

# Binding of an *N*-Ethylmaleimide-sensitive Fusion Protein to Golgi Membranes Requires Both a Soluble Protein(s) and an Integral Membrane Receptor

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**Abstract.** An *N*-ethylmaleimide (NEM)-sensitive fusion protein (NSF) has recently been purified on the basis of its ability to restore transport to NEM-inactivated Golgi membranes in a cell-free transport system. NSF is a peripheral membrane protein required for the fusion of transport vesicles. We now report the existence of two novel components that together bind NSF to Golgi membranes in a saturable manner. These components were detected by examining the requirements for reassociation of purified NSF with Golgi membranes *in vitro*. One component is an integral membrane receptor that is heat sensitive, but

resistant to Na<sub>2</sub>CO<sub>3</sub> extraction and to all proteases tested. The second component is a cytosolic factor that is sensitive to both proteases and heat. This soluble NSF attachment protein (SNAP) is largely resistant to NEM and is further distinguished from NSF by chromatography. SNAP appears to act stoichiometrically in promoting a high-affinity interaction between NSF and the membrane receptor. Because NSF promotes vesicle fusion, it seems likely that these two new factors that allow NSF to bind to the membrane are also part of the fusion machinery.

**T**HE movement of proteins from the ER to the cell surface via the Golgi stack involves multiple cycles of vesicle budding and fusion. A cell-free system that reconstitutes transport within the Golgi stack (1, 2, 8, 15) has been used to purify a protein needed for fusion of transport vesicles termed *N*-ethylmaleimide (NEM)<sup>1</sup>-sensitive fusion protein (NSF). Here, we report that NSF binds to Golgi membranes in a specific and saturable manner. Binding requires an integral membrane receptor and a distinct cytoplasmic protein as cofactor. This soluble NSF attachment protein (SNAP) and the receptor may function with NSF in the fusion process as a part of the multisubunit complex we have postulated (13).

The cell-free transport system measures the vesicular movement of vesicular stomatitis virus (VSV)-encoded glycoprotein (VSV-G) from the *cis* compartment of a donor Golgi population to the *medial* compartment of an acceptor Golgi population. The donor Golgi are prepared from a VSV-infected, mutant Chinese hamster ovary (CHO) cell (clone 15B) that lacks the enzyme *N*-acetylglucosamine (GlcNAc) transferase I in its *medial* Golgi cisternae. The acceptor

Golgi complexes are prepared from uninfected, wild-type CHO cells (housing an active GlcNAc transferase I). Transport from donor to acceptor Golgi stacks is measured by the transport-coupled incorporation of [<sup>3</sup>H]GlcNAc (from UDP-[<sup>3</sup>H]GlcNAc) into VSV-G protein (1), requires ATP and cytosol (the high speed supernatant from cell homogenates), and can be completely inhibited by pretreating donor and acceptor membranes with the alkylating agent NEM (3, 11).

The NEM-sensitive factor, NSF, was first described as a membrane-associated factor that could restore transport activity to NEM-treated Golgi membranes (11). This factor could be released from membranes by incubation with ATP and Mg<sup>2+</sup> (11) and it was subsequently found that the soluble form of NSF required adenine nucleotides for maintenance of its activity (4). NSF was purified from cytosol that was prepared from homogenates treated with ATP and Mg<sup>2+</sup> such that the NSF activity was largely recovered in a stable, soluble form (4). The restorative, NEM-sensitive activity of this extract was found to reside in a single tetrameric protein of identical 76-kD subunits.

Two intermediates in the pathway of processing transport vesicles for fusion have been previously described (2, 20). Biochemical and morphological evidence suggests that NSF is required for a step occurring before the formation of these intermediates but after transport vesicles have attached to the Golgi cisternae and have lost their cytoplasmic coats (13, 16). Because of its critical role in the fusion process we classify NSF as a fusion protein, although this classification does not

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1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; GlcNAc, *N*-acetylglucosamine; NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide sensitive fusion protein; SNAP, soluble NSF attachment protein; STI, soybean trypsin inhibitor; VSV, vesicular stomatitis virus; VSV-G, VSV-encoded glycoprotein.

necessarily imply that NSF is in and of itself a lipid bilayer fusogen (in the sense of many viral spike proteins). By investigating the reassociation of purified NSF with Golgi membranes *in vitro*, we present evidence for at least two additional fusion components.

## Materials and Methods

### Materials

NEM, BSA, soybean trypsin inhibitor (STI), polyethyleneglycol 4000, creatine phosphokinase, and PMSF were purchased from Sigma Chemical Co. (St. Louis, MO). DTT, Tris, ATP, creatine phosphate, UTP, sucrose, and proteinase K were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). IgG, Hepes, and pronase were purchased from Calbiochem-Behring Corp. (La Jolla, CA). Trypsin was from Worthington Biochemical Corp. (Freehold, NJ). All other reagents and chemicals were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ).

### Purification of NSF

NSF was purified from CHO cell homogenates as described by Block et al. (4). This procedure yielded a preparation of NSF that was >95% homogeneous as judged by Coomassie blue-stained SDS-polyacrylamide gels. The purified enzyme was stored at a concentration of ~0.15 mg/ml in 20 mM Hepes, pH 7.0, 100 mM KCl, 0.5 mM ATP, 2 mM MgCl<sub>2</sub>, 2 mM DTT, 25% glycerol at -80°C. Protein concentration was determined by the method of Bradford (5) using IgG as standard. The specific activity of purified NSF ranged from 500 to 1,800 U per μg.

### Preparation of Golgi-enriched Membranes

Golgi membranes were prepared from cell homogenates by sucrose density gradient flotation as described by Balch et al. (1). For the NSF-dependent transport assays, donor and acceptor membranes were mixed and treated with NEM as described by Block et al. (4).

