A Possible Predocking Attachment Site for N-Ethylmaleimide-sensitive Fusion Protein

INSIGHTS FROM IN VITRO ENDOSONE FUSION*

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N-Ethylmaleimide-sensitive fusion protein (NSF) is an ubiquitous protein required for multiple vesicular transport events. We have investigated the role of the two nucleotide-binding regions of NSF in endosomal fusion by analyzing NSF mutants in a cell-free system. Our results indicate that mutations on the first ATP-binding domain, that render a protein defective in either ATP binding or ATP hydrolysis, results in almost complete inhibition of endosomal fusion. A mutation in the second ATP-binding site of NSF was only slightly inhibitory. The inhibitory effect was observed only when the mutant proteins were added at early times during the fusion reaction indicating that NSF may be required for an early step during the docking/fusion process. Binding studies using Western blotting reveal that the binding of NSF mutants to endosomal membranes is differentially affected by Ca\(^2^+\). Our results indicate that NSF, depending on its nucleotide state, may interact with membranes via an alternate mechanism. Our findings suggest the existence of a predocking binding site either independent of the docking complex or a site that leads to the formation of the SNAP-SNARE complex (e.g. 20 S particle).

The N-ethylmaleimide (NEM)\(^{-}\)-sensitive fusion protein NSF was originally identified as the factor required to restore transport activity of Golgi membranes treated with the alkylating agent NEM (Block et al., 1988). Studies using other cell-free systems revealed that NSF is required not only for intra-Golgi transport but also for other intracellular transport events (reviewed by Rothman (1994) and Whiteheart and Kubalek (1995)). For example it has been clearly shown that NSF is required for endosome fusion, ER to Golgi transport and transcytotic fusion (Diaz et al., 1989; Rodriguez et al., 1994; Beckers et al., 1989; Sztul et al., 1993) suggesting that NSF is a general component of the fusion machinery. NSF requires SNAPs (soluble NSF attachment proteins) for binding to membranes (Weidman et al., 1989). NSF-SNAP interacts with membrane-associated receptors known as SNAREs (Söllner et al., 1993). Support for SNARE involvement in vesicle docking/fusion is based on in vitro reconstitution studies and on genetic experiments (Rothman, 1994; Bennett, 1995; Rothman and Warren, 1994). The SNARE hypothesis postulates that each kind of transport vesicle is endowed with its own vesicle-SNARE (v-SNARE) which forms a specific complex with its cognate target-SNARE (t-SNARE) that is present only on the target membrane (Söllner et al., 1993; Rothman; Bennett, 1995). When membrane bound NSF-SNAP-SNARE complexes are solubilized with detergent, they sediment as a multisubunit particle at 20 S (Wilson et al., 1992; Söllner et al., 1993). Stable 20 S particles are formed in the presence of Mg-ATP\(^{\gamma}\)S (a non-hydrolyzable analogue of ATP). In the presence of Mg-ATP the 20 S particle rapidly disassembles. It is believed that the ATPase activity of NSF allows for the disassembly of the SNARE complex, an event that may lead to membrane fusion (reviewed by Whiteheart and Kubalek (1995)).

NSF is a homotrimer of 76-kDa subunits, each of which contain three distinct domains: an amino-terminal domain and two homologous ATP-binding domains (Wilson et al., 1989; Tagaya et al., 1993). Mutants of NSF have been produced in which either the ATP binding or ATP hydrolysis of each nucleotide-binding region was altered (Whiteheart et al., 1994; Szymida et al., 1994).

In the present study we have investigated the role of the two nucleotide-binding regions of NSF in endosome fusion by analyzing NSF mutants in an assay that reconstitutes fusion among endosomes (Diaz et al., 1988). Our results indicate that mutations in the first ATP-binding domain result in almost complete inhibition of endosome fusion, whereas a mutant in the second ATP-binding site was only slightly inhibitory. The inhibitory effect was only observed when the mutant protein was added at early times during the fusion reaction. Earlier work indicated that NSF is required for endosomal fusion (Diaz et al., 1989). Our present results demonstrate a critical role for ATP binding and hydrolysis by NSF in endosome fusion. Additionally, our data indicate that NSF is required for an early step during the docking/fusion reaction. We have also observed that binding of the NSF mutants to endosomal membranes is differentially affected by Ca\(^2^+\). Taken together, our results suggest that NSF may associate with endosomal membranes by interactions that are different from those involved in the formation of the 20 S particle.

