Multiple N-ethylmaleimide-Sensitive Components Are Required for Endosomal Vesicle Fusion

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This report examines the inhibition of endosomal vesicle fusion by the alkylating agent N-ethylmaleimide (NEM). The concentration of NEM required to inhibit vesicle fusion depended upon whether membrane and cytosolic fractions were treated separately or together, enabling the resolution of at least two components to the inhibition. The first component is inactivated at low levels of NEM when cytosolic and membrane fractions are treated together. On the contrary, inhibition of the second component required higher levels of NEM but was achieved by treating cytosol and membranes separately. Reconstitution studies indicated that both components were cytosolic and that neither corresponded to the ubiquitous NEM-sensitive fusion protein (NSF). The role of NSF in this fusion reaction was further examined using salt-washed membranes depleted of NSF protein. Under these conditions the fusion reaction was fully dependent upon added NSF whose activity, in this context, was sensitive to NEM treatment. From these data we conclude that NSF activity during endosomal vesicle fusion can be dissected into several steps, only a subset of which (perhaps attachment of NSF to the membrane) are sensitive to NEM. Fusion between salt-washed endosomal membranes was also dependent on soluble NSF attachment proteins.

INTRODUCTION

Fusion of endocytic vesicles with their target membrane is a central event in the process of receptor-mediated endocytosis. To ensure that receptor internalization and recycling occurs efficiently, vesicle fusion must be tightly controlled so that it occurs only with specific compartments. Such biochemical specificity also allows endosomal vesicle fusion to be reconstituted in cell-free systems (Davey *et al.*, 1985) as has been demonstrated for a variety of intracellular transport events, most notably intra-Golgi protein transport (Balch *et al.*, 1984; Rothman and Orci, 1992). This type of analysis, where the components of the reaction are open to biochemical manipulation, has enabled the mechanism of endosomal vesicle attachment and fusion to be investigated in some detail (Warren *et al.*, 1988; Gruenberg and Howell, 1989).

Research from several laboratories has established that fusion between endocytic compartments shares broad features with other intracellular transport events. Chiefly, fusion requires Mg-ATP and proteins from both cytosol and membrane fractions (Gruenberg and Howell, 1986; Braell, 1987; Woodman and Warren, 1988; Diaz *et al.*, 1989). Hydrolysis of GTP is also required for a number of reactions that either regulate or mediate endosomal vesicle fusion (Mayorga *et al.*, 1989), and three classes of GTP-binding proteins have been implicated in the reaction. Heterotrimeric G proteins play an as yet undefined role in regulation of fusion, judging by the effects of various G protein effectors and inhibitors (Colombo *et al.*, 1992), whereas other studies have shown that an ADP ribosylation factor is also required (Lenhard *et al.*, 1992). More specifically, the small GTP-binding protein rab5, which is localized to the peripheral endosomal system *in situ* (Chavrier *et al.*, 1990), is an essential component of the fusion apparatus (Gorvel *et al.*, 1991).

Endosomal vesicle fusion is also controlled by protein phosphorylation. Intracellular transport is inhibited as

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mammalian cells enter mitosis (Fawcett, 1965), and this effect can be reproduced *in vitro* by the inclusion in the fusion reaction of the mitosis-specific kinase, cdc2 (Tuomikoski *et al.*, 1989). Both cyclin A- and cyclin B-cdc2 kinase can inhibit the reaction (Thomas *et al.*, 1992; Woodman *et al.*, 1993), although it is not yet clear whether the two kinases act at the same site. Protein phosphorylation events distinct from those involving cdc2 kinase also appear to regulate fusion, because the phosphatase inhibitor okadaic acid is a potent inhibitor of the reaction (Woodman *et al.*, 1992).

A particularly useful reagent for manipulation of intracellular transport *in vitro* has been *N*-ethylmaleimide (NEM).¹ Inactivation of intra-Golgi transport by NEM (Glick and Rothman, 1987) led to the identification of the first "fusion" protein (NEM-sensitive fusion protein [NSF]) based on its ability to restore transport activity to NEM-treated incubations (Block *et al.*, 1988). NEM sensitivity is a feature of virtually all reconstituted membrane fusion reactions, and for some of these additional evidence has shown that NSF is an essential component (Beckers *et al.*, 1989; Sztul *et al.*, 1993). NSF interacts with Golgi membranes via soluble NSF attachment proteins (α -, β -, and γ -SNAPs) (Clary and Rothman, 1990; Clary *et al.*, 1990) that bind to integral membrane receptors within the Golgi complex (Whiteheart *et al.*, 1992). The assembly of the NSF/SNAP/receptor complex is tightly regulated by ATP turnover (Wilson *et al.*, 1992), and this property has been exploited to identify putative NSF/SNAP receptors from brain membranes. Isolation thereby of three previously identified components of the synaptic vesicle/presynaptic membrane targeting machinery (synaptobrevin, SNAP-25, and syntaxin) (Söllner *et al.*, 1993) implies that the NSF/SNAP complex forms a key component in all membrane fusion events in the cell (Warren, 1993).