Membranes used in the binding assay were prepared from wild-type CHO cell homogenates and treated with NEM (1 mM for 15 min at 0°C). Residual NEM was quenched by adding DTT to 2 mM. The membranes were then washed and concentrated by diluting fivefold into 10 mM Tris, pH 7.4 (0.2 M sucrose final), and sedimenting through 5 ml of 0.5 M sucrose (in 10 mM Tris, pH 7.4) onto a 1-ml cushion of 1 M sucrose (in 10 mM Tris, pH 7.4; 45,000 rpm for 30 min in a SW 50.1 rotor, Beckman Instruments, Inc., Fullerton, CA). The membranes were recovered from the top of the 1 M sucrose cushion in one quarter of the original volume (1-2 mg protein/ml) and then aliquoted, frozen, and stored in liquid nitrogen. Protein concentration was determined using Peterson's modification of the Lowry assay (18) with BSA as standard.

### Preparation of Cytosol Fractions

CHO 15B cell homogenates were prepared according to Balch and Rothman (3). Yeast and bovine tissue homogenates were prepared as described by Dunphy et al. (6), with the exception that a strain of yeast having a deleted invertase gene (SEY 2102.1, a gift of Scott Emr, California Institute of Technology, Division of Biology, Pasadena, CA) was used to circumvent inhibition of the transport assay by invertase. NSF activity-free cytosol used in the transport assay was prepared as described by Block et al. (4). The same procedure was used to prepare cytosols for the binding assays except that the cytosol was equilibrated in 20 mM Hepes, pH 7.4, 100 mM KCl. Protein concentration was determined by the method of Bradford (5). Aliquots were frozen in liquid nitrogen and stored -80°C. In experiments where cytosols were subjected to various treatments, details are given in the figure legend.

### The NSF-Membrane Binding Assay

NSF binding to Golgi membranes was measured by incubating purified NSF with NEM-treated Golgi membranes and then measuring the amount of NSF bound to the reisolated membranes using an NSF-dependent transport assay. For each binding incubation, 5 μl NEM-treated Golgi membranes (0.5-14.0 μg protein in 1 M sucrose, 10 mM Tris, pH 7.4) were added to 20 μl of binding buffer containing purified NSF (5-40 ng) and cytosol (0-100 μg protein). Unless otherwise indicated, the binding buffer consisted

of 20 mM Hepes (pH 7.4), 2 mM EDTA, 100 mM KCl, 250 μM ATP, 1 mM DTT, 1% polyethyleneglycol 4000, and 250 μg/ml carrier protein (STI). After a 2-min incubation on ice, the membranes were pelleted by centrifugation for 5 min at 4°C in an Eppendorf centrifuge (Brinkman Instruments Co., Westbury, NY). After removing the supernatant, the pellets were homogenized in 25 μl of resuspension buffer (20 μl binding buffer, 5 μl 1 M sucrose) using a Pellet Pestle (Kontes Co., Vineland, NJ). Duplicate 5-μl aliquots of the resuspended membranes or supernatants were assayed for NSF activity in a 50 μl NSF-dependent transport assay. In some experiments, the binding incubation was increased to 50 μl in order to accommodate larger volumes.

### The NSF-dependent Transport Assay

NSF activity bound to membranes was measured by using a modified Golgi transport assay as described by Block et al. (4). In this assay, the amount of [<sup>3</sup>H]GlcNAc incorporated into VSV-G is linearly dependent on the amount of NSF added until ~80% saturation is reached. All results reported in this paper were obtained within this linear range. 1 U of NSF activity is defined as the amount of NSF that supports the incorporation of 1,000 cpm of [<sup>3</sup>H]GlcNAc (~0.3 pmol) into VSV-G protein in a 1-h incubation at 37°C. For each set of assays, a blank sample (the resuspended "pellet" of a binding incubation without Golgi membranes) was used to obtain a suitable value for the background in the transport assay that is due to residual NSF on the donor and acceptor membranes. This value (typically 500-900 cpm) was subtracted from the total cpm [<sup>3</sup>H]GlcNAc incorporated into VSV-G protein for calculation of NSF activity bound.

### Golgi Membrane Treatments

KCl-extracted Golgi membranes were prepared by diluting NEM-treated membranes (in 1 M sucrose, all sucrose solutions also contain 10 mM Tris, pH 7.4) fivefold with 10 mM Tris, pH 7.4, 0.625 M KCl (0.5 M KCl final). Control membranes were diluted into 10 mM Tris, pH 7.4. After a 30-min incubation on ice, the membranes (0.5 ml) were washed by sedimentation through 0.75 ml of 0.5 M sucrose onto a 0.24-ml cushion of 1 M sucrose (50,000 rpm for 20 min in a TLS-55 rotor, Beckman Instruments, Inc.) and recovered in the original volume (0.1 ml) from the top of the 1 M sucrose cushion using a pipetter (Pipetman; Gilson Medical Electronics, Inc., Freehold, NJ). Na<sub>2</sub>CO<sub>3</sub>-extracted Golgi membranes were prepared by diluting KCl-extracted membranes 10-fold in 0.11 M freshly prepared Na<sub>2</sub>CO<sub>3</sub>, pH 11.5 (9). Control membranes were diluted into 10 mM Tris, pH 7.4, 0.11 M KCl. After incubating for 30 min on ice, the membranes were washed and collected as described above.