**EXPERIMENTAL PROCEDURES**

Cells and Materials—J774 E-celone (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. HDP-1, a mouse IgG1 monoclonal antibody specific for dinitro-
and intact cells. The samples (200–300 μg) 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 (Colombo et al., 1992). Homogenates were centrifuged at 800 g for 5 min to eliminate nucleic and intact cells. The samples (200–μl aliquots) were quickly frozen in liquid nitrogen and stored at −80°C. In vitro Fusion Assay—Postnuclear fractions containing each probe were quickly thawed, diluted with homogenization buffer, and preincubated for 5 min at 37°C to release or inactivate some membrane-associated proteins. The samples were then pelleted for 1 min at 37,000 × g in a Beckman L100 centrifuge. The supernatants were centrifuged for an additional 5 min at 50,000 × g. The pellets of this second centrifugation were enriched with 5-mn endosomes. Endosomal fractions were mixed with fusion 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, 8 mM creatine phosphate, 31 units/ml creatine phosphokinase, and 0.25 mg/ml DNP-BSA as scavenger), supplemented with gel-filtered cytosol. The samples were incubated at 37°C for 60 min and the reaction was stopped by cooling on ice. To measure the immune complexes formed, the vesicles were solubilized by adding 50 μl of lysis buffer (1% Triton X-100, 0.2% β-mercaptoethanol, 1 mM EDTA, 0.1% BSA, 0.15 mM NaCl, 30 mM Tris-HCl, pH 7.4) containing 50 μg/ml DNP-BSA. For immunoprecipitation the samples were transferred to multwell plates coated with rabbit anti-mouse IgG. After 30–45 min of incubation at room temperature, the wells were washed three times with 300 μl of solubilization buffer, and β-glucuronidase activity was measured using 4-methylumbelliferyl β-D-glucuronide as substrate in a Microplate fluorometer 7600, Cambridge Technology, Inc. (Colombo et al., 1992). Fusion was expressed as a percentage of control fusion.

NSF Binding to Endosomes—Postnuclear fractions were quickly thawed, diluted with homogenization buffer containing 0.5 mM KCl, and incubated 15 min at 4°C followed by a 5-min incubation at 37°C to renature the probe in NSF. An enriched fraction of the second centrifugation was enriched by differential centrifugation as described above. The endosomes were incubated in fusion buffer containing 0.8–1 mg of cytosolic proteins supplemented with either wt NSF or mutant NSF (60 ng/assay). In some assays, the ATP-regenerating system present in the fusion buffer was replaced by an ATP-depleting system (5 mM mannose, 25 units/ml hexokinase). After 20 min of incubation at 37°C, the endosomal membranes were washed with homogenization buffer and sedimented by centrifugation. The samples were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes using standard techniques. NSF was detected by Western blot analysis using either a polyclonal antibody raised against its amino-terminal domain (Nagiec et al., 1995) or at a 1:500 dilution, or the mouse monoclonal antibody 6E6 (Tagaya et al., 1993). Visualization was performed with a horseradish peroxidase-coupled goat anti-rabbit antibody (1:5000) or horseradish peroxidase-rabbit anti-mouse (1:10,000) using the enhanced chemiluminescence reagents (Amersham Corp.) according to the manufacturer’s instructions.

