opening (Fisher et al., 2001).

Recent reports have shown that NSF also interacts with many other proteins involved in membrane dynamics, such as Rab effectors (McBride et al., 1999), AMPA receptors (Hanley et al., 2002), and β-arrestin (McDonald et al., 1999). NSF rearranges the conformational or oligomeric state of these proteins, implying that it may have a broader chaperone-like role within cells.

Taken with our findings, we suggest that NSF may serve a dual cellular role during membrane fusion as both a foldase and unfoldase. Unfolding, such as SNARE disassembly, would occur in an ATPase-dependent manner, whereas folding events, such as GATE-16–GOS-28 complex formation, would occur in an ATPase-independent manner, perhaps similar to protein folding by GroEL (Makino et al., 1993; Rye et al., 1997). Interestingly, other AAA proteins also have similar chaperone-like activities. The archaebacterial homologue of p97/CDC48, termed VAT, displays differential chaperone activity depending on its ATP hydrolysis rate. Similar to findings with NSF, VAT accelerates unfolding of a test substrate in its high ATPase activity state, whereas refolding is accelerated in the low activity state (Golbik et al., 1999). Similarly, p97 ATPase activity mediates the unfolding of polypeptide substrates as they are dislocated from the lumen of the ER to the cytosol for degradation. When p97 ATPase activity is inhibited, the substrate is no longer unfolded and remains attached to the ER membrane (Ye et al., 2001). By extension, it may be that the NSF *comatose* mutant provides a foldase activity (ATPase independent) but is defective in terms of unfoldase activity (ATPase dependent).

Our analysis of the original *comatose* NSF mutant revealed its functional and structural similarities to the mammalian temperature-sensitive NSF counterpart. The dNSF-1 *comatose* mutant lacks ATPase and SNARE-dissociating activity, nevertheless it promoted membrane fusion at 25°C. At the restrictive temperature of 37°C, the mutant is irreversibly denatured. This is consistent with the irreversible translocation of the dNSF-1 *comatose* protein from the cytosol into a Triton X-100–insoluble fraction in *comatose* flies at 37°C (Mohtashami et al., 2001). This supports the idea that new NSF biosynthesis is required for *comatose* flies to recover at 25°C, rather than indicating at which stage during membrane fusion NSF is involved (Morgan, 1996).

In total, our findings suggest that the ATPase-independent activity of NSF is an evolutionarily conserved aspect of NSF function and raise new considerations. Given the importance of SNARE disassembly, how do *comatose* flies survive at 25°C given that the *comatose* dNSF-1 mutant is highly compromised in its ATPase and SNARE-dissociating activity? This is curious since the dNSF-1 wt protein has no detectable ATPase activity in vitro (unpublished data) yet is competent to disassemble SNAREs. One possibility is that other proteins compensate the deficiency of dNSF-1 in *comatose* flies. One candidate is the second NSF gene of *D. melanogaster*, dNSF-2. Despite functional similarities of both dNSF isoforms, expression studies in flies suggested that both genes have a mutually exclusive temporal and spatial expression pattern (Golby et al., 2001). However, whether the expression pattern of dNSF-2 is altered in *comatose* flies is unknown. Lastly, given that dNSF-1 has two conserved activities it is clear that further analysis of NSF ATPase mutants within the context of the fly may help distinguish the relative contributions of NSF foldase and unfoldase activities in membrane fusion.

Materials and methods

Plasmids

A PCR-based, site-directed mutagenesis approach (Quik change site-directed mutagenesis Kit; Stratagene) was used to introduce a G274E mutation into the cDNA encoding dNSF-1 (from L. Pallanck, University of Washington, Seattle, WA) (Müller et al., 1999).

Pure proteins

NSF (*Drosophila* and CHO wt or G274E), α-SNAP (*Drosophila* and CHO), γ-SNAP, and GATE-16 were expressed and purified from *E. coli* as His-tagged proteins. His_6–GATE-16 was biotinylated with NHS-LC-biotin (Pierce Chemical Co.) to obtain an average of 1 biotin moiety per GATE-16 molecule as determined with the HABA (2-[4′-hydroxyazobenzene]-benzoic acid) reagent (Pierce Chemical Co.). GST–syntaxin-5 and His_6–GOS-28 were purified as in Shorter et al. (2002). p115 was purified from rat liver cytosol as in Nakamura et al. (1997).