Protease-treated membranes were prepared by adding 1/10 vol of 10 mg/ml trypsin, 1 mg/ml proteinase K, or 1 mg/ml pronase plus 1 mM CaCl<sub>2</sub> to NEM-treated membranes (0.1 ml; 1-2 mg membrane protein/ml). After a 2-h incubation on ice, the enzymes were inactivated by adding 1/10 vol of 10 mg/ml STI, 5 mM PMSF in 90% ethanol, or 40 mM EDTA, respectively. Control membranes were prepared by mixing enzymes and inhibitors at the beginning of the incubation. The membranes were then washed by diluting fourfold with 20 mM Tris, pH 7.4, and centrifugation on a discontinuous sucrose gradient consisting of 0.3 ml 0.5 M sucrose, 0.2 ml 0.7 M sucrose, and 0.1 ml 1 M sucrose (TLS-55 rotor; 50,000 rpm for 30 min). Protease-treated membranes were recovered at the 0.5-0.7 M sucrose interface as described above. Control membranes were recovered at the 0.7-1.0 M sucrose interface. Heat-treated membranes were prepared by incubating the membranes at 75°C for 15 min. The membranes were then chilled on ice and used without further processing. The treated membranes were no more inhibitory than control membranes when tested in the transport assay.

### Estimation of the Apparent Dissociation Constant ( $K_d$ ) for NSF and the SNAP/Receptor Complex

The data in Fig. 2 B was used to estimate the upper limit for the dissociation constant as follows. We assume, since the concentration of cytosol in these assays is in excess of the concentration required to saturate the membrane receptors present, that we are measuring the apparent dissociation constant ( $K_d$ ) for the interaction between NSF and the SNAP/membrane receptor complex: NSF + SNAP/receptor  $\rightleftharpoons$  NSF/SNAP/receptor. When half of the receptor sites are occupied,  $K_d = \text{NSF}_{\text{free}/2} = \text{NSF}_{\text{total}/2} - \text{NSF}_{\text{bound}/2}$ ; where  $\text{NSF}_{\text{free}/2}$  is the concentration of NSF at half saturation. Since  $\text{NSF}_{\text{total}/2}$  and  $\text{NSF}_{\text{bound}/2}$  are the same within experimental error (see Results), the difference between them should be  $\leq 1$  SD of the mean NSF activity. The

average standard error for NSF activities (from six independent titrations) was  $\sim 20\%$ . Thus, the upper limit for  $K_d$  is given by  $0.2 \text{ NSF}_{\text{total}}^{1/2}$  for the titration carried out at the lowest membrane concentration. At this concentration ( $20 \mu\text{g/ml}$  membrane protein; Fig. 2 B), half-maximal binding occurs at  $\sim 0.1 \mu\text{g/ml}$  NSF ( $3.2 \times 10^{-10} \text{ M}$ ), giving an upper limit of  $\sim 6 \times 10^{-11} \text{ M}$  ( $60 \text{ pM}$ ) for the  $K_d$ .

### Estimation of the Cellular Content of NSF, SNAP, and the Membrane Receptor

The morphometric data obtained for baby hamster kidney cells (12, 19) were used to estimate the volume of cytosol ( $\sim 1.0 \times 10^{-12}$  liter) and the concentration of Golgi membrane protein ( $\sim 6 \times 10^{-8} \text{ mg}$ ) for a CHO cell. Careful quantitation of NSF activity recovered during purification indicates that NSF comprises a maximum of 0.2% of cytosolic protein when most of the NSF is present in soluble form (4). Using the protein concentration of cytosol from which NSF was purified, we estimate the cellular concentration to be  $25 \text{ mg/ml}$ . Therefore, the maximum cellular concentration of NSF is estimated to be  $50 \mu\text{g/ml}$  or  $1.6 \times 10^{-7} \text{ M}$  for a tetramer of 76-kD subunits. At  $10^{-12}$  liter per cell, this gives  $1.0 \times 10^5$  molecules of NSF per cell.

The amount of the membrane receptor per cell was estimated from the data in Table I. From the specific activity of purified NSF, we calculate that there are  $\sim 1.5 \times 10^{13}$  molecules of NSF bound per mg of membrane protein. Assuming that one tetramer of NSF binds to one receptor on the Golgi membranes and that our Golgi fractions are 50% pure, we obtain a receptor concentration of  $\sim 3.0 \times 10^{13}$  receptors/mg or  $52 \text{ pmol/mg}$  Golgi membrane protein. At  $6 \times 10^{-9} \text{ mg}$  of Golgi membrane protein per cell, this gives a value of  $\sim 1.8 \times 10^5$  receptor molecules per cell.

The amount of SNAP present in cytosol was estimated from the data in Fig. 2 A. We find that a cytosol to membrane protein ratio of 20:1 is required to saturate the receptors. Assuming that essentially all of the SNAP in cytosol is bound until saturation is achieved and that one molecule of SNAP is bound per receptor, then there are  $\sim 1.5 \times 10^{13}$  molecules of SNAP per 20 mg of cytosol. The concentration of this cytosol ( $\sim 10 \text{ mg/ml}$ ), adjusted for a fivefold dilution at homogenization, predicts that maximum cytosolic concentration of SNAP in the cell is  $6 \times 10^{-8} \text{ M}$ . At  $10^{-12}$  liter per cell, there are  $\sim 4 \times 10^4$  molecules of soluble SNAP per cell. Since  $\sim 50\%$  of the SNAP activity in cell homogenates is recovered in soluble form (data not shown), the total number of SNAP molecules in the cell is  $\sim 0.8 \times 10^5$ .

For all of the calculated abundances, the absolute values depend on the assumption that all molecules of purified NSF are active for binding and for transport. The relative abundance of these components (NSF, SNAP, and the receptor), however, will be the same even if some of the NSF is inactive.

## Results

### The Binding of NSF to Golgi Membranes Requires Cytosol

We have measured the binding of NSF to Golgi membranes by incubating purified NSF with Golgi membranes lacking NSF activity, and then reisolating the membranes to measure the amount of NSF activity bound.

