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Purification of an *N*-ethylmaleimide-sensitive protein catalyzing vesicular transport

(Golgi stack/intracellular protein transport)

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ABSTRACT ***N*-Ethylmaleimide (NEM) inhibits protein transport between successive compartments of the Golgi stack in a cell-free system. After inactivation of the Golgi membranes by NEM, transport can be rescued by adding back an appropriately prepared cytosol fraction. This complementation assay has allowed us to purify the NEM-sensitive factor, which we term NSF. The NEM-sensitive factor is a tetramer of 76-kDa subunits, and appears to act catalytically, one tetramer leading to the metabolism of numerous transport vesicles.**

The movement of proteins between membrane-bound compartments is carried out by transport vesicles (1). Elucidation of the molecular mechanisms involved in vesicle budding and fusion will require that the components of the transport machinery be isolated in functional form. Here, we report the purification to essential homogeneity of one such protein component. This protein, the *N*-ethylmaleimide-sensitive factor (NSF), is needed for biosynthetic transport between Golgi cisternae in cell-free systems (2, 3).

Biosynthetic protein transport occurs in two distinct phases. First, a chain of nonselective "bulk-carrier" vesicles move proteins from the endoplasmic reticulum to the Golgi, from cisternae to cisternae across the Golgi stack, and into the trans-Golgi network (4). Then, in the trans-Golgi network, the proteins are separated according to their destinations (5–7). Much of the first part of this pathway has been reconstituted in a cell-free system that measures the transport of the vesicular stomatitis virus-encoded glycoprotein (VSV-G protein) between successive cisternae of the Golgi stack (8–13). Transport requires ATP as well as cytosol (high-speed supernatant), and appears to be mediated by non-clathrin-coated vesicles that are the bulk carriers mentioned above (12, 14).

We have reported (2) that treatment with *N*-ethylmaleimide (NEM) under mild conditions (1 mM, 15 min, 0°C) selectively inactivated Golgi membranes in the cell-free transport assay. Transport could be restored to NEM-treated Golgi membranes by adding back the ATP extract of untreated Golgi membranes. This restorative factor was itself sensitive to NEM. The activity of NSF is stimulated by long chain fatty acyl-CoA (2, 3). NSF is required for transport at multiple levels of the Golgi stack (3). Here we report the purification of NSF from CHO cytosol prepared in the presence of ATP.

MATERIALS AND METHODS

Preparation of Golgi Membranes and NSF-Free Cytosol. Donor and acceptor membranes were prepared as described (8, 15) from VSV-infected 15B CHO and wild-type uninfected CHO cells, respectively. A mixture of equal volumes of acceptor and donor Golgi membranes (in 1 M sucrose/10 mM Tris·HCl, pH 7.4) was incubated at 0°C for 15 min with 1 mM NEM (final concentration) added from a fresh stock solution (50 mM). Then dithiothreitol was added to 2 mM from a 0.1 M stock. This mixture of NEM-treated donor and acceptor membranes was refrozen in liquid N_2 and stored in aliquots at −80°C for use in NSF assays.

15B CHO cells were grown and homogenized (15). Cytosol (8 mg/ml of protein) was prepared and desalted on a Bio-Gel P6-DG column essentially as described (8, 15), incubated for 20 min at 37°C in the absence of ATP to inactivate any NSF, frozen in liquid nitrogen, and stored at −80°C.

Assay of NSF Activity. The assay used is a variation on the standard transport assay (8). Incubation mixtures (50 μl) contained NEM-treated donor (5 μl) and acceptor (5 μl) membranes, 5 μl of NSF-free 15B CHO cytosol, 10 μM palmityl-CoA, 50 μM ATP, 2 mM creatine phosphate, creatine kinase (7.3 international units/ml), 250 μM UTP, and 0.4 μM UDP-[^{3}H]GlcNAc (0.5 μCi; 1 Ci = 37 GBq) in an assay buffer containing 25 mM Hepes·KOH (pH 7.0), 15 mM KCl, 2.5 mM $Mg(OAc)_2$, and 0.2 M sucrose (derived from the Golgi fractions). The NSF fraction to be tested (up to 20 μl) was added last. After incubation at 37°C for 1 hr, the VSV-G protein was immunoprecipitated at 4°C for at least 6 hr as described (8). Assays of all fractions were performed in a predetermined linear range (0–0.2 mg/ml for crude ATP-stabilized cytosol).