Antibodies

The following mAbs were used against GOS-28, GS15, Bet1p (Transduction Labs), α-SNAP (ExAlpha), dsyntaxin-1A (S. Benzer, California Institute of Technology, Pasadena, CA), RGS-His (QIAGEN), and Myc tag (9E10). Rabbit polyclonal antibodies used were against syntaxin-5, NSF (A. Price, Yale Medical School), GOS-28 (T. Söllner, Memorial Sloan Kettering Cancer Center, New York, NY), Ykt6p (W. Hong, Institute of Molecular and Cell Biology, Singapore), and GATE-16 (Z. Elazar, Weizmann Institute of Science).

Negative staining

dNSF-1 proteins were viewed by EM as in Müller et al. (1999).

ATPase activity assay

NSF proteins (0.4 μM) were mixed with or without different combinations of GATE-16 (1.7 μM), α-SNAP (1.4 μM), GOS-28 (1.4 μM), and the ATPase activity measured as in Müller et al. (1999). In some reactions [γ-^{32}P]ATP was replaced with [γ-^{35}S]ATPγS.

Reassembly reactions

Reassembly reactions were performed as described in Müller et al. (1999). In some reactions, ATP (2 mM) was omitted or replaced with ATPγS (2 mM), AMP-PNP (2 mM), AMP-PCP (2 mM), or ADPβS (2 mM). In others the ATP regeneration system was replaced with an ATP depletion system comprising either hexokinase (5 U/ml; Sigma-Aldrich) and glucose (10 mM) or apyrase (5 U/ml; Sigma-Aldrich). In some reactions, RLGs and mitotic cytosol were pretreated with NEM (2.5 mM) for 15 min on ice, and the NEM was quenched with DTT (5 mM) for 15 min on ice before mixing for mitotic fragmentation. Fragmentation reactions were then supplemented with α-SNAP (0.7 μM) and either wt or G274E NSF (1.3 μM) and incubated at 25°C for 10 min. NSF/α-SNAP was then inactivated with NEM, and the reaction continued at 37°C for 20 min. Recovered MGFs were then either reassembled or solubilized for GOS-28 or GS15 immunoprecipitation (see

below). Reassembly reactions were supplemented with His_6–GATE-16 (0–3.1 μM). 3 μl anti–GATE-16 serum or 3 μl anti–GATE-16 serum that was treated with 3 μg GATE-16 for 15 min on ice was also added. In kinetic analyses, reactions were treated with NEM (2.5 mM for 5 min on ice and 5 mM DTT for 5 min on ice), anti–GATE-16 serum (3 μl), or anti–GOS-28 serum (3 μl) at the designated times (0, 5, 15, 23, 30, 38, 45, and 60 min) and incubated for a total time of 1 h. Other reactions were treated with buffer or terminated by fixation at these times. All reactions were terminated by fixation with 2% glutaraldehyde and processed for EM, and the extent of cisternal regrowth was determined (Nakamura et al., 1997).

SDS-resistant SNARE complexes

RLGs or MGFs (40 μg) were incubated in SDS-sample buffer (31.25 mM Tris HCl, pH 6.8, 1% SDS, 5% sucrose, 2.5% β-mercaptoethanol) at 37 or 100°C for 7 min and processed for immunoblot. Fly heads were homogenized in 20 mM Hepes-KOH pH 7.4, 0.2 M sucrose, 2.5 mM NEM, 0.5 mM PMSF, 2 mM benzamidine, 10 μg/ml leupeptin, and a postnuclear supernatant (PNS) was prepared by centrifugation at 1,000 *g* for 10 min. The PNS was centrifuged at 12,000 *g* for 10 min to isolate light membranes and mixed with 2.5 mM NEM for 20 min followed by 10 mM DTT for 20 min. This supernatant (40 μg) was incubated with 3 μg dNSF-1 (wt or mutant) and 3 μg dSNAP in 20 mM Hepes-KOH, pH 7.8, 100 mM KCl, 2 mM DTT, 2 mM EDTA, 0.5 mM PMSF, 2 mM benzamidine, 10 μg/ml leupeptin, 0.5 mM ATP, 8 mM $MgCl_2$, and ATP regeneration system for 40 min at 25°C. In some reactions, NSF was preinactivated with NEM. Samples were then mixed with SDS-sample buffer at 23 or 100°C for 10 min and processed for immunoblot.