Although there is substantial evidence for NSF acting at multiple stages of the secretory pathway both in mammalian cells (Davidson and Balch, 1993) and in yeast (Graham and Emr, 1991), where the *SEC 18* gene product has been identified as the functional homologue of NSF (Wilson *et al.*, 1989), evidence for NSF involvement on the endocytic pathway is equivocal. It has been reported that the activity that restores fusion capacity to NEM-treated endosomal vesicles is also NSF (Diaz *et al.*, 1989), judged by its reactivity toward a monoclonal antibody specific for NSF. However, this conclusion has been disputed in a more recent study (Wessling-Resnick and Braell, 1990) using vesicles containing fluid phase endocytic markers.

Given that transport between an endosomal compartment and the trans-Golgi network requires an NEM-sensitive protein distinct from NSF (Goda and Pfeffer,

1991) and that an NSF-like activity is not implicated in the recycling of transferrin receptor to the cell surface (Podbilewicz and Mellman, 1990), we decided to reexamine the inactivation of endosomal vesicle fusion by NEM. Our findings indicate that the NEM sensitivity of endosomal vesicle fusion is conferred by at least two components that are distinct from NSF. However, dependency of vesicle fusion on NSF activity, with evidence of a role for α - or γ -SNAP, is revealed by use of salt-washed membranes that are depleted of peripheral components.

MATERIALS AND METHODS

Materials

Recombinant NSF (His₆ NSF; 1.3 mg/ml in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]/KOH pH 7.0, 200 mM KCl, 2 mM 2-mercaptoethanol, 0.5 mM ATP, 10% glycerol, 250 mM imidazole), recombinant α -SNAP (His₆ α -SNAP; 0.5 mg/ml in 25 mM tris(hydroxymethyl) aminomethane [Tris]-HCl pH 7.8, 75 mM KCl, 2 mM 2-mercaptoethanol, 10% glycerol, 250 mM imidazole), and recombinant γ -SNAP (His₆ γ -SNAP; 0.78 mg/ml in 25 mM Tris-HCl pH 7.8, 1 mM dithiothreitol [DTT], 10% glycerol, 280 mM KCl) were kindly provided by Professor James Rothman (Sloan-Kettering Institute for Cancer Research, New York). For some studies recombinant NSF bearing a carboxy-terminal tag of 10 amino acids from the myc protein (NSF-myc) was purified from cultures of *Escherichia coli* (strain provided by J. Rothman) according to the procedure of Wilson and Rothman (1992).

Cells

A431 cells were maintained in Dulbecco's minimal essential medium containing 10% (vol/vol) fetal calf serum (FCS), 100 U/ml of both penicillin and streptomycin. Suspension HeLa (sHeLa) cells were grown in Eagle's minimum essential medium (MEM) modified for suspension cultures (GIBCO, Paisley, Scotland) containing 5% (vol/vol) FCS, 100 U/ml of both penicillin and streptomycin and nonessential amino acids.

Membrane Preparations

Donor preparations were made by internalizing radio-iodinated transferrin into sHeLa cells. Human transferrin was radiolabelled with 125-iodine (Amersham International, Amersham, U.K.) to a specific activity of $\sim 10^7$ cpm/ μ g exactly as described (Woodman and Warren, 1991), except for the experiments described in Figure 10 where a specific activity of 4×10^7 cpm/ μ g was used. Cells (a 1-l culture of $\sim 10^6$ cells per ml) were washed three times in phosphate-buffered saline and resuspended in 5 ml of binding medium (MEM containing 20 mM HEPES pH 7.4 and 2 mg/ml bovine serum albumin). The cells were warmed to 37°C, and [¹²⁵I]transferrin was added to 10 μ g/ml. After 5 min, the suspension was diluted to 50 ml with ice-cold binding medium, and the cells were washed three times in STM buffer (250 mM sucrose, 10 mM Tris-HCl pH 7.4, 1 mM MgAcetate). The cell pellet was resuspended in 5 ml of STM supplemented with protease inhibitors (1 μ g/ml chymostatin, 1 μ g/ml pepstatin A, 2 μ g/ml E64, 1 μ g/ml antipain, 40 μ g/ml phenylmethylsulfonyl fluoride). Cells were homogenized, and a membrane fraction prepared on 20/40% (wt/vol) sucrose gradients in 10 mM Tris-HCl pH 7.4 as described previously (Woodman and Warren, 1989). Acceptor preparations were made from A431 cells containing internalized anti-transferrin antibodies. Full details for the internalization of antibodies provided elsewhere (Woodman and Warren, 1991). Cells were homogenized and membranes prepared as for donors.