To obtain membranes with suitably little NSF activity for initial binding studies, we used a mild NEM-treatment of the membranes ( $1 \text{ mM}$ ,  $0^\circ\text{C}$  for 15 min) to inactivate most of the endogenous NSF (4). ATP was included in all binding assays because adenine nucleotides are needed to stabilize soluble NSF (4). In addition, DTT, polyethyleneglycol, and a carrier protein (STI) were included to further stabilize NSF. A magnesium-free (EDTA-containing) incubation buffer was used because preliminary experiments showed that this improves the extent and reproducibility of binding. Magnesium is not needed for the ATP-dependent stabilization of soluble NSF (4).

The binding assay is done in two stages. In stage 1 (in which binding occurs), purified NSF is mixed with NEM-treated membranes. After a short incubation (typically at  $0^\circ\text{C}$ ), the membranes are collected by centrifugation and resuspended in the original volume of incubation buffer. These membranes are added to a second stage, NSF-depen-

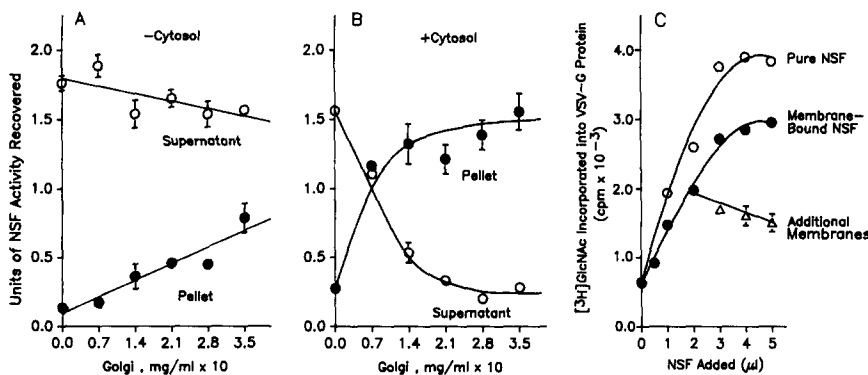


Figure 1. Assay of NSF binding to Golgi membranes. (A and B) NSF activity bound to Golgi membranes in the absence and presence of cytosol. Pure NSF in  $20 \mu\text{l}$  of binding buffer (see Materials and Methods) or binding buffer containing cytosol ( $2.0 \text{ mg/ml}$ ) was mixed with  $5 \mu\text{l}$  of NEM-treated Golgi membranes (in  $1 \text{ M}$  sucrose) to give the indicated concentrations of Golgi membrane protein and  $0.2 \mu\text{g/ml}$  NSF. After a 2-min incubation on ice, the membranes were collected by centrifugation, the supernatant removed, and the membrane pellet homogenized in  $25 \mu\text{l}$  of resuspension buffer. Duplicate  $5\text{-}\mu\text{l}$

aliquots of supernatant and homogenate were assayed for NSF activity in the NSF-dependent transport assay. The amount of NSF activity in  $5 \mu\text{l}$  of incubation mix was within the linear range of the transport assay (not shown). (A) No cytosol in the binding incubation; (B) cytosol in the binding incubation. (○) Units of NSF activity per  $5 \mu\text{l}$  of supernatant; (●) units of NSF activity per  $5 \mu\text{l}$  of membrane homogenate. (C) Quantitation of soluble and membrane-bound NSF in the NSF-dependent transport assay. Membrane-bound NSF (●) was prepared by mixing pure NSF in binding buffer containing cytosol with NEM-treated Golgi membranes (in  $1 \text{ M}$  sucrose) to give final concentrations of  $1.7 \mu\text{g/ml}$  NSF,  $3.8 \text{ mg/ml}$  cytosol, and  $180 \mu\text{g/ml}$  membrane protein. After 2 min, the membranes were collected by centrifugation and homogenized in  $50 \mu\text{l}$  of resuspension buffer. The indicated volumes of homogenate, balanced to  $5 \mu\text{l}$  with the same buffer, were added to a  $50 \mu\text{l}$  NSF-dependent transport assay. To quantitate the effect of membranes alone on the assay, membrane homogenates were prepared under identical conditions but without NSF. This homogenate ( $\Delta$ ), mixed with  $2 \mu\text{l}$  of NSF-containing homogenate to give the indicated total volume of homogenate, was added to the transport assay. Purified NSF (○) was diluted to a concentration of  $0.8 \mu\text{g/ml}$  in resuspension buffer and the indicated volumes, balanced to  $5 \mu\text{l}$  with the same buffer, were added to a  $50\text{-}\mu\text{l}$  transport assay. The assays were incubated at  $37^\circ\text{C}$  for 60 min, and the amount of [ $^3\text{H}$ ]GlcNAc incorporated into VSV-G protein was determined by immunoprecipitation.

dent, Golgi transport assay (4) to quantitate the amount NSF bound to the membranes in terms of its activity.

Fig. 1 *A* illustrates the results of a binding assay in which NSF was incubated with membranes for 2 min on ice. After centrifugation, the NSF activity in both the supernatant and the membrane pellet was quantitated (see below). As the concentration of membranes in the binding assay is increased, the amount of NSF activity appearing in the pellet increases and the amount in the supernatant decreases proportionally, demonstrating that NSF indeed binds to the membranes. The amount of NSF bound per microgram of Golgi membrane (0.5 U/ $\mu$ g) however, is fivefold lower than might be expected based on the amount of NSF activity associated with freshly isolated Golgi membranes (typically 2.5–3 U/ $\mu$ g; Weidman, P., unpublished observations). The amount of NSF bound does not increase after longer incubations or when the incubation temperature is increased to 37°C (not shown). When cytosol (free of NSF activity; see Materials and Methods) is added to an otherwise identical binding assay (Fig. 1 *B*), the amount of NSF appearing in the pellet is dramatically increased and most of the activity in the supernatant is removed. We conclude that purified NSF binds to NEM-treated Golgi membranes and that this binding is greatly enhanced by cytosol.