Recombinant GATE-16–SNARE binding assays

His_6–GOS-28 (75 nM) was incubated for 1 h at 4°C with 0–3,750 nM His_6–GATE-16 and 75 nM GST–syntaxin-5 in binding buffer (20 mM Hepes-KOH, pH 7.3, 150 mM KCl, 2 mM $MgCl_2$, 30 mM imidazole, 5% glycerol, 0.5% Triton X-100, 0.1 mM DTT, 0.5 mg/ml STI). His_6–GOS-28 alone or His_6–GOS-28 plus 3,750 nM His_6–GATE-16 served as controls. GST–syntaxin-5 was retrieved by incubation with 5 μl glutathione-sepharose for 30 min at 4°C. Alternatively, His_6–GOS-28 was immunoprecipitated with 10 μl anti–GOS-28 beads for 30 min at 4°C. In which case, GST–syntaxin-5 alone or GST–syntaxin-5 plus 3,750 nM His_6–GATE-16 served as controls. Washed beads were eluted with SDS-PAGE sample buffer. Eluates were processed for immunoblot.

Immunoprecipitations

RLGs or MGFs were resuspended at 0.2 mg/ml in Triton X-100 buffer (20 mM Hepes-KOH, pH 7.4, 200 mM KCl, 1% (wt/vol) Triton X-100, 1 mM DTT, 1 mM pepstatin, 10 μg/ml leupeptin) and incubated for 30 min at 4°C with agitation. Extracts were clarified by centrifugation at 20,000 *g* for 30 min at 4°C.

Extracts (200 μl) were incubated for 1 h with anti–GOS-28 antibodies or anti-GS15 antibodies coupled to Affi-Gel 10 (BioRad Laboratories, Inc.). Washed beads were eluted with SDS-PAGE sample buffer and processed for immunoblot. In some experiments, the extract (200 μl) was supplemented with 1 mM ATP, 5 mM $MgCl_2$, and 1 μg His_6–GATE-16, and reactions were incubated with anti–GOS-28 beads for 16 h at 4°C to immunoisolate complexes containing GOS-28.

Other reactions (200 μl) were supplemented with 1 mM ATP, 5 mM $MgCl_2$, and 0.3 μM His_6–GATE-16 and incubated for 1 h at 4°C in the presence or absence of either α-SNAP (0.1 μM) alone, NSF (wt or G274E; 0.03 μM) alone, or α-SNAP and NSF (wt or G274E). Anti–GATE-16 antibodies covalently coupled to protein A–sepharose (Sagiv et al., 2000) were then added (20 μl) and incubated for 4 h at 4°C. Washed beads were eluted with SDS-PAGE sample buffer. Eluates were processed for immunoblot.

bio–GATE-16 pull down assays

MGF detergent extract (0.2 mg/ml) was supplemented with 1 mM adenine nucleotide (ATP, ATPγS, or ADPβS), 5 mM $MgCl_2$, and 0.3 μM bio–GATE-16 and incubated for 1 h at 4°C in the presence or absence of α-SNAP (0.1 μM) and NSF (wt or G274E; 0.03 μM). Monomeric avidin beads (10 μl; Pierce Chemical Co.) were added and incubated for 30 min at 4°C. Washed beads were eluted with 100 μl Triton X-100 buffer plus 2 mM biotin. Eluted proteins were precipitated with 12% (wt/vol) trichloroacetic acid. Precipitated proteins were fractionated by SDS-PAGE and processed for immunoblot.

Other binding reactions (200 μl) were performed using pure proteins in Triton X-100 buffer plus 5 mM $MgCl_2$ and 2 mM ATP. bio–GATE-16 (0.5 μM) was incubated with His_6–GOS-28 (0.5 μM) in the presence or absence of α-SNAP (0.1 μM) and NSF (wt or G274E; 0.03 μM) for 1 h on ice. In some reactions, the adenine nucleotide was omitted or replaced by 2 mM ATPγS, AMP-PNP, AMP-PCP, ADP, or ADPβS. In others, bio–GATE-16 was immobilized on monomeric avidin beads and incubated with His_6–GOS-28 (0.5 μM), α-SNAP (0.1 μM), and NSF (wt or G274E; 5 nM) in the presence of 2 mM ATP or 2 mM AMP-PCP for 2 min–2 h on ice. bio–GATE-16 was retrieved, and bound proteins were eluted and precipitated as above. Precipitated proteins were fractionated by SDS-PAGE and stained with Coomassie brilliant blue.

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