¹Abbreviations used: NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein.

Salt-washed membranes were prepared by a modification of Clary and Rothman (1992). Postnuclear supernatants were diluted with an equal volume of 2 M KCl and incubated with DNase I (10 μ g/ml) for 10 min at 37°C. After passing the suspension through a fine-gauge needle, membranes were isolated on sucrose cushions as described above. Fresh membranes were combined with equal volumes of water and 3M KCl (to give 1 M KCl final), incubated for 10 min at 37°C, then reisolated as before.

Carbonate-washed membranes were prepared from pig brain membranes, isolated in the buffers described above. One volume of membranes was incubated with 4 vol of 125 mM sodium carbonate for 30 min on ice, then reisolated on 20–40% sucrose gradients in 10 mM Tris-HCl pH 7.4.

Cytosol

Cytosol was prepared from sHeLa cells. Usually, 1 l of cells ($\sim 10^6$ cells/ml) were harvested by centrifugation, washed three times in KEHM buffer (50 mM KCl, 10 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid [EGTA], 50 mM HEPES pH 7.2, 2 mM $MgCl_2$), and resuspended to 5 ml in KEHM-containing protease inhibitors. Cells were homogenized as for membrane preparations, and the extracts were centrifuged at $400\,000 \times g$ for 30 min in a TL100 centrifuge. Cytosol was frozen and stored in liquid nitrogen. Cytosols were not usually desalted. To prepare cytosol depleted of NSF activity, cytosol was desalted into KEHM buffer using Biogel P6 columns (Bio-Rad Laboratories, Cambridge, MA) then incubated for 30 min at 37°C to inactivate NSF (Block *et al.*, 1988).

To deplete cytosol of SNAP activity, desalted cytosol (10 mg in 1 ml) was incubated for 30 min at 37°C to destroy NSF then mixed with 20 mg carbonate-treated membranes that had been washed twice in KEHM buffer. After 10 min on ice, the sample was centrifuged at $400\,000 \times g$ for 10 min in a TL100 centrifuge. The membrane pellet was washed with a further 0.5 ml KEHM buffer, and after centrifugation the supernatants were combined. These were incubated with a second batch (20 mg) of carbonate-treated membranes, and the final supernatant was stored in liquid nitrogen.

Fusion Assays

Vesicle fusion assays were carried out essentially as described elsewhere (Woodman and Warren, 1989). Incubations included cytosol (generally 200 μ g), membranes (5 μ l donor and 7 μ l acceptor), unlabeled transferrin (10 μ g), and an ATP-regenerating cocktail (5 mM creatine phosphate, 1 mM Mg-ATP, 8 IU/ml creatine phosphokinase). Incubations were for 2 h at 37°C, and reactions were stopped by addition of ice-cold immunoprecipitation buffer (1 ml) containing *Staphylococcus aureus* cells (5 μ l of a 10% suspension). Unless indicated, fusion experiments contained a control sample lacking cytosol, that provided a background for the immunoprecipitation. This value, always <10% of the maximum signal, was subtracted from all readings, unless shown.

NEM Treatment

All experiments were performed using the same stocks of NEM and DTT. Both reagents were dissolved to 100 mM in water and stored at -20°C . Samples were thawed immediately before use. Membranes and cytosol were incubated with the indicated final concentration of NEM for 30 min at 4°C. Unreacted NEM was quenched with an equal concentration of DTT and left for ≥ 10 min at 4°C before other components of the incubation were mixed.

Western Blotting

For detection of NSF protein, samples containing membrane or cytosol fractions were run on 10% sodium dodecyl sulfate gels and transferred to nitrocellulose using a Hoefer semidry transfer apparatus. Membranes were probed with a monoclonal antibody to NSF (2E5) (Tagaya *et al.*, 1993) diluted 1:2000, followed by a horse radish peroxidase-

conjugated rabbit anti-mouse antibody (DAKO, 1:2000). Peroxidase activity was detected using enhanced chemiluminescence (ECL) reagent (Amersham International).

Protein Determination

Protein concentrations were determined by the method of Bradford (1976).