In vitro Fusion Assay—NSF on Endosomes by Negative Staining—Endosomes were labeled by a 5-min internalization of 20-nm colloidal gold particles coated with mannosylated BSA as described previously (Colombo et al., 1992). After internalization, cells were homogenized and the postnuclear supernatants prepared as described above were quickly frozen in liquid nitrogen and stored at −80°C. Postnuclear fractions were quickly thawed, diluted with homogenization buffer containing 0.5 mM KCl, and incubated 10 min at 4°C, followed by a 5-min incubation at 37°C to remove endogenous NSF. An enriched endosomal fraction was prepared by differential centrifugation as described above. The endosomes were incubated in fusion buffer containing 1 mg/ml cytosolic proteins supplemented with wt NSF (600 ngassay). After 20 min of incubation at 37°C, the endosomal membranes were washed with homogenization buffer and sedimented by centrifugation. Samples were then processed for immunolabeling using the negative staining technique as follows: glow-discharged Formvar and carbon-coated nickel grids were placed on a drop of vesicle suspension for 2 min. Excess fluid was removed from the grid with filter paper. The specimen was quickly rinsed twice on homogenization buffer (HB) and incubated for 30 min on HB containing 23% goat serum and 0.1% gelatin (blocking buffer). The samples were then incubated for 2 h with the anti-NSF antibody 6E6 (Tagaya et al., 1993), diluted 1:50 in blocking buffer. After rinsing three times (5 min each) with blocking buffer, the specimens were incubated for 1 h with the secondary antibody (goat anti-mouse coupled to 12 nm gold) at 1:25 dilution. The samples were then rinsed twice with HB and fixed in 1% glutaraldehyde in HB for 10 min. The samples were rinsed sequentially with HB and distilled water, stained with 0.5% aqueous uranyl acetate for 1 min, blotted on filter paper, and air-dried.

RESULTS

NSF Mutants Inhibit Endosome Fusion—NSF is a member of a family of ATP-binding proteins delineated by one or more conserved regions corresponding to ATP-binding sites (Erdmann et al., 1991). NSF has three distinct domains: an amino-terminal domain and two homologous ATP-binding domains (D1 and D2). It has been shown that specific mutations in the first ATP-binding site (D1) lead to a completely inactive form of NSF. Two different mutants, K266A (D1KA) and E329Q (D1EQ), that affect binding and hydrolysis respectively, result in NSF mutants that fail to support intra-Golgi transport. Mutations in the second ATP-binding site (D2DQ) result in an attenuated form of NSF that only partially supports Golgi transport (Whiteheart et al., 1994; Sumida et al., 1994).

Given that NSF is an ubiquitous protein required for multiple vesicular transport events, we were interested in determining whether the same domains involved in intra-Golgi transport were also required for endosomal fusion. Therefore, we tested the effect of NSF mutants in an in vitro assay that reconstitutes fusion among endosomes. Fig. 1 shows that increasing concentrations of the D1EQ mutant (closed circles) inhibit endosome fusion (EC₅₀ of 50 ng/10 μl assay). It has been shown that the ATP hydrolysis defective mutant participates in the formation of the 20 S particle. However, particle disassembly is inhibited because of the inability of this mutant to hydrolyze ATP (Nagiec et al., 1995). Since particle disassembly seems to be a crucial step for vesicular transport, it is likely that fusion among endosomes is inhibited because the ATPase activity of the D1 domain is an absolute requirement for this event. Similar to the results obtained in the in vitro Golgi-transport assay, a mutant in the second ATP-binding region (D2DQ) was only slightly inhibitory when tested in the in vitro endosome fusion assay (open circles). Interestingly, Fig. 1 shows that D1KA, the ATP-binding deficient mutant (closed triangles) also inhibited fusion with a potency similar to the D1EQ mutant. This was a surprising finding since in the Golgi transport assay, D1KA showed only 10% of the inhibitory activity of the ATP hydrolysis mutant D1EQ (Whiteheart et al., 1994). Although the significance for this differential activity in both assays remains to be elucidated, our results suggest that the D1KA mutant is competing for NSF association with one or more of its interacting factors. The fact that the ATP-binding mutant D1KA does not assemble into the 20 S particle (Nagiec et al., 1995) suggests that this mutant competes for NSF binding to another site, perhaps an independent site or a site that leads to the formation of the SNAP-SNARE complex.