In stage 2 of the binding assay, we add NSF to the NSF-dependent transport assay in a membrane-bound form. Fig. 1 *C* demonstrates that this assay is linearly dependent on the amount of NSF added until saturation is reached. This is true for both soluble (Fig. 1 *C*, open circles) and membrane-bound (Fig. 1 *C*, closed circles) NSF. These data also demonstrate that NSF readily transfers between membranes under the conditions of the transport assay, as shown before (10). The membranes to which NSF had bound are themselves slightly inhibitory (Fig. 1 *C*, open triangles). This explains why the amount of [<sup>3</sup>H]GlcNAc incorporated into VSV-G protein at saturation is lower for NSF bound to membranes than for pure NSF. In all subsequent experiments,

NSF binding was measured as the NSF activity associated with the membrane pellet. All reported NSF activity measurements were obtained within the linear range of the transport assay.

### NSF Binding to Golgi Membranes Is Saturable

The fact that cytosol stimulates the reassociation of NSF with Golgi membranes suggests that a factor(s) present in cytosol is required for this association to occur. If the mechanism of stimulation by cytosol is an enzymatic one, then the extent of binding should depend on the length and temperature of incubation when cytosol is limiting. On the other hand, if cytosol stimulation reflects the formation of a stoichiometric complex of the cytosolic factor(s) with NSF, the extent of binding will be limited by the amount of cytosol present rather than the length of the incubation. The amount of NSF bound to membranes (when NSF is in excess) should then be strictly proportional to the concentration of cytosol in the binding incubation until a plateau is reached at which all available binding sites are titrated (e.g., saturation). In addition, if binding involves specific receptor sites on the Golgi membranes, the maximum extent of binding with excess cytosol should be proportional to the membrane concentration.

To test these possibilities, the relationship between NSF binding and cytosol concentration was examined for two concentrations of membranes (Fig. 2 *A*). At a fixed concentration of Golgi membranes and with excess NSF (a fourfold higher concentration of NSF relative to the experiments shown in Fig. 1), NSF binding is linearly dependent on the concentration of cytosol in the incubation mixture until a plateau is reached. Binding is indeed saturable with respect to cytosol and the amount of NSF bound at saturation (the plateaus in Fig. 2 *A*) is proportional to the membrane concentration. At subsaturating cytosol concentrations, binding is the same at 0°C as at 37°C and is independent of the length

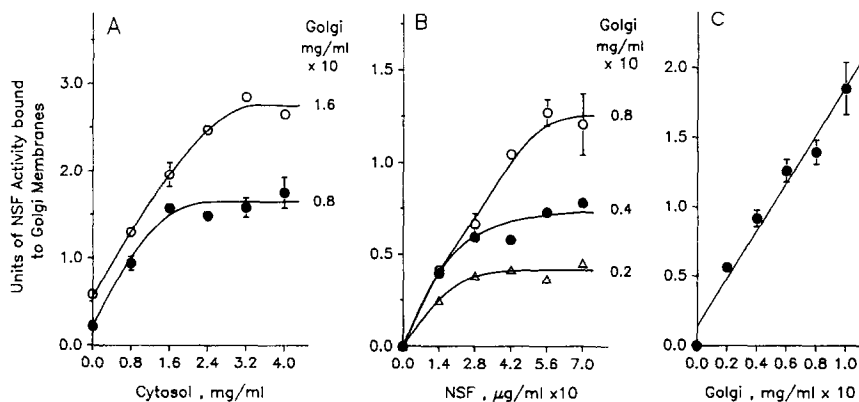


Figure 2. The binding of NSF to Golgi membranes is a saturable process. (A) Cytosol titration. Pure NSF in 10  $\mu$ l of binding buffer was mixed with 10  $\mu$ l of cytosol in binding buffer and 5  $\mu$ l of Golgi membranes (in 1 M sucrose) to give 0.8  $\mu$ g/ml NSF and the indicated concentration of cytosolic protein. The titrations contained either 80 ( $\bullet$ ) or 160 ( $\circ$ )  $\mu$ g/ml membrane protein. After a 2-min incubation, the membranes were collected by centrifugation and resuspended by homogenization in 25  $\mu$ l of resuspension buffer. Duplicate 5- $\mu$ l aliquots of the resuspended membranes were assayed for NSF activity in a 50- $\mu$ l transport assay. Values are reported as units of NSF

activity per 5  $\mu$ l of resuspended membranes. (B) NSF titration. Pure NSF was diluted to the indicated concentrations in a 25- $\mu$ l binding incubation mixture containing either 20 ( $\Delta$ ), 40 ( $\bullet$ ), or 80 ( $\circ$ )  $\mu$ g/ml membrane protein and 2.5 mg/ml cytosol which, according to the data in A, is in excess of the amount required to saturate the membranes. The samples were processed as described above. Total NSF activity added was determined for identical samples containing 40  $\mu$ g/ml membrane protein that were not centrifuged (data not shown). At NSF concentrations below saturation, no significant difference was detected between the total and membrane-bound NSF activities. (C) Membrane titration. Each 25- $\mu$ l binding assay contained the indicated concentration of membrane protein, 2  $\mu$ g/ml NSF, and 2.5 mg/ml cytosol. These concentrations, according to the data in A and B, are more than sufficient to saturate the membranes. Samples were processed as described in A.