RESULTS

NEM-Inactivation of Endosomal Vesicle Fusion Has Distinct Components

Fusion between endosomal vesicles was measured by an indirect method, described in detail elsewhere (Woodman and Warren, 1989). Briefly, endosomal vesicles containing radioiodinated transferrin were incubated with vesicles containing an anti-transferrin antibody. Fusion occurred under appropriate conditions, leading to exposure of labelled antigen to the antibody, and the resulting immunocomplex was precipitated on *Staphylococcus aureus* cells. Fusion proceeded efficiently when an ATP-regenerating cocktail and a cytosol fraction were supplied.

The central theme of this study was to establish whether a component inactivated by NEM treatment of fractions was identical to, or distinct from, the vesicle fusion protein NSF (Block *et al.*, 1988). Initially, we were struck by our inability to inhibit vesicle fusion reproducibly when membrane and cytosol fractions were preincubated separately with a low concentration (1 mM) of NEM, sufficient to destroy NSF activity (Glick and Rothman, 1987). We therefore examined in more detail the effect of NEM at different concentrations (Figure 1). When vesicles and cy-

Figure 1. NEM inactivation of fusion has distinct components. Membrane fractions (combined) and cytosol fractions were treated together or separately with 1 mM or 3 mM NEM. After quenching with equimolar DTT, incubation mixes were completed and incubated for 2 h at 37°C. A control sample was not treated with NEM. Fusion efficiency is expressed as [^{125}I]transferrin (cpm) immunoprecipitated in a cytosol-dependent fashion. Results are from a representative experiment.

tosol were incubated separately with 1 mM NEM, then combined after quenching with equimolar DTT, only slight inactivation of the subsequent fusion reaction was found. In contrast, the same concentration of NEM caused inactivation to a much greater extent when incubated with combined membrane and cytosol fractions. At higher concentrations of NEM (3 mM), inactivation was complete whether membranes and cytosol were exposed to NEM together or separately. At each concentration, prior mixing of NEM with equimolar DTT blocked its inhibitory effect.

NEM action often inactivates cytosolic components of intracellular transport steps, but in some instances membrane proteins are also affected (see for example, Goda and Pfeffer, 1991). Some components, such as NSF, are localized predominantly to membranes (Glick and Rothman, 1987) but can also be isolated in soluble form. We examined, therefore, whether inhibition of endosomal vesicle fusion by different concentrations of NEM could be reversed by addition of untreated cytosolic or membrane fractions. All components sensitive to NEM over the range tested were almost exclusively cytosolic, because complete recovery of fusion activity followed addition of untreated cytosol (Figure 2A) but not untreated membranes. The distribution of restor-

ative activity was quite different from that of NSF protein, which was found mainly in the membrane fractions (Figure 2B). Restorative activity of cytosol toward both concentrations of NEM resided predominantly in macromolecules, as all restorative functions were substantially reduced by heat treatment of cytosols.

NSF Is Not Sufficient to Restore Activity to NEM-treated Incubations

The results described above suggested that the NEM-restorative components were distinct from NSF. We therefore investigated whether NSF alone could restore activity to NEM-treated incubations. As demonstrated in Figure 3, recombinant NSF that is fully functional in intra-Golgi transport assays (Wilson and Rothman, 1992) had limited ability to restore activity to incubations exposed to 1 mM NEM. Even 500 ng NSF, 50 times that sufficient to saturate intra-Golgi transport assays, restored only ~15% activity. In control incubations fusion activity was almost fully restored by a cytosol fraction.

NSF possessed slight restorative activity toward complete incubations treated with 1 mM NEM. To investigate this further we examined the ability of saturating amounts of NSF to restore activity to incubations treated with different concentrations of NEM. NSF enhanced the activity of control incubations to a small extent, and

Figure 3. NSF does not restore activity to NEM-treated reactions. Membranes and cytosol were treated together with 1 mM NEM then quenched with DTT. Incubations were supplemented with the indicated amounts of cytosol or recombinant His₆ NSF. Reactions were continued for 2 h, and fusion efficiency was determined. A control sample, not treated with NEM, is shown (■).

this enhancement was maintained, or slightly increased, toward incubations treated with 0.75–1 mM NEM (Figure 4A). In contrast, incubations treated with 3 mM NEM were devoid of activity, even when supplemented with NSF. It was possible that low or high molecular weight components in cytosol were required to associate with NSF for it to restore fusion activity in full. However, inclusion of heat-inactivated cytosol did not enhance the ability of NSF to restore activity, and the ability of untreated cytosol to restore activity was not increased by inclusion of NSF.