Our data indicate that both ATP binding and hydrolysis by the first ATP binding domain of NSF are absolutely required for its function in endosomal fusion, whereas the ATPase ac-
The inhibitory effect of the second ATP-binding domain does not appear to be essential.

NSF has three distinct domains: an amino-terminal domain and two ATP-binding domains (D1 and D2). To study the role of the different NSF domains in endosome fusion, we examined a series of truncated forms of NSF (Nagiec et al., 1995). NSF binding to the SNAP-SNARE complex seems to be mediated by the N domain. A truncated mutant D1D2, that lacks the N domain, is unable to assemble into the SNAP-SNARE complex and does not have activity in the Golgi transport assay (Nagiec et al., 1995). Consistent with these findings, D1D2 did not display inhibitory activity in the endosomal fusion assay (Fig. 2). It has been shown that the D2 domain is important for trimerization of NSF (Whiteheart et al., 1994). The ND1 mutant lacking the D2 domain, although monomeric, assembles into the 20 S particle but with a much lower affinity than wt NSF. Since ND1 has some ATPase activity, it is released from the 20 S particle upon addition of Mg-ATP (Nagiec et al., 1995). As shown for the Golgi transport assay, this mutant did not inhibit endosome fusion (Fig. 2), probably because it can be released from the 20 S particle. Interestingly, Fig. 2 shows that the ND2 truncation mutant and the N fragment partly inhibit endosome fusion. The low level of inhibitory activity observed with these truncated proteins is not surprising since the isolated N fragment is monomeric and likely a poor competitor of the trimeric NSF. The ND2 mutant, although capable of forming a trimer when salt conditions are at least 300 mM, binds to the SNAP-SNARE complex with a much lower affinity than wt NSF (Nagiec et al., 1995). The weak inhibitory effect of this mutant suggests that it is a poor competitor of native NSF.

Taken together, our results indicate that the amino domain of NSF is important for endosome fusion. However, the partial inhibitory effect of the truncated mutants indicates that other domains beside the amino-terminal region may be important for association of NSF with its interacting proteins.

The Inhibitory Effect of the NSF Mutants Is Prevented by a Short Incubation at 37 °C—In order to determine whether the NSF mutants inhibit an early or late step in the fusion reaction, we tested the NSF mutants using the following protocol. Vesicles were incubated in a standard fusion assay at 37 °C for various periods of time. The samples were either stopped at 4 °C or supplemented with the mutants D1EQ or D1KA and further incubated at 37 °C for the remainder of the 60-min time period. The samples were then cooled on ice and vesicle fusion was determined. As shown in Fig. 3, both NSF mutants inhibited endosome fusion only when added at early times during the assay. When the reaction was allowed to proceed for a few minutes before adding the mutants, virtually no inhibitory effect was observed.

The results suggest that NSF is incorporated early in the docking/fusion machinery because the inhibitory effect of the NSF mutants was rapidly lost with preincubation. Therefore, NSF might be required for an early stage in the docking/fusion mechanism, although it is likely that its function is completed in a later step (e.g. 20 S particle disassembly).

In order to show that the inhibitory effect observed with the NSF mutants was specific for NSF activity, we studied the reversibility of the inhibition by adding recombinant wild type NSF. As shown in Fig. 4 (panels A and B), the inhibitory effect of both NSF mutants D1KA and D1EQ was reversed by wt NSF.

Interestingly, wt NSF reversed the inhibitory effect of the mutants only when added at the beginning of the incubation. When NSF was added after a short preincubation at 37 °C (5–10 min) no protective effect was observed (Fig. 5). The results indicate that once the mutants have been incorporated in the docking/fusion machinery, the multisubunit protein complex becomes resistant to exogenously added NSF. These data are consistent with the results described above, i.e. that NSF mutants only inhibit when added at early times during the fusion reaction.