**Table 1. The Effect of Membrane Extraction on NSF Binding**

	NSF activity bound to Golgi membranes (units/ $\mu$ g membrane protein)	
	+ Cytosol	- Cytosol
NEM treated (control)	3.5 $\pm$ 0.5 (6)	1.1 $\pm$ 0.5 (8)
KCl extracted	3.1 $\pm$ 0.9 (10)	0.3 $\pm$ 0.2 (6)
Sodium carbonate extracted	4.9 $\pm$ 1.5 (8)	-0.1 $\pm$ 0.1 (6)

NEM-treated membranes were diluted into either 100 mM KCl (*control*), 500 mM KCl (pH 7.0), or 100 mM Na<sub>2</sub>CO<sub>3</sub> (pH 11.5), and incubated on ice for 30 min. The membranes were washed and concentrated by sucrose density centrifugation (see Materials and Methods for details). NSF binding was quantitated in membrane titration assays as in Fig. 2 C. Incubation mixtures contained 30–160  $\mu$ g/ml membrane protein and 0.8  $\mu$ g/ml NSF with or without 2.0 mg/ml cytosol. Units of NSF activity bound per microgram of membrane protein were determined over the linear portion of the titration curves by least squares analysis. Values are reported  $\pm$  SE with the number of data points indicated in parentheses.

of the incubation (from 2 to 15 min at 0°C; not shown), indicating that cytosol-stimulated binding most likely involves a stoichiometric rather than enzymatic mechanism. The binding curves have essentially the same slopes independent of the membrane concentration and there is an abrupt transition when the plateau is reached. It is as though all of the cytoplasmic binding factor added is complexed with NSF on the membranes and, when cytosol is in excess, binding is limited only by the quantity of membranes present. This strongly suggests that there is a high-affinity, stoichiometric interaction between the cytosolic factor, a membrane receptor, and NSF.

The complementary experiment, titration of NSF in the presence of excess cytosol, was also performed (Fig. 2 B). NSF binding increases with increasing concentration of NSF until a plateau level is reached. At NSF concentrations below saturation, essentially all of the added NSF was recovered bound to membranes (not shown) indicating a high-affinity interaction.

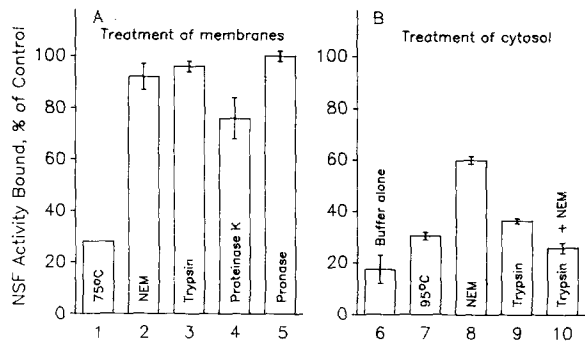
Fig. 2 C confirms that, in the presence of excess cytosol and NSF, binding is indeed strictly proportional to the membrane concentration, as expected when there are a limited number of saturable sites on the membrane. The specific binding capacity of Golgi membranes, estimated from the slope of the line in Fig. 2 C and the specific activity of purified NSF, is  $\sim$ 8 ng of NSF bound per microgram of membrane protein ( $1.5 \times 10^{10}$  NSF tetramers of 76-kD subunits). The data suggest that there is only one type of receptor. Since our Golgi membrane preparations are only 30–50% pure, we can not rule out the possibility that the receptor is found on non-Golgi membranes as well.

In summary, these data demonstrate saturable binding of NSF to Golgi membranes, indicating the existence of a specific receptor for NSF. In addition, a cytosolic cofactor(s) is required for binding to this receptor. This cofactor, SNAP, appears to act stoichiometrically rather than enzymatically to promote interaction of NSF with its receptor. We suggest that the binding of NSF to Golgi membranes under our assay conditions reflects the formation of a high-affinity NSF/SNAP/membrane receptor complex.

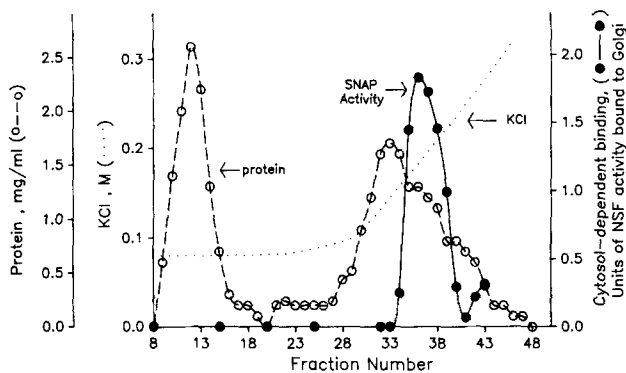
### **The Membrane Receptor for NSF is an Integral Membrane Component**

We have used various pretreatments to study the properties

of the membrane receptor that participates in the binding reaction. When NEM-treated membranes are preextracted with 0.5 M KCl, binding in the presence of cytosol is essentially unaffected (Table I), however, cytosol-independent binding is reduced. When membranes are preextracted with 0.1 M sodium carbonate (pH 11.5), a procedure that removes peripheral but not integral membrane proteins (9), the NSF binding capacity in the presence of cytosol actually increased and binding in the absence of cytosol was reduced below the level of detectability. These results strongly suggest that the receptor is an integral membrane component. The background of cytosol-independent binding observed with NEM-