A recent report showed that some activities of α -SNAP are particularly sensitive to NEM (Wattenberg *et al.*, 1992). Moreover, other studies have shown that intra-Golgi transport has a strong dependency for palmitoyl coenzyme A under conditions where NSF is limiting (Glick and Rothman, 1987; Pfanner *et al.*, 1990). It was possible that inclusion of either, or both, of these activities could enhance the restorative activity of NSF. However, over a range of NEM treatments, α -SNAP added at a concentration sufficient to saturate intra-Golgi transport (Clary *et al.*, 1990) had only a marginal effect in addition to NSF (Figure 4B). Palmitoyl coenzyme A, when included at a concentration optimal for intra-Golgi transport (Glick and Rothman, 1987), appeared to remove any restorative activity of NSF (Figure 4B). Additionally, inclusion of γ -SNAP, which also associates with NSF during intra-Golgi transport (Clary *et al.*, 1990), did not increase the restorative ability of NSF and α -SNAP (Figure 4C).

Neither NEM-Restorative Component Is Dependent upon NSF Activity

Because NSF, alone or in combination with associated factors, could not restore activity to NEM-treated incubations, we tested whether that component that restored activity to incubations treated with 1 mM NEM (sufficient to inactivate NSF) required functional NSF for its activity. A singular feature of NSF is its dependence on ATP for its stability. Desalting of cytosol to remove ATP leads to gradual destruction of NSF activity, and warming to 37°C accelerates this process (Block *et al.*, 1988). In contrast, the restorative activity of cytosol toward incubations treated with 1 mM NEM was affected only slightly by depletion of NSF activity (Figure 5). The small proportion of the restorative activity af-

Figure 4. Neither NSF nor associated factors can restore activity to NEM-treated reactions. Membranes and cytosol were inactivated with the indicated concentrations of NEM. After quenching, reactions were supplemented with the following: (A) buffer, His₆ NSF (50 ng), His₆ NSF + cytosol (200 μ g), cytosol, or His₆ NSF + heat-treated cytosol (10 min at 95°C). (B) Buffer, His₆ NSF (50 ng), His₆ NSF + His₆ α -SNAP (50 ng), or His₆ NSF + His₆ α -SNAP + palmitoyl coenzyme A (10 μ M). (C) Buffer, His₆ NSF (50 ng) + His₆ α -SNAP (50 ng), or His₆ NSF + His₆ α -SNAP + His₆ γ -SNAP.

ected by NSF depletion was recovered upon addition of recombinant NSF. Similar experiments demonstrated that restoration of activity to samples treated with 3 mM NEM was similarly insensitive to NSF depletion.

NSF and SNAP Activities Are Required for Fusion Between Salt-washed Endosomal Vesicles

The results described above show that only a fraction of the NEM-sensitive activity required for endosomal fusion could be attributed to NSF. It was possible that two fusion activities were being measured in parallel, and only the minor activity required NSF. Alternatively, fusion may have been totally dependent upon NSF but was not revealed by NEM treatment. To distinguish between these possibilities, we treated fractions in an alternative manner to remove NSF activity. Membranes washed with 1M salt at 37°C were depleted of NSF, as shown by Western blot analysis (Figure 6A). Residual NSF should be inactive after this treatment (Clary and Rothman, 1992), and salt-washed membranes had poor fusion activity when combined with NSF-depleted cytosol (Figure 6B). Substantial activity could be restored by addition of recombinant NSF but not by α - or γ -SNAP. The restorative activity of NSF was specific, because it was saturable (Figure 7A) and sensitive to NEM treatment (Figure 7B).

The different abilities of the two assay systems (NEM-inactivation or salt-washing of membranes) to reveal NSF function during endosomal vesicle fusion is most likely a consequence of the distribution of NSF activity between membrane and cytosolic forms, and this is suggested by the distribution of NSF protein. To confirm that NSF activity is distributed in this way, we examined what contribution the cytosolic or membrane-bound pools of NSF made to fusion activity when the other pool was removed. When membrane-bound NSF was removed by salt-washing, control cytosol, though more effective than NSF-depleted cytosol, could support only a fraction (40%) of the fusion activity found when NSF was provided in excess (Figure 8A). This contrasted with the marginal effect of addition of NSF to complete incubations (see Figure 4, A and B) and showed that the cytosolic pool of NSF had limited ability to promote fusion. By comparison, efficient fusion (>65% of the total [125 I]transferrin available for immunoprecipitation) occurred whether NSF was present or removed from cytosol by ATP-depletion (Figure 8B). Hence, the membrane-bound pool of NSF is sufficient to drive fusion to completion.