Differential Interaction of NSF with Membranes Depends...
upon the Nucleotide State of NSF—Our biochemical data indicate that both the ATP-binding and ATP hydrolysis defective NSF mutants inhibit endosome fusion. The ATP hydrolysis defective mutant (D1EQ) binds to the 20 S SNAP-SNARE complex particle but the latter can not be disrupted in the presence of the mutant and vesicle fusion is inhibited. D1KA, a mutant that fails to bind ATP, can not assemble into the 20 S particle. However, this mutant also inhibits endosome fusion in a reversible fashion, suggesting that the D1KA mutant competes for binding of NSF in an ATP-independent fashion.

In order to address the possibility that NSF is capable of binding to membranes in the absence of ATP, we examined the binding of cytosolic NSF to an endosome enriched fraction under different nucleotide conditions. Endosomes were washed with 0.5 M KCl to remove endogenous NSF attached to the membranes. The endosomal fraction was then incubated with cytosol for 20 min at 37 °C. After washing and pelleting the membranes, bound NSF was detected by Western blot analysis. Fig. 6 (panel A) shows that in the presence of ATP and an ATP-regenerating system, NSF was readily detected on endosomal membranes. Previous work has shown that stable NSF-SNAP-SNARE complexes (e.g. 20 S particle) are formed in the presence of Mg and ATPγS, a non-hydrolyzable analog of ATP (Wilson et al., 1992; Söllner et al., 1993). As expected, incubation in the presence of ATPγS resulted in a marked increase in the amount of NSF bound to the endosomal membranes. This is consistent with the previous observations that ATP hydrolysis is required for SNAP complex disassembly (Söllner et al., 1993). However, when an ATP-depleting system (e.g. mannose-hexokinase) was included in the incubation, the amount of membrane-bound NSF was also greatly increased. Similar results were obtained in the presence of ADP (data not shown). These results suggest that NSF may interact with the membranes in its ADP-bound form. Interestingly, addition of Ca2+ in the presence of the ATP-depleting system, markedly reduced the amount of NSF bound to the membranes (Fig. 6, panel A). A moderate effect was also observed in the presence of a regenerating system. Strikingly, Ca2+ did not affect NSF binding when ATPγS was present. The fact that Ca2+ regulates the association of NSF in its ADP-bound form rules out a possible nonspecific association of this protein with the membranes. Moreover, the differential effects of Ca2+ suggest that NSF, depending on its nucleotide state, may interact with the membrane by an alternate mechanism.

Since both ATPγS and ATP depletion may affect other molecules in addition to NSF, we next studied the binding of wt NSF and the NSF mutants D1EQ and D1KA to endosomal membranes in the presence of ATP. Fig. 6 (panel B) shows that the D1EQ mutant associated with the membranes to a greater extent than wt NSF. The D1KA mutant also bound to the membranes, indicating that ATP binding to D1 is not an absolute requirement for binding. In agreement with the above results, Ca2+ decreased the association of both wt NSF and D1KA, but did not affect D1EQ binding.

Our results suggest that NSF, besides its ATP-dependent interaction with the 20 S particle may interact with other molecules, in an ATP-independent fashion. The fact that Ca2+ differentially modulated NSF association with endosomes depending on the nucleotide state of NSF further supports the idea that NSF binds to endosomal membranes by interactions in addition to those required for the formation of the 20 S particle.

In order to confirm the interaction of NSF with endosomal membranes, we studied the membrane association of NSF by immunogold electron microscopy. For this purpose endosomes were labeled by a 5-min internalization of 20 nm colloidal gold particles coated with mannansylated BSA (Diaz et al., 1989). An enriched endosomal fraction was salt-washed and incubated with cytosol supplemented with wt NSF. Control endosomes were incubated with buffer alone. After the incubation, the endosomal membranes were washed with buffer and sedimented by centrifugation. Samples were then processed for immunolabeling using an anti-NSF antibody. As shown in Fig.
7, when endosomes were incubated with cytosol supplemented with NSF, labeling was observed not only on docked endocytic vesicles (panels B and C) but also on undocked endosomes (panel A). No significant labeling was observed when the endosomes were incubated with buffer alone (data not shown). The fact that NSF was localized on the endosomal surface and not only at sites where vesicle docking was observed further suggests that NSF may interact with other membrane sites (e.g. receptors) in addition to docking complexes.

