N-Ethylmaleimide-Sensitive Factor-dependent α-SNAP Release, an Early Event in the Docking/Fusion Process, Is Not Regulated by Rab GTPases*

(Received for publication, September 19, 1997, and in revised form, October 31, 1997)

Maria I. Colombo‡, S. Courtney Gelberman§, Sidney W. Whiteheart¶, and Philip D. Stahl‡

From the ‡Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110 and the §Department of Biochemistry, University of Kentucky College of Medicine, Chandler Medical Center, Lexington, Kentucky 40536

The N-ethylmaleimide-sensitive factor (NSF) is required for multiple intracellular vesicle transport events. In vitro biochemical studies have demonstrated that NSF, soluble NSF attachment proteins (SNAPs), and SNAP receptors form a 20 S particle. This complex is disassembled by the ATPase activity of NSF. We have studied particle disassembly in a membrane environment by examining the binding of recombinant SNAPs and NSF to endosomal membranes. We present evidence that α-SNAP is released from the membranes in a temperature- and time-dependent manner and that this release is mediated by the ATPase activity of NSF. Our results indicate that NSF mutants in the first ATP binding domain completely abrogate α-SNAP release, whereas no inhibitory effect is observed with a mutant in the second ATP binding domain. Interestingly, neither β-SNAP nor γ-SNAP are released by the ATPase activity of NSF, indicating that these proteins are retained on the membranes by interactions that differ from those that retain α-SNAP. Although the small Rab GTPases are known to play a role in SNARE complex assembly, our results indicate that these GTPases do not regulate the NSF-dependent release of α-SNAP.

Membrane association of NSF requires accessory proteins termed α-, β-, and γ-SNAPs (soluble NSF attachment proteins) (20, 21). α- and γ-SNAPs function synergistically and are widely distributed among tissues, whereas β-SNAP seems to be a brain specific isoform of α-SNAP. SNAPs bind to SNAREs (SNAP receptors), specific proteins present in the vesicle and in the target membrane. A set of SNAREs from bovine brain extracts was first identified by Rothman and colleagues (22). These receptors are the neuronal proteins synaptobrevin or VAMP (vesicle associated membrane protein), syntaxin and SNAP 25 (synaptosomal associated protein of 25 kDa) involved in synaptic transmission (for a review see Ref. 23). In vitro biochemical studies have demonstrated that NSF, SNAPs, and SNAREs form a 20 S complex (22, 24) that is proposed to function in vesicle targeting, docking, or fusion. To form a stable 20 S particle, a nonhydrolysable analog of ATP, e.g. ATPγS, is required. In the presence of ATP, hydrolysis of this nucleotide by the ATPase activity of NSF results in the dissociation of the 20 S complex into its component subunits (22). Although the significance of this disassembly remains controversial, an attractive possibility is that the energy of ATP hydrolysis drives a conformational change in one or more of the SNARE proteins. It is important to emphasize that even though NSF is widely recognized as an essential factor in multiple transport events this protein's exact function is still unknown.

SNARE complex disassembly has been studied mainly using recombinant purified components and detergent extracts (22, 25, 26). To gather further insight into the molecular mechanism of particle disassembly in a membrane environment, we studied the binding of recombinant SNAPs and NSF to endosomal membranes. We present evidence that α-SNAP is released from the membranes in a temperature- and time-dependent manner and that this release is mediated by the ATPase activity of NSF. Our results indicate that certain NSF mutants (in the first ATP binding domain) completely abrogate α-SNAP release, whereas no inhibitory effect is observed with another NSF mutant (in the second ATP binding domain). Interestingly, neither β-SNAP nor γ-SNAP were released by the ATPase activity of NSF, indicating that these proteins are retained on the membranes by interactions that are different from those that retain α-SNAP. Our results also indicate that NSF-dependent release of α-SNAP is not regulated by GTPases.

EXPERIMENTAL PROCEDURES

Cells and Materials—J774 E-clone (mannose receptor positive), a macrophage cell line, was grown to confluence in minimum essential medium containing Earle’s salts and supplemented with 10% fetal calf serum. Cytosol from J774 was the high speed supernatant of a cell homogenate obtained as described (27, 28) and stored at −80 °C. Cy-
tosol samples (200 μl) were gel filtered through 1-ml G-25 Sephadex spin columns just before use in the fusion assay. Protein concentration after filtration was 3–5 mg/ml. Recombinant NSF wild type and mutants were prepared and purified essentially as described (29, 30). Recombinant α-, β-, and γ-SNAPs were prepared and purified as described previously (31). The monoclonal antibody, CI 77.1 anti-α/βSNAP, was a generous gift from Dr. Reinhard Jahn (Yale University, New Haven, CT). All other chemicals were obtained from Sigma.