Large-Scale Preparation of ATP-Containing Cytosol for NSF Purifications. A washed pellet of CHO cells (1 vol) was resuspended with 4 vol of swelling buffer containing 20 mM Pipes·KOH (pH 7.2), 10 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM *o*-phenanthroline, leupeptin (10 μg/ml), and 1 μM pepstatin. The cells were allowed to swell 20 min on ice and then were disrupted in a Waring Blendor at high speed for 30 sec. Then, KCl was added slowly to the homogenate while stirring to achieve a final concentration of 0.1 M from a 2.5 M stock solution. After low-speed centrifugation at 800 × *g* for 15 min, the postnuclear supernatant was frozen in 45-ml aliquots in liquid nitrogen and stored at −80°C. When cytosol was required, postnuclear supernatant (rapidly thawed at 37°C) was spun at 45,000 rpm in a 45 Ti rotor for 90 min. This supernatant was the cytosol used for purification.

Purification of NSF. Fresh dithiothreitol was added to all buffers immediately before use. The purification described here is a standard one designed for 450 ml of cytosol and employs tandem DE-52 (Whatman) and S Sepharose Fast Flow (Pharmacia) columns. The purification described in the text and Table 1 and Figs. 2 and 3 employed 120 ml of cytosol (600 mg of protein) and is meant to illustrate each step

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Abbreviations: NEM, *N*-ethylmaleimide; NSF, NEM-sensitive factor; VSV-G protein, vesicular stomatitis virus-encoded glycoprotein.

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Table 1. Purification of NSF from CHO cell cytosol

Step	Yield of activity, % of step 1	Relative specific activity normalized to step 1	Yield of protein, mg
1. Cytosol	100	1	600
2. PEG precipitation	70	2.5	163
3. DE-52 flow through	70	12.5	54
4. S Sepharose Fast Flow flow through	35	29	11
5. Glycerol gradient	28	206	1.2
6. Mono S FPLC	12	1000	0.14

separately. The various steps can be scaled up or down in a straightforward proportional manner as needed.

Polyethylene Glycol (PEG) Precipitation. The protein concentration of the cytosol was adjusted by dilution to 5.0 mg/ml. The temperature was set at 0°C and the pH of the cytosol was precisely adjusted to 7.0 with 1 M KOH (pH electrode calibrated at 20°C). The appropriate volume of 50% (wt/vol) PEG 4000 (Sigma) was added very slowly with stirring until a final concentration of 8.0% (wt/vol) was reached. After 30 min with stirring, the precipitate was pelleted in 15 min at 10,000 rpm in a JA-20 rotor (Beckman). The pellet was suspended in one-third of the initial volume in a solution of 100 mM KCl, 20 mM Pipes·KOH (pH 7), 2 mM $MgCl_2$, 2 mM dithiothreitol, and 0.5 mM ATP. The suspension was homogenized with a Dounce homogenizer and then sonicated with three 10-sec bursts in a waterbath sonicator at 0°C. Undissolved protein was then removed by a second centrifugation at 10,000 rpm in the JA-20 rotor for 15 min. These conditions must be carefully followed for reproducible results.

Ion-Exchange Chromatography (Tandem DE-52 and S Sepharose Fast Flow Columns). DEAE-cellulose resin (DE-52) was swollen in a solution of 1 M Pipes·KOH (pH 7) and 0.5 M KCl and then poured into a 120-ml column (2.5 cm × 24 cm). S Sepharose Fast Flow was equilibrated with a 2 M KCl solution and then poured into a 50-ml glass column (2.5 cm × 10 cm). The columns were connected in series, equilibrated with a solution of 20 mM Pipes (pH 7), 100 mM KCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, and 0.5 mM ATP, and run at 2.5 ml/min. Fractions (8 ml) were collected. The protein concentration of fractions was measured by the Bradford method (5). The flow through protein peak (typically fractions 12–28) was concentrated 20-fold by ultrafiltration on an Amicon XM300 membrane at 0°C. The concentrate was centrifuged 15 min at 25,000 × *g* to remove precipitated material. ATP will flow through the DE-52 column at the concentration of KCl (0.1 M) used.