**DISCUSSION**

NSF and SNAPs are essential components of the intracellular fusion machinery. The SNARE hypothesis originated with the observation that membrane proteins on transport vesicles (known as v-SNAREs) and membrane proteins on target membrane (known as t-SNAREs) form stable complexes that serve as binding sites for the general membrane trafficking factors α-SNAP and NSF (reviewed by Bennett (1995) and Rothman and Warren (1994)). The ATPase activity of NSF putatively allows for the disassembly of the SNARE complex, an event that may lead to membrane fusion. This model predicts the transient binding of NSF from the cytosol to the SNARE complex. However, it is unclear whether NSF exists on the surface of the vesicles or is recruited from a cytosolic pool followed by transient binding to the docking site.

In the present study, using NSF mutants, we present evidence that both ATP binding and ATP hydrolysis by the first ATP binding domain of NSF are critical for endosome fusion. We also found that NSF mutants inhibit fusion only when added at early times during the fusion assay. Likewise, the inhibitory effect of NSF mutants was fully reversed by wt NSF only when NSF was added at the beginning of the fusion assay. Our kinetic studies suggest that NSF is incorporated into vesicles, probably as a predocking intermediate before the assembly of the 20 S particle containing SNAREs. This predocking complex seems to be assembled at an early stage and once formed, the NSF binding site becomes inaccessible to exogenously added NSF. Our observation of an early requirement for NSF is consistent with previous observations with in vitro Golgi transport (Wattenberg et al., 1992; Sumida et al., 1994). Indeed, it has previously been suggested that NSF is incorporated early into forming vesicles and cannot be exchanged by exogenously added NSF (Wattenberg et al., 1992). Since our assay most likely measures fusion of preformed vesicles, our results suggest that NSF is required for an earlier function in the docking/fusion process. A prefusion role for NSF has previously been postulated by Morgan and Burgoyne (1995). Data have been presented indicating that α-SNAP, perhaps in association with NSF, acts in an earlier priming stage during exo-
cytosis and may have no effect on the later Ca\(^{2+}\)-dependent triggering of secretion (Chamberlain et al., 1995).

It is possible that the predocking intermediate may accommodate SNARES, but perhaps in an inactive conformation or associated with other proteins that render the fusion apparatus inactive. A conformational change or the release of some of the components that form this complex would allow the actual docking/fusion event to occur. The existence of a predocking intermediate is also consistent with the findings that NSF is associated with predockend synaptic vesicles (Hong et al., 1994) and isolated clathrin-coated vesicles (Steel et al., 1996) implying that NSF may interact with either v- or t-SNAREs alone. Alternatively, both proteins may be present in the same vesicle. Indeed, the t-SNAREs, syntaxin I and SNAP 25, have been localized to synaptic vesicles (Walch-Solimena et al., 1995). Moreover, it was recently demonstrated that NSF and \(\beta\)-SNAP can form a complex with the individual t-SNARE syntaxin (Hanson et al., 1995). Alternatively, NSF may bind to membranes via a different protein-protein interaction. In a recent paper it has been shown that NSF binds to synaptotagmin with high affinity and that this interaction is mediated by \(\beta\)-SNAP (Schiavo et al., 1995). Synaptotagmin is a Ca\(^{2+}\)-binding protein, which may function as a calcium sensor in exocytosis (Südhof, 1995; Geppert et al., 1994). It has been shown that the in vitro association among NSF, \(\beta\)-SNAP, and synaptotagmin is not affected by Ca\(^{2+}\) (Schiavo et al., 1995). However, it is possible that in the in vitro system other membrane components involved in the Ca\(^{2+}\)-mediated regulation of synaptotagmin-NSF association, may be absent. Interestingly, our results indicate that Ca\(^{2+}\) differentially modulates the association of D1KA and D1EQ mutants with membranes. Whether this observation is related to NSF-synaptotagmin association remains to be determined.