Preparation of Endosomal Membranes—3774 macrophages (1 × 10^6 cells) were washed sequentially with 150 mM NaCl, 5 mM EDTA, 10 mM phosphate buffer, pH 7.0, and with 250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes-KOH, pH 7.0 (homogenization buffer) and homogenized in the latter buffer (2 ml) using a cell homogenizer (28). Homogenates were centrifuged at 800 × g for 5 min to eliminate nuclei and intact cells. Postnuclear fractions were pelleted for 1 min at 37,000 × g in a Beckman L 100 centrifuge. The supernatants were centrifuged for an additional 5 min at 50,000 × g. The pellets of this second centrifugation were enriched with 5 min endosomes. Endosomal fractions were washed with homogenization buffer to remove cytosolic proteins, pelleted again and resuspended in the same buffer. The samples (200-μl aliquots) were frozen quickly in liquid nitrogen and stored at −80 °C.

NSF/SNAP Binding to Endosomes—Endosomal fractions (200-μl aliquot) were thawed quickly, diluted with homogenization buffer containing 1 M KCl and supplemented with trypsin inhibitor and p-amidophenylmethylsulfonyl fluoride hydrochloride as protease inhibitors. Samples were incubated for 10 min at 4 °C followed by a 5–10 min incubation period at 37 °C to remove endogenous NSF and SNAP. The stripped endosomal fractions were collected by centrifugation at 45,000 rpm for 15 min using a TL 100 rotor (Beckman). The membranes (15 μg/tube) were incubated in the same buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes-KOH, pH 7.0, 1 mM dithiothreitol, 1.5 mM MgCl₂, 50 mM KCl, 1 mM ATP, 5 mM creatine phosphate, 31 units/ml creatine phosphokinase) used in a standard endosome fusion assay (27, 28) containing a mixture of protease inhibitors. Binding assays were carried out in fusion buffer in a final volume of 30 μl supplemented with 100 nM recombinant NSF and/or SNAPs. In some assays, the ATP-regenerating system was replaced by an ATP-depleting system (5 mM ATP, 50 mM KCl, and supplemented with trypsin inhibitor and 1 M KCl, and supplemented with recombinant NSF and SNAPs were prepared and purified as described previously (31). The monoclonal antibody, CI 77.1 anti-α/βSNAP, was a generous gift from Dr. Reinhard Jahn (Yale University, New Haven, CT). All other chemicals were obtained from Sigma.

**RESULTS**

**NSF-dependent Release of α-SNAP Requires ATP Hydrolysis**—It has been shown previously that ATP hydrolysis by NSF disassembles the SNARE complex (22, 25). However, the formation and disruption of this complex has been studied mainly with recombinant purified components and detergent extracts (22, 25, 26, 44, 45). To gain further insight into the molecular mechanism involved in particle disassembly in a membrane environment, we studied the binding of recombinant SNAPs and NSF to endosomal membranes. For this purpose, an endosomal fraction was prepared as described under "Experimental Procedures." The membranes were incubated with 1 mM KCl in homogenization buffer to strip off the endogenous peripheral membrane-associated proteins. Membranes then were incubated in fusion buffer supplemented with recombinant NSF and/or α-SNAP for 5–10 min at 4 °C to allow for binding of the proteins. Subsequently, the samples were incubated for an additional 20 min at either 4 or 37 °C. The endosomal membranes were then sedimented by centrifugation, and bound proteins were detected by Western blotting. Because α-SNAP binds to plastic tubes (35), we precoated all of the tubes used in the binding experiments with 10% ovalbumin in phosphate-buffered saline. Very little binding of α-SNAP and NSF was detected when ovalbumin-coated tubes were incubated with these proteins in the absence of endosomal membranes (data not shown).