Salt-washed membranes should be depleted of SNAPs, in addition to NSF (Clary *et al.*, 1990). They should therefore be ideal reagents to test the dependency of endosomal vesicle fusion on SNAPs, which are essential for transport through the Golgi complex (Clary *et al.*, 1990). Firstly, although cytosol depleted of NSF should retain SNAP activity, we examined

Figure 5. NEM-restorative activity does not require NSF activity. Membranes and cytosol were treated together with 1 mM NEM then supplemented with the following: buffer, cytosol (200 μ g), cytosol (200 μ g) desalted into KEHM including 1 mM ATP and incubated for 30 min at 37°C [DS + ATP], or cytosol (200 μ g) desalted into KEHM lacking ATP and incubated for 30 min at 37°C [DS-ATP]. Parallel samples contained His₆ NSF (100 ng) or buffer.

whether SNAP availability might limit fusion efficiency. Titration of cytosol showed that, though NSF could support cytosol-dependent fusion, little activity was seen below a threshold of 20 μ g cytosol (Figure 9). This threshold was reduced significantly by inclusion of recombinant α - and γ -SNAP, though fusion remained dependent upon other cytosolic components.

To provide further evidence of SNAP dependency, we took advantage of the findings of Söllner *et al.* (1993) that brain membranes are rich in receptors for SNAPs. NSF-depleted cytosol was incubated with carbonate-washed brain membranes. This treated cytosol revealed, first, a much greater SNAP-dependency than control cytosol (Figure 10A). Fusion activity was increased threefold by addition of SNAPs to carbonate membrane-treated cytosol, compared to a 1.1-fold increase in the case of control cytosol. Second, fusion efficiency was reduced substantially even when SNAPs were included, indicating the depletion of other fusion factors. Either α - or γ -SNAP could restore fusion activity to SNAP-depleted incubations (Figure 10B). Some additional activity was observed when both SNAPs were added, though not sufficient to confirm a synergistic action between the two proteins as observed in another context (Whiteheart *et al.*, 1993).

It was possible that removal of peripheral proteins from endosomal membranes by salt-washing allowed nonspecific fusion, promoted by NSF and SNAPs. This appeared unlikely, because fusion of salt-washed membranes required NEM-sensitive component(s) distinct from NSF and α -SNAP (Figure 11), and thus involved protein(s) distinct from those identified as being involved in intra-Golgi transport.

DISCUSSION

In this report we have examined in detail the sensitivity of endocytic vesicle fusion to the alkylating agent NEM.

Figure 6. Fusion of salt-washed membranes requires NSF. (A) Abundance of NSF in control membranes (lane 1) and salt-washed membranes (lane 2) was determined by Western blotting using a monoclonal antibody to NSF and detected by ECL. (B) Salt-washed donor and acceptor membranes were combined with cytosol (200 μ g) treated to inactivate NSF and the indicated additional components. (NSF, 100 ng NSF-myc; α -SNAP, 100 ng His₆ α -SNAP; γ -SNAP, 100 ng His₆ γ -SNAP). Fusion reactions were continued for 2 h, and fusion activity was measured.

In common with previous studies (Braell, 1987; Woodman and Warren, 1988), we have found that fusion activity is abolished by treatment with low concentrations of NEM, and that activity is restored by supplementing the incubation with untreated cytosol. A previous report has found that the restorative activity of cytosol can be attributed to the general fusion protein, NSF (Diaz *et al.*, 1989). Our findings, however, contrast with this interpretation in a number of respects.

First, the pattern of inhibition we describe is not consistent with inactivation of NSF alone. Inactivation of NSF required for intra-Golgi transport has been characterized in some detail, and separate incubation of membrane and cytosolic pools of the protein with a low concentration (1 mM) of NEM for a short time (15 min on ice) was sufficient to destroy all NSF activity (Block *et al.*, 1988). We have found that 1 mM NEM will abolish endocytic vesicle fusion only when membrane and cytosolic fractions are treated together. Higher concentrations (3 mM) of NEM are required for full inactivation when membrane and cytosolic fractions are treated separately.

These results can most easily be explained in two ways. First, fusion activity might require the activity of two NEM-sensitive cytosolic components. One component, sensitive to 1 mM NEM, is exposed only when it interacts with membranes. The second component (contained on either the same or a different polypeptide) is exposed to the alkylating agent whether or not membranes are present and is sensitive to 3 mM NEM. Alternatively, fusion might require only one NEM-sensitive component (in addition to NSF); the affinity of NEM for this component would be low but would increase substantially when it interacted with membranes.