Sedimentation on Glycerol Gradients. Linear 10–35% (wt/vol) glycerol gradients (38 ml) were formed from the bottom of 40-ml Beckman quick-seal tubes. The buffer throughout the gradient was 20 mM Hepes·KOH (pH 7), 100 mM KCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, and 0.5 mM ATP. The concentrated DE-52/S Sepharose Fast Flow flow-through peak was layered on the top of each gradient in 2-ml portions by using a peristaltic pump. The gradients were centrifuged in a Beckman VTi 50 rotor at 50,000 rpm for 2.5 hr by using a slow acceleration and deceleration program. Typically, three gradients were required to process NSF derived from 450 ml of cytosol. Fractions (2 ml) were collected from the bottom of the tube. The protein concentration and NSF activity (in 0.1 μl samples) of each fraction were measured. The most active fractions (typically fractions 9–13, see Fig. 2) were pooled, yielding ≈1.5 mg of protein per ml.

Mono S FPLC Chromatography. The pooled glycerol gradient fraction was concentrated 2-fold by ultrafiltration (XM300 membrane) and dialyzed in Spectrapor 2 tubing (Spectrum Medical Industries, Los Angeles) against 100 vol of a solution of 20 mM Hepes·KOH (pH 7), 10% (wt/vol) glycerol, 2 mM $MgCl_2$, 2 mM dithiothreitol, and 0.5 mM ATP for a total of 2 hr at 4°C (with one buffer change). The dialysate was centrifuged for 10 min at 10,000 rpm in the JA-20 rotor, and 8-ml portions of the clear supernatant were loaded onto a 1-ml Mono S column (preequilibrated with the dialysis buffer) at a flow rate of 0.5 ml/min. After a 15-ml wash, NSF activity was eluted with a 10-ml linear gradient of 0–100 mM KCl. Activity was mainly found in two 0.5-ml fractions at ≈60 mM KCl. Then the column was "stripped" with 2 M KCl before reuse. Two runs were necessary to process the entire preparation. NSF (≈0.15 mg/ml) was frozen in aliquots in liquid N_2 and stored at −80°C. NSF could be repeatedly frozen in liquid N_2 with little loss of activity.

Monoclonal Antibody Production and Screening. Pure NSF (20 μg) in complete Freund's adjuvant was injected into the peritoneal cavity of a BALB/c mouse, followed after 4 weeks by a similar injection in incomplete Freund's adjuvant. After 10 weeks, 20 μg of purified NSF was injected into the tail vein on 4 consecutive days. Forty-eight hours after the last injection, spleen cells were fused with Sp2/0 myeloma cells and hybridomas were selected in hypoxanthine/aminopterine/thymidine (HAT) medium by using modifications of standard procedures (16). Colonies were selected according to their ability to react with native NSF in a standard RIA in which 0.5 μg of purified NSF was added to each microtiter well. These colonies were expanded in hypoxanthine/thymidine (HT) medium and subcloned by limiting dilution. The hybridoma (4A6) that gave the strongest signal in the RIA (60,000 cpm with saturating antibody, over background of 300 cpm when NSF was omitted) was selected for further study. It was found to secrete an IgM type monoclonal antibody that was purified from ascites fluid by 50% saturated ammonium sulfate precipitation, followed by gel filtration on a Sepharose CL-6B column equilibrated with 20 ml of 20 mM Tris·HCl, pH 8/150 mM NaCl/1 mm EDTA/0.02% NaN_3. The IgM peak was precipitated by dialysis against 5 mM Tris·HCl (pH 7.4) and the precipitate was dissolved in the above Tris/NaCl/EDTA/NaN_3 buffer. The IgM preparation was homogeneous as judged by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis. IgM antibodies were tested for inhibition under high-salt conditions in which they were freely soluble (see Fig. 5).

RESULTS

Extraction and Stabilization of NSF. The standard assay for NSF consists of the fraction to be tested incubated with NEM-pretreated donor and acceptor membranes, cytosol free of NSF activity (referred to as "NSF-free" cytosol), ATP, an ATP-regenerating system, and UDP-[^{3}H]GlcNAc for 1 hr at 37°C. To prepare NSF-free cytosol, cytosol was preincubated for 20 min at 37°C in the absence of ATP.

We decided to purify NSF from the supernatant (cytosol) fraction of CHO cells to avoid the necessity of preparing large amounts of Golgi membranes as starting material. NSF is released from Golgi membranes upon incubation with ATP (2) and the efficiency of release is improved by including 0.1 M KCl (data not shown). Cytosol prepared from CHO cells homogenized in a solution containing 100 mM KCl and 5 mM ATP typically had an NSF specific activity of ≈2500 cpm of [^{3}H]GlcNAc incorporated into VSV-G protein per μg of cytosolic protein. All of this NSF activity was eliminated by 1 mM NEM at 0°C for 15 min (data not shown).