Endosomal membranes were incubated with 100 nm α-SNAP in the presence (Fig. 1A, lanes 2 and 4) or the absence (lanes 1 and 3) of recombinant NSF. Neither NSF nor α-SNAP were detected (data not shown) in membranes incubated in fusion buffer in the absence of added proteins, indicating that the endogenous proteins were removed by the high salt treatment. Fig. 1, panel A shows that when the membranes were incubated in the presence or absence of recombinant NSF at 4 °C, α-SNAP remained bound to the endosomal fraction (Fig. 1, lanes 1 and 2). In samples incubated for 20 min at 37 °C, after the initial binding at 4 °C, α-SNAP remained bound to the membranes only in the absence of added NSF (lane 3). In contrast, in the presence of NSF, almost no bound α-SNAP was detected (lane 4). A concomitant increase in α-SNAP was found in the supernatant (data not shown), indicating α-SNAP release from the membranes. These results suggest that NSF mediates the release of α-SNAP in a temperature-dependent fashion.

Interestingly, we observed that in samples incubated at 37 °C, most of α-SNAP was released from the membranes, whereas only a fraction of NSF was released. Quantification by densitometry indicates that while approximately 80% of
**NSF-dependent α-SNAP Release**

α-SNAP was released, only 45% of NSF was released under the same experimental conditions. Moreover, a substantial amount of NSF bound to the endosomal membrane fraction in the absence of added α-SNAP (data not shown). It is possible that this binding was due to aggregated inactive NSF. Using the *in vitro* endosome fusion assay, our results indicate that NSF bound to membranes in the absence of α-SNAP is active and capable of restoring endosome fusion activity in N-ethylmaleimide-treated endosomes (data not shown). To determine whether or not NSF was interacting with a protein factor, the endosomal fraction was either heat-inactivated or preincubated for 40–60 min at room temperature with protease K (0.7 mg/ml) in the presence of a small amount of Triton X-100 (0.002%). Both treatments result in almost complete inhibition of NSF binding in the absence of α-SNAP, indicating that NSF associates with a protein factor (data not shown).

To study the kinetics of NSF-mediated α-SNAP release, we analyzed the retention of proteins after incubating membranes at 37 °C for different periods of time. Fig. 1, panel B shows that after a 5 min incubation period at 37 °C, a significant amount of α-SNAP was released. After 20 min, the release was almost complete, while a substantial amount of NSF still remained associated with the membranes.

Next, we studied the nucleotide requirement for the process. As shown in Fig. 2, panel A, in the presence of NSF and an ATP-regenerating system, nearly complete release of α-SNAP was observed (lane 1). This release was abrogated by an ATP-depleting system (lane 2) or by ATPγS (lane 3). These results are consistent with earlier data using isolated purified proteins and suggest that in our system NSF drives the release of α-SNAP in a process that requires ATP hydrolysis. Since it has been shown that NSF has ATPase activity, it is likely that ATP hydrolysis by NSF catalyzes the release of α-SNAP from the membranes. NSF is a homotrimer of 76-kDa subunits that contains an amino-terminal domain and two homologous ATP-binding domains (32, 36). Mutants of NSF have been produced in which either the ATP binding or ATP hydrolysis of each nucleotide-binding region was altered (29, 37). In a recent report, we have shown that mutations in the first ATP binding domain of NSF block endosome fusion (14). To rule out the possibility that another ATPase present on the endosomal fraction is involved in α-SNAP release and to confirm the requirement of NSF's ATPase activity in the process, we tested the effect of NSF mutants in the binding assay. As shown in Fig. 2, panel B, D1EQ, an NSF construct with a point mutation in the first ATP binding domain lacking ATPase activity, and D1KA, a mutant that does not bind ATP, completely blocked α-SNAP release. In contrast, D2DQ, a construct with a point mutation in the second ATP binding domain of NSF, permitted α-SNAP release to an extent similar to that permitted by wild-type NSF (Fig. 3, lane 5), indicating that the second ATP-binding domain of NSF is not required for the NSF-mediated release of α-SNAP. As expected, the isolated amino-terminal domain of NSF (N fragment) and a truncated NSF molecule lacking the D1 domain (ND2) did not release α-SNAP (Fig. 3, lanes 6 and 8). These results indicate that the ATPase activity of the D1 domain of NSF is absolutely required for the NSF-dependent release of α-SNAP. Interestingly, although the ND1 truncated form contains the D1 domain, this mutant was unable to release α-SNAP (lane 7). The lack of activity observed with this truncated protein is not surprising as the ND1 mutant, although capable of dissociating an *in vitro* SNAP-SNARE complex, binds to this complex with a much lower affinity than wild-type NSF (30).