Figure 7. Action of NSF is specific. (A) Salt-washed donor and acceptor membranes were combined with cytosol (200 μ g) treated to inactivate NSF and the indicated amount of NSF-myc. Fusion activity was measured after 2 h incubation. (B) Salt-washed membranes and NSF-inactivated cytosol were combined and assayed for fusion activity in the presence of the following components: none, 100 ng NSF-myc (NSF), 100 ng NSF-myc preincubated with 3 mM NEM then quenched with 3 mM DTT (NSF-NEM), and 100 ng NSF-myc preincubated with 3 mM NEM and 3 mM DTT together (NSF-NEM/DTT).

Figure 8. NSF activity fractionates to membranes. (A) Fusion between salt-washed membranes was monitored using different concentrations of control cytosol or NSF-inactivated cytosol[DS(-ATP)], with or without 100 ng NSF-myc. (B) Fusion activity was measured without cytosol or with 200 μ g cytosol that had been desalted and incubated for 30 min in the presence or absence of 1 mM ATP. For estimation of fusion efficiency, the result of an incubation lacking unlabeled transferrin but containing 1% Triton X-100 is shown.

At present, we cannot distinguish between these possibilities, but either characteristic would distinguish these component(s) from NSF.

Second, we have found that purified NSF is not sufficient by itself to restore activity to NEM-treated incubations. The extent of restoration upon addition of NSF depended on the conditions of the NEM treatment. When incubations were treated with high (3 mM) concentrations of NEM, recombinant NSF had virtually no restorative activity. At lower concentrations of NEM (0.75–1 mM), a significant degree of restoration was observed (~15–20% on average) when NSF sufficient to saturate intra-Golgi transport assays (Block *et al.*,

1988) was added. Indeed, it is not clear to what extent this effect can be attributed to a true restorative function, because NSF also stimulates control incubations, and its effect was seen only under conditions when inhibition of fusion was not complete. We note that Stahl and colleagues could restore ~20% of fusion activity to incubations treated with 1 mM NEM using an excess of conventionally purified NSF (Diaz *et al.*, 1989).

Third, the distribution of the NEM-sensitive factor that we describe differs from that of NSF. NSF is found mainly in membrane fractions (Glick and Rothman, 1987), although some activity is found within the cytosol (Block *et al.*, 1988). We have confirmed the distribution of NSF protein and have shown that NSF activity is similarly distributed. Indeed, the cytosolic pool of NSF appears insufficient to complement inactivation of fusion caused by removal of NSF activity. Despite this, NEM-treated endocytic vesicle incubations can be restored to full activity upon addition of cytosol, whereas addition of membranes does little to restore activity.

Fourth, the restorative activity of mammalian cytosol is largely insensitive to manipulations that destroy soluble NSF activity. NSF is an ATP-binding protein that requires ATP for its stability. When cytosol is desalted to remove ATP, then incubated for a short period at 37°C, NSF activity is destroyed (Block *et al.*, 1988). However, we have found that treatment of cytosol in this way results in the loss of only 10–20% of its restorative activity. Additionally, we have found that incubations treated with 0.75–1 mM NEM can be restored using yeast cytosol. Preliminary experiments have shown that the restorative activity is unchanged

Figure 9. Fusion efficiency of salt-washed membranes is limited by SNAP concentration. Salt-washed membranes were assayed for fusion activity in the presence of the indicated amounts of NSF-inactivated cytosol, supplemented with the following: buffer (control), 100 ng His₆ NSF (+NSF), 100 ng His₆ NSF, 100 ng His₆ α -SNAP, and 100 ng His₆ γ -SNAP (+NSF, +SNAPs).

Figure 10. Vesicle fusion is SNAP-dependent. (A) Salt-washed membranes were assayed for fusion activity in parallel incubations containing NSF-myc (100 ng) or NSF-myc (100 ng) plus 100 ng each of His₆ α-SNAP and His₆ γ-SNAP. Incubations contained either no cytosol (none), 100 μg of NSF-depleted cytosol preincubated with carbonate-washed membranes (carbonate), or NSF-depleted cytosol (control). (B) Fusion incubations contained salt-washed membranes, cytosol preincubated with carbonate-washed membranes (60 μg), NSF-myc (100 ng), and different combinations of α-SNAP or γ-SNAP (100 ng of each).

whether yeast cytosols are prepared from wild-type or *sec18-1* strains, which carry a temperature-sensitive mutation in the *SEC18* locus and are therefore deficient in NSF activity (Novick *et al.*, 1981; Wilson *et al.*, 1989). These data provide corroborative evidence that the NEM-restorative activity is not dependent on NSF.

At present, it is not clear why our findings differ from those of Diaz *et al.* (1989), who demonstrated that NSF was the NEM-sensitive component by using a monoclonal antibody against NSF to inactivate the restorative function of cytosol. One possibility, however, is that the NEM-sensitive component(s) associates with NSF as part of a multisubunit fusion complex. Under these

circumstances, binding of an anti-NSF antibody might lead to the inactivation of other components of the complex, thereby removing the NEM-restorative activity indirectly.