**Figure 3.** The effect of various treatments on NSF binding. (A) Treatment of Golgi membranes. Golgi membranes were treated as follows: (experiment 1) incubation at 75°C for 15 min; (experiment 2) incubation in 1 mM NEM at 37°C for 15 min followed by addition of DTT to 2 mM; (experiment 3) digestion with 1 mg/ml trypsin at 4°C for 2 h followed by the addition of STI to 1 mg/ml; (experiment 4) digestion with 0.1 mg/ml proteinase K at 4°C for 2 h followed by addition of PMSF to 5 mM; and (experiment 5) digestion with 0.1 mg/ml pronase at 4°C for 2 h followed by the addition of EDTA to 2 mM. For each treatment, mock-treated membranes were prepared by adding protease and inhibitor simultaneously at the beginning of the incubation. Protease-treated membranes were reisolated by sucrose density centrifugation (see Materials and Methods for specific details). Each binding assay contained treated or mock-treated membranes (80  $\mu$ g/ml protein), 1.0  $\mu$ g/ml NSF, and 2.0 mg/ml cytosol. Results are expressed as NSF activity bound to treated membranes relative to mock-treated membranes. In all cases, NSF binding to mock-treated membranes was within 15% of the binding observed for untreated membranes. (B) Treatment of the cytosol. Cytosol was treated as follows: (experiment 6) incubation at 95°C for 10 min followed by removal of precipitated protein by centrifugation; (experiment 7) incubation with 1 mM NEM at 4°C for 30 min followed by the addition of DTT to 2 mM; (experiment 8) digestion with 1 mg/ml trypsin at 4°C for 60 min followed by addition of STI to 1 mg/ml; and (experiment 9) a combined treatment with NEM (1 mM for 15 min at 4°C) followed by trypsin (1 mg/ml for 1 h). For each treatment, mock-treated samples were prepared by adding NEM plus DTT or trypsin plus STI simultaneously at the beginning of the incubation. Each binding incubation contained 1.65  $\mu$ g/ml NSF, and 120  $\mu$ g/ml Golgi membrane protein and either buffer alone (experiment 6) or 2.0 mg/ml cytosol (or equivalent). Results are expressed in B. No significant difference in NSF binding was observed between control cytosols and untreated cytosol. Membranes incubated with NEM or trypsin-treated cytosols were more inhibitory in the NSF-dependent transport assay than membranes incubated with the control cytosols (10% for NEM and 25% for trypsin and trypsin plus NEM; not shown). This effect, however, is much smaller than the decrease in NSF activity bound observed with these cytosols (50–85%).



**Figure 4.** Fractionation of cytosol by ion-exchange chromatography. Cytosol (2.0 ml, 7 mg/ml protein) in 20 mM Hepes, pH 7.0, 80 mM KCl, 1 mM DTT, and 10% glycerol was applied to a 5-ml column of DE-52 cellulose equilibrated in the same buffer. The column was washed with 4 ml of starting buffer and then eluted with a 15-ml linear KCl gradient (100–300 mM KCl) generated by a three-channel pump. Protein concentration (○) was determined by the method of Bradford (5). KCl concentration (dotted line) was determined by comparing the conductance of selected fractions to the conductance of buffer standards containing 80–400 mM KCl. Fractions containing SNAP activity (●) were identified using a 50- $\mu$ l binding assay. Each assay contained 10  $\mu$ l of fractionated cytosol (adjusted to 100 mM KCl in binding buffer), 80  $\mu$ g/ml membrane protein, and 1.5  $\mu$ g/ml NSF.

treated membranes may be explained in part by the presence of endogenous SNAP on the membrane.

Several procedures were used to further establish the nature of the membrane receptor. When membranes are heated to 75°C for 15 min before the binding assay, NSF binding is greatly reduced (Fig. 3 A, experiment 1), as would be expected if the receptor is a protein. However, extensive digestion of membranes with a variety of proteases (sufficient digestion to cause a noticeable shift in membrane buoyant density; see Materials and Methods) had little or no effect on NSF-binding capacity in the presence of cytosol (Fig. 3 A, experiments 3–5). Treatment with NEM under harsh conditions (1 mM, 37°C for 15 min) also failed to inhibit cytosol-dependent binding of NSF to the membrane receptor (Fig. 3 A, experiment 2).

We conclude that the membrane receptor for NSF is an integral membrane component. Although the nature of this component remains unclear, its sensitivity to thermal denaturation suggests that it is a protein rather than a lipid. The resistance of this component to proteolytic degradation is consistent with the evidence that it is integral membrane protein.

#### ***SNAP is a Polypeptide That Can Be Separated from Bulk Protein***

We have used an analogous approach to investigate the nature and specificity of the cytosolic cofactor (SNAP) that is required for NSF to bind to its membrane receptor. As shown in Fig. 3 B, experiments 7 and 9, the activity of SNAP in cytosol is sensitive to inactivation by heat and trypsin, but largely (60%) resistant to NEM (Fig. 3 B, experiment 8), even after 30 min at 37°C (not shown). The NEM-resistant activity can be removed by trypsin digestion (Fig. 3 B, experiment 10). These data demonstrate that SNAP is a protein

distinct from the integral membrane receptor. Its relative resistance to NEM treatment also contrasts with the complete sensitivity of NSF to NEM.

Fractionation of cytosolic protein by chromatography on DE-52 cellulose (Fig. 4) demonstrates that SNAP activity is resolved from bulk protein and elutes at  $\sim$ 0.2 M KCl. Fractions containing SNAP activity (fractions 34–49) were pooled and assayed in the linear range of a cytosol titration assay with excess NSF and membranes (as in Fig. 2 A). Greater than 95% of the SNAP activity applied to the column was recovered in these pooled fractions, resulting in a tenfold increase in specific activity. The sensitivity of SNAP activity in these fractions to heat, NEM, and trypsin parallels the sensitivity of the activity in unfractionated cytosol (not shown).

Cytosol prepared from a variety of plant, fungal, and animal sources will substitute for CHO cytosol in our standard transport assay (where NSF is provided by the Golgi membranes), indicating that many of the proteins involved in intracellular transport are conserved in evolution (6, 17). As a further demonstration of the specificity of SNAP in NSF binding, we examined cytosols prepared from yeast, bovine brain, bovine kidney, and bovine liver for activity that stimulates binding of CHO NSF to CHO Golgi membranes. All of the cytosols, with the exception of those prepared from yeast, are able to stimulate NSF binding to levels that are comparable to those of CHO cytosol (not shown). In mixing experiments, yeast cytosol did not inhibit binding mediated by CHO cytosol. Bovine brain SNAP activity chromatographs on DE-52 cellulose very similarly to CHO SNAP (not shown). The molecular weight of bovine brain SNAP, as estimated by Superose-12 gel permeation chromatography, is  $\sim$ 35 kD (not shown).