Our results indicate that the D1KA mutant inhibits endosome fusion, an effect that is reversed by wt NSF. We postulate that D1KA competes for the binding of wt NSF to the predocking intermediate. The existence of a predocking intermediate would explain why the D1KA mutant, which is unable to bind ATP and does not assemble in the 20 S particle (Nagiec et al., 1995), inhibits fusion. Indeed, our data indicate that ATP is not absolutely required for NSF binding to the membranes. It may also not be required for the formation of the predocking intermediate. However, fusion is inhibited because ATP binding and hydrolysis by NSF is likely required for the next step, the assembly and subsequent disassembly of the 20 S particle, which, in turn, would allow fusion to occur. Our observation

**Fig. 7. Immunolocalization of NSF on endosomes.** Endosomes were labeled by a 5-min internalization of 20 nm colloidal gold particles coated with mannosylated BSA. An endosomal enriched fraction was incubated in fusion buffer containing cytosol (1 mg/ml) and supplemented with recombinant NSF (600 ng/assay). After 20 min of incubation at 37°C the endosomal membranes were washed with homogenization buffer and sedimented by centrifugation. Samples were then processed for immunolabeling using the negative staining technique described under "Experimental Procedures." NSF was detected with the monoclonal antibody 6E6 (Tagaya et al., 1993) and a goat anti-mouse coupled to 12 nm gold, as a secondary antibody. Panel A shows that NSF (arrows) is present on the surface of an undocked endosome labeled with 20 nm colloidal gold particles (arrowheads). Panels B and C show clusters of endosomes. NSF is present on the surface of endosomes and at the sites were vesicles dock (arrows). Bar, 80 nm.
that NSF binds to membranes in its ADP-form is consistent with the findings of Morgan and Burgoyne (1995) that ATP depletion prevented the leakage of both NSF and α-SNAP from permeabilized chromaffin cells, implying that in the absence of ATP, the two proteins remain associated with membranes. Moreover, in a recent publication it has been shown that treatment of clathrin-coated vesicles with Mg-ATP, a conditions that favor ATP hydrolysis, did not result in release of NSF from the membranes (Steel et al., 1996).

Another set of proteins that are involved in multiple membrane trafficking pathways are the small GTPase Rabs (Zerial and Stenmark, 1993). One mechanism by which Rab may function is through the regulation of SNARE complex formation. Indeed, it has been shown that active Rab(Ypt1p) protein is required for the formation of the SNARE complex (Lim et al., 1994; Sogaard et al., 1994). However, neither Rab3A nor Ypt1p are found in the 20 S SNARE-containing complexes (Söllner et al., 1993; Sogaard et al., 1994). We have previously shown that, similar to the NSF mutants, a Rab5 dominant negative mutant also inhibits at an early time point in the in vitro endosome fusion reaction (Barbieri et al., 1994). Given that both Rab5 and NSF are required at an early stage in the docking/fusion process, we speculate that these proteins are involved in the formation of the pre-docking intermediate. Rab5, after performing its function, probably dissociates from the complex or more likely, associates with other proteins allowing the actual docking/fusion event to occur (e.g. the assembly of the 20 S particle).

In this report we describe two findings that may have important connotations for understanding how the ubiquitous protein NSF acts during endosome fusion in particular, and in vesicular transport in general. First, that NSF seems to be required for an early step in the docking/fusion process. Second, that NSF interacts with the membranes in its ADP-form suggesting the existence of a pre-docking binding site either independent of or a precursor to the 20 S particle. Furthermore, the fact that Ca$^{2+}$ decreased the association of both wt NSF and D1KA, but did not affect D1EQ binding suggests that NSF, depending on its nucleotide state may interact with membranes via an alternate mechanism. Our immunolabeling studies indicate that NSF binds to endosomes diffusely and not only at sites were vesicle dock. These results further support the idea that NSF may interact with other membrane receptors that are not part of the docking complex (e.g. 20 S particle).

Acknowledgments—We are grateful to Marilyn Levy for excellent technical assistance with the immunolocalization experiments. We thank Drs. Luis Mayorga, John Heuser, and Guangpu Li for critical reading of this manuscript and for helpful discussions and insights.

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