**NSF Regulates α-SNAP but not β- or γ-SNAP Release**

Three different forms of SNAPs, α-, β- and γ-SNAP (35, 36 and 39 KDa, respectively), have been purified from bovine brain (21). α- and γ-SNAP are widely distributed among tissues, whereas β-SNAP seems to be a brain specific isoform of α-SNAP (α- and β-SNAP are 80% identical to each other). Therefore, we were interested in determining whether β- and γ-SNAP were released from the membranes in a manner similar to that of α-SNAP. As shown in Fig. 4, panel A, neither β-SNAP nor γ-SNAP were released from the membranes by the ATPase activity of NSF, suggesting that they are retained by the membranes through interactions that are different from those that retain α-SNAP. Interestingly, when α-SNAP and γ-SNAP were added together, α-SNAP was released by wild-type NSF while γ-SNAP remained bound (Fig. 4, panel B). This result suggests that, in contrast to what is observed in the in
Fig. 4. NSF regulates α-SNAP but not β- or γ-SNAP release. Panel A, endosomal membranes were incubated in the presence of 1 mM ATP and an ATP-regenerating system. Recombinant α-, β-, or γ-SNAPs were added to the binding assay. Samples were preincubated with wild-type NSF (NSFwt), DIEQ, or D1KA (100 nM each) as indicated. Bound proteins were detected as described in Fig. 1. Data represent one of three independent experiments. Panel B, membranes were supplemented with wild-type NSF, α-, and γ-SNAP. Samples were incubated either at 4 or 37 °C. Bound proteins were detected as described in Fig. 1. Data represent one of three independent experiments.

vitro particle disassembly reaction (35), γ-SNAP is not released by the ATPase activity of NSF when monitored in a membrane environment.

NSF-dependent Release of α-SNAP Is Not Regulated by Rab GTPases—Genetic interactions and biochemical studies have demonstrated that Rab proteins, a class of small GTPases, play a role in the assembly of specific SNARE complexes (38, 39). We were interested in determining the role of GTPases in the NSF-mediated release of α-SNAP. For this purpose, salt-washed endosomal membranes were preincubated for 30 min at 25 °C in the absence (control) or presence of 100 μM GDP or GDPβS or GDPγS. Samples then were incubated for 20 min at either 4 or 37 °C in fusion buffer containing 100 nM α-SNAP with or without recombinant NSF. Fig. 5, panel A shows that neither GDPβS nor GDPγS blocked the NSF-mediated release of α-SNAP.

Next, we examined the effect of recombinant GDI (Rab GDP dissociation inhibitor), which is known to remove Rab proteins specifically from membranes (40, 41). The endosomal fraction was preincubated for 30 min at 25 °C in the absence (control) or presence of 1 mM GDP and 10 μM GDI. Samples were supplemented with 100 nM α-SNAP with or without recombinant NSF and subsequently were incubated for 20 min at either 4 or 37 °C. Fig. 5, panel B shows that preincubation of the membranes with GDI did not affect the NSF-driven release of α-SNAP, suggesting that Rab proteins do not regulate the disassembly process.

DISCUSSION

An important conceptual highlight regarding the molecular machinery involved in intracellular transport and neurotransmitter release came from the discovery of the SNAREs (for a review see Ref. 42), membrane receptors for SNAP (22). NSF binds through SNAPs to a complex of neuronal proteins comprised of synaptobrevin, syntaxin, and SNAP 25. Disassembly of this complex by NSF occurs in the presence of ATP and is thought to be critical for membrane fusion not only at the synaptic level but also in intracellular transport events (reviewed in Refs. 1, 3, 4, and 42).

In the present report, we present evidence that α-SNAP is released from the membranes in a temperature- and time-dependent manner. This release is absolutely dependent on the ATPase activity of NSF, as mutations in NSF that render the protein defective in either ATP binding or hydrolysis result in almost complete inhibition of α-SNAP release. Our data indicate that the release of α-SNAP takes place after a few minutes of incubation at 37 °C and is almost complete by 15–20 min. Our results agree with those of Wickner and collaborators (43) showing that Sec18p (yeast NSF homologue) drives the release of Sec17p (yeast α-SNAP homologue) after a short incubation at 37 °C. Interestingly, we have observed previously that endosome fusion becomes insensitive to NSF mutants after 15 min (14). However, at that point only 20% of the measurable fusion has occurred. Therefore, α-SNAP is released long before the fusion reaction is complete. Both results are consistent with the idea of an early action for NSF. This hypothesis receives independent support from studies in regulated exocytosis that show NSF acting at a prefusion ATP-dependent step but not at the Ca2+ triggered fusion step (16, 18). The recent finding that Sec18p/Sec17p are required in a priming step before the actual docking event (44) provides additional support for the idea that NSF is required for early steps.