When CHO cells are homogenized under our standard conditions (in 50 mM KCl in the absence of ATP [15]), some NSF activity was usually found in the cytosol fraction but was lost within an hour on ice. This cytosolic NSF activity was inactivated during the time required for gel-filtration, explaining our inability to detect it previously (2).

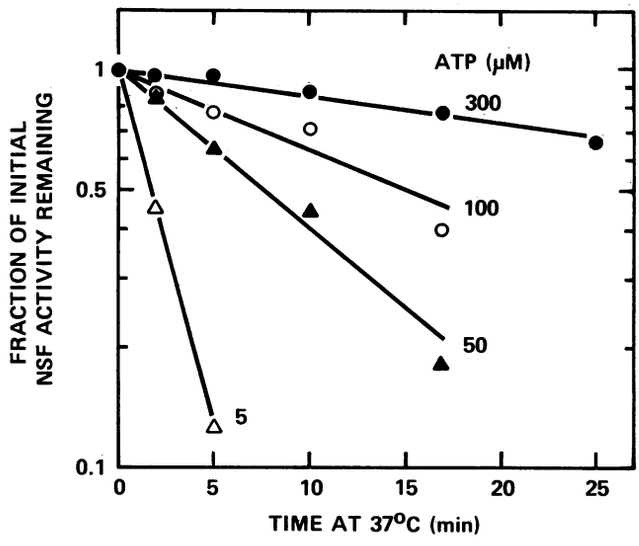

FIG. 1. ATP stabilizes NSF activity. NSF-containing CHO cytosol (prepared with 50 μM ATP instead of the standard 5 mM ATP) was diluted 1:10 with a buffer containing 100 mM KCl, 20 mM Pipes·KOH (pH 7), 2 mM $MgCl_2$, and 2 mM dithiothreitol, and ATP was added as needed to achieve the indicated final concentration. These mixtures were then preincubated at 37°C and samples were removed (and chilled on ice) for assaying the amount of NSF activity remaining as a function of time at 37°C.

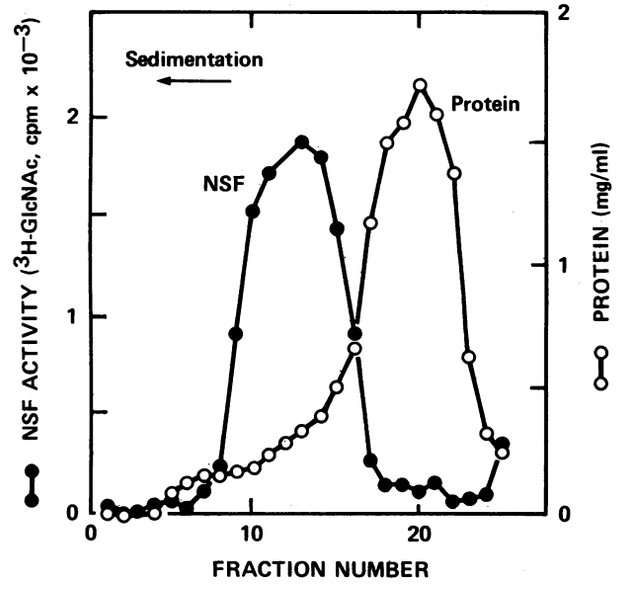

FIG. 2. Glycerol gradient step in the purification of NSF. A 10–35% (vol/vol) glycerol gradient was prepared and centrifuged. Then 0.1 μl and 5 μl of each 2-ml fraction were assayed for NSF activity (●) and protein concentration (○), respectively.

Crucial to the success of the purification was the finding that ATP stabilizes extracted NSF activity, in addition to its role in extraction. Fig. 1 shows that ATP markedly protects NSF against thermal denaturation at 37°C. Activity is lost within a few minutes upon incubation with 5 μM ATP but is almost fully retained after 20 min in the presence of 300 μM ATP. Half-maximal protection occurred at ≈50 μM ATP. dATP or ADP substitute for ATP in stabilizing NSF activity against thermal denaturation. Mg^{2+} is not required for ATP-dependent stabilization. NSF is not stabilized by AMP, GTP, palmityl-CoA, or CoA (data not shown). The ability of the adenine nucleotides to stabilize NSF strongly suggests that this molecule possesses binding sites for both ADP and ATP. These sites may be related to the effect of nucleotide upon extraction from Golgi membranes.

NSF was further stabilized by including either 1% PEG 4000 or 10% (vol/vol) glycerol as well as a reducing agent, such as dithiothreitol. NSF activity is lost in phosphate buffer