As well as NSF, other candidates for the NEM-sensitive activity have been tested. Addition of α-SNAP, γ-SNAP, and palmitoyl coenzyme A did little to enhance the ability of NSF to restore activity to NEM-treated incubations. Therefore, none of these components alone or together are sufficient to restore activity, though we do not rule out the possibility that they are necessary, in combination with other unidentified components. Another NEM-sensitive factor, required for recycling of the mannose-6-phosphate receptor to the trans-Golgi network, has been partially characterized (Goda and Pfeffer, 1991). However, this factor is sensitive to much lower concentrations of NEM (0.25 mM; which does not inhibit our assay) for it to be the same as the activity that we describe.

Although our findings show that NSF is neither necessary nor sufficient to restore the bulk of activity to NEM-inactivated endosomal vesicle fusion incubations, they confirm a role for the protein in the fusion event. Fusion activity blocked by low concentrations of NEM is partly restored by NSF, and even basal activity is enhanced slightly by addition of the protein. More significantly, fusion between salt-washed endosomal membranes using cytosol specifically depleted of NSF activity was largely dependent on exogenous NSF. Furthermore, use of salt-washed membranes revealed that

Figure 11. Fusion of salt-washed membranes is NEM-sensitive. Salt-washed membranes were combined with NSF-inactivated cytosol and incubated with the indicated concentrations of NEM. After quenching with DTT, incubations were supplemented with NSF-myc (100 ng) and His₆ α-SNAP (100 ng) and continued as normal.

SNAP proteins contribute to fusion between endocytic membranes, thus it is likely that the same basic mechanism allows fusion between endocytic membranes as between secretory compartments.

How can we reconcile these observations? The most likely explanation is that intra-Golgi transport and endocytic vesicle fusion measure different types of event. This being so, the differential effects of NEM on each pathway could be exploited to dissect the mechanism by which NSF participates in vesicular transport. Reconstitution of intra-Golgi transport requires the formation of nascent vesicles from the donor cisterna, followed by the fusion of these vesicles with the acceptor cisterna. Therefore, the fusion complex (containing NSF) must be attached to each vesicle *de novo* during the incubation. Inactivation of any portion of NSF activity would completely abolish transport activity. On the other hand, the event that we monitor is a simple fusion event. Although fusion is strictly cytosol-dependent, it is likely that the endocytic membranes that participate in this reaction already contain many of the components of the fusion complex, including NSF. We have confirmed that the major contribution toward NSF activity is derived from the membrane-bound pool. Therefore, only a small portion of the total fusion signal would depend on the recruitment of new complexes to the membranes. If the NEM-sensitive portion of NSF activity were recruitment of NSF to the membrane (indeed, NEM-treated NSF does not bind to membranes [Wilson *et al.*, 1992]) or another activity of NSF already fulfilled upon membrane attachment, then the majority of NSF-dependent vesicle fusion would be insensitive to NEM treatment. Such an argument is consistent in the main with the reasoning of Wattenberg *et al.* (1992) who suggest that NSF activity required for intra-Golgi transport is needed for vesicle formation and that, once formed, functional vesicles containing NSF are resistant to NEM-inactivation.

We also show for the first time a significant contribution toward endosomal vesicle fusion by the SNAP proteins, which form a complex with NSF required for intra-Golgi transport (Clary *et al.*, 1990). SNAP dependency of fusion between salt-washed membranes was usually seen only at low cytosol concentrations suggesting that, unlike NSF, SNAP activity in cytosol is relatively high. Further incubation of cytosol with carbonate-extracted membranes revealed significant SNAP dependency, though this was not absolute. It is possible that SNAPs are required simply to stabilize NSF or to potentiate its activity in some other way, but the extent of SNAP dependency would suggest it plays a direct role in the fusion reaction. To our surprise, we found that the contribution of γ -SNAP was at least as great as that of α -SNAP, though it has much lower activity than α -SNAP during intra-Golgi transport (Clary *et al.*, 1990; Whiteheart *et al.*, 1993). This may have biological significance, but we feel it is more likely a consequence

of our inability to completely remove endogenous SNAP activity.

In summary, we have shown that NEM-sensitive component(s) in addition to NSF and SNAPs play a role in endosomal vesicle fusion. Identification of these factor(s) and investigation into how they interact with the NSF/SNAP machinery that is also required for vesicle fusion should provide further insight into the mechanism of membrane attachment and fusion.

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