NSF-SNAP-SNARE complex formation requires the presence of ATP, and disassembly is driven by conditions that favor ATP hydrolysis (22). It has been shown that in the presence of ATP NSF bound to syntaxin through α-SNAP catalyzes a conformational change in the syntaxin molecule that induces the release of both NSF and α-SNAP (26). However, most of these studies have been carried out with recombinant proteins and/or deter-
gent extracts (22, 25, 26, 33, 34). Our binding studies with membranes indicate that upon incubation with Mg²⁺-ATP most α-SNAP is released, whereas NSF is released only partially from the membranes. These data suggest that although part of NSF is bound to membranes in an α-SNAP-dependent manner there is a fraction of NSF that is retained by the membranes through an α-SNAP-independent mechanism. However, this NSF binding in the absence of SNAP was sensitive to proteinase K and heat treatment, indicating that NSF associates with a protein factor perhaps distinct from the classical NSF/SNAP receptors. Our results are consistent with the observations of Mayer et al. (43). They showed that in yeast, although the release of Sec17p is rapid and complete, little Sec18p is released from the membranes. This finding indicates that Sec18p must be bound to the membranes via a protein distinct from Sec17p. Moreover, in a recent publication, it has been shown that Sec18p (yeast NSF) binds to GST-Pep12p, a putative yeast SNARE, in a Sec17p (yeast SNAP) independent manner (45). Interestingly, Woodman and co-workers have shown that treatment of coated vesicles with Mg²⁺-ATP did not result in release of NSF (46). Therefore, it appears that NSF is retained on coated vesicles by interactions that are different from the classical NSF/SNAP-SNARE complexes. In addition, it has been shown previously that NSF associated with synaptic vesicles (47) and with growth cones (48) was not released upon incubation with Mg²⁺-ATP. Considered together, these results indicate that in the presence of Mg²⁺-ATP there is a pool of NSF that remains associated with membranes perhaps in an α-SNAP independent manner.

In contrast to α-SNAP, neither β-SNAP nor γ-SNAP were released from the membranes by the ATPase activity of NSF, suggesting that they are retained by interactions that are different from those that retain α-SNAP. Indeed, it has been shown that β-SNAP unlike α-SNAP binds synaptotagmin and recruits NSF (49). In agreement with our data, this complex is unaffected by Mg²⁺-ATP. Interestingly, when α- and γ-SNAP were added together, α-SNAP was released by NSF whereas γ-SNAP was not released. Although cross-linking experiments indicate that α- and γ-SNAP interact directly when bound to membranes (31), it is possible that this interaction is transient and occurs only under certain experimental conditions (e.g. in the absence of Mg²⁺-ATP). Moreover, it has been shown that γ-SNAP binds to a different membrane receptor protein or at a site that is distinct from the α/β-SNAP-binding site (34, 35). This differential interaction is consistent with our observation that the nearly complete release of α-SNAP from membranes is not accompanied by γ-SNAP release.

Among the proteins that have been implicated in the regulation of intracellular membrane transport are the members of the Rab family of monomeric GTPases (for review see Refs. 50–53). Although Rab proteins do not seem to be core components of the SNARE complex, genetic evidence supports a role for the Rab proteins in the assembly of this complex (38, 39). In addition, evidence for a functional link between Rab3 and the SNARE complex has been presented recently (54). Our data indicate that NSF-driven release of α-SNAP is not regulated by GTPases. Therefore, although complex assembly relies on Rab function, it is likely that the NSF-mediated disassembly does not. Our results are consistent with previous results showing that the release of sec17p in yeast was not influenced by GTP-γS or by antibodies against the Rab protein homologue Ypt7p (43).

We believe that binding studies using a membrane environment, such as those described in this report, should prove to be useful for further biochemical analyses and for the identification of other molecules that regulate the assembly and disassembly of the docking/fusion machinery.