We conclude that SNAP activity is associated with a specific protein(s) that is distinct both from NSF and the membrane receptor.

#### ***Discussion***

Transport in the Golgi stack is mediated by a population of nonclathrin-coated vesicles which bud from one cisternal compartment, are uncoated, and then fuse with the next cisternal compartment in the stack (16). Biochemical and morphological studies indicate that NSF is required for the fusion of the uncoated transport vesicles (13). Proteins that interact with NSF can thus be expected to play a role in the fusion process. In this paper we have reported the existence of an integral membrane component and a cytoplasmic factor (SNAP) that together promote the binding of NSF to Golgi membranes. The shapes of the binding curves (Fig. 2) suggest that NSF, SNAP, and the integral membrane receptor assemble with high affinity to form a stoichiometric complex. It therefore seems likely that this ternary complex forms part of the multisubunit enzyme catalyzing membrane fusion that was proposed earlier (13). Purification of SNAP and the receptor will, of course, be necessary to test this hypothesis directly.

The amount of NSF activity associated with Golgi membranes at isolation (2.5–3.0 U/ $\mu$ g of membrane protein) is  $\sim$ 80% of the maximum binding capacity of Golgi membranes as measured in the *in vitro* binding assay (3.5 U/ $\mu$ g membrane protein; Table I). The number of binding sites that we observe *in vitro* is therefore reasonable and most likely

pertinent to the mechanism of binding *in vivo*. The fact that a significant amount of NSF remains bound to Golgi membranes during their isolation is consistent with the high affinity of this interaction in the *in vitro* binding assay (Fig. 2).

Can we estimate the affinity of this binding interaction? Since essentially all of the NSF is bound to membranes at subsaturating concentrations of NSF (Fig. 2B), it is not possible to determine the equilibrium constant by Scatchard analysis. An upper limit for the apparent dissociation constant for NSF binding to the SNAP/receptor complex,  $K_d$ , can be estimated, however, from the experimental error in the binding assays (20%). From an error analysis as described in Materials and Methods,  $6 \times 10^{-11}$  M (60 pM) is estimated as the upper limit for  $K_d$  under the conditions of the binding assay (in the absence of  $Mg^{2+}$ ).

How abundant are NSF, SNAP, and the membrane receptor in the cell? The methods that we have used to estimate these values are detailed in Materials and Methods. Using data from the purification of NSF (4), we estimate that there are  $\sim 1.0 \times 10^5$  molecules of NSF per cell. From our *in vitro* binding data (Table I and Fig. 2A), we estimate that there are  $\sim 0.8 \times 10^5$  molecules of SNAP and  $1.8 \times 10^5$  molecules of receptor per cell, assuming that NSF forms a one-to-one complex with SNAP and the receptor on Golgi membranes. Thus, the estimated number of molecules of NSF per cell is very similar to the number of molecules of SNAP and membrane receptors in a cell. This calculation assumes that NSF acts within the Golgi stack only. If the NSF receptor is present on other organelles, the ratio of receptor to NSF molecules would be greater. These estimates are consistent with the notion that membrane-bound NSF is the functional species of NSF during catalysis of vesicle fusion. It is noteworthy that the receptor, at a concentration of  $\sim 50$  pmol/mg of Golgi membrane protein, is an abundant protein, in the range of such major organelle proteins as ribophorin I (43 pmol/mg total microsomal protein; see reference 14) and porin (100 pmol/mg total mitochondrial protein; see reference 7). This suggests that it will be feasible to attempt to identify this component.

The involvement of a specific cofactor (SNAP) in the interaction of NSF with membranes is indicated by the observation that SNAP activity is separated from bulk cytosolic protein during chromatography (Fig. 4). The fact that SNAP activity is found in cytosol from several different bovine tissues and that bovine brain SNAP behaves similarly to CHO SNAP during chromatography is suggestive of a conserved function for this protein in cellular metabolism. Yeast cytosol will support transport between CHO Golgi stacks in the cell-free system when NSF and presumably SNAP are provided by the membranes (6). The apparent lack of SNAP activity in yeast cytosol does not rule out the possibility that homologous components (NSF, SNAP, and the receptor) are involved in intracellular transport in yeast. Indeed, NSF activity can be demonstrated in cytosol fractions from yeast (Flynn, G., C. Wilcox, and J. E. Rothman, unpublished observations). It is possible, for example, that yeast SNAP is not stable or extractable from membranes under the conditions used to prepare cytosol or that, since at least three specific components are involved, minor protein variations between different species may prevent the formation of a functional unit from heterologous components.

In conclusion, from the data presented in this paper and

the correlations discussed above, we suggest that the formation of the NSF/SNAP/receptor complex is necessary for the function of NSF and thus for catalysis of vesicle-membrane fusion. It may well be that an additional level of complexity is superimposed upon the assembly and/or disassembly of this complex. ATP promotes the release of endogenous NSF from Golgi membranes (11) in the presence but not the absence of magnesium (10). The use of EDTA in our binding assays has eliminated these effects from the present study. However, it remains an intriguing possibility that ATP binding and/or hydrolysis may be an important part of these complex reactions.

We thank Lyne Paquet for expert technical assistance.

This research was supported by a Bank of America, Giannini Foundation Fellowship and a National Institutes of Health Fellowship (7F32-GM 12042-02; to P. J. Weidman); a Canadian Medical Research Council Fellowship (to P. Melançon); a California American Cancer Society Senior Fellowship (to M. R. Block); and National Institutes of Health Grant DK 27044 (to J. E. Rothman).

Received for publication 21 November 1988 and in revised form 10 January 1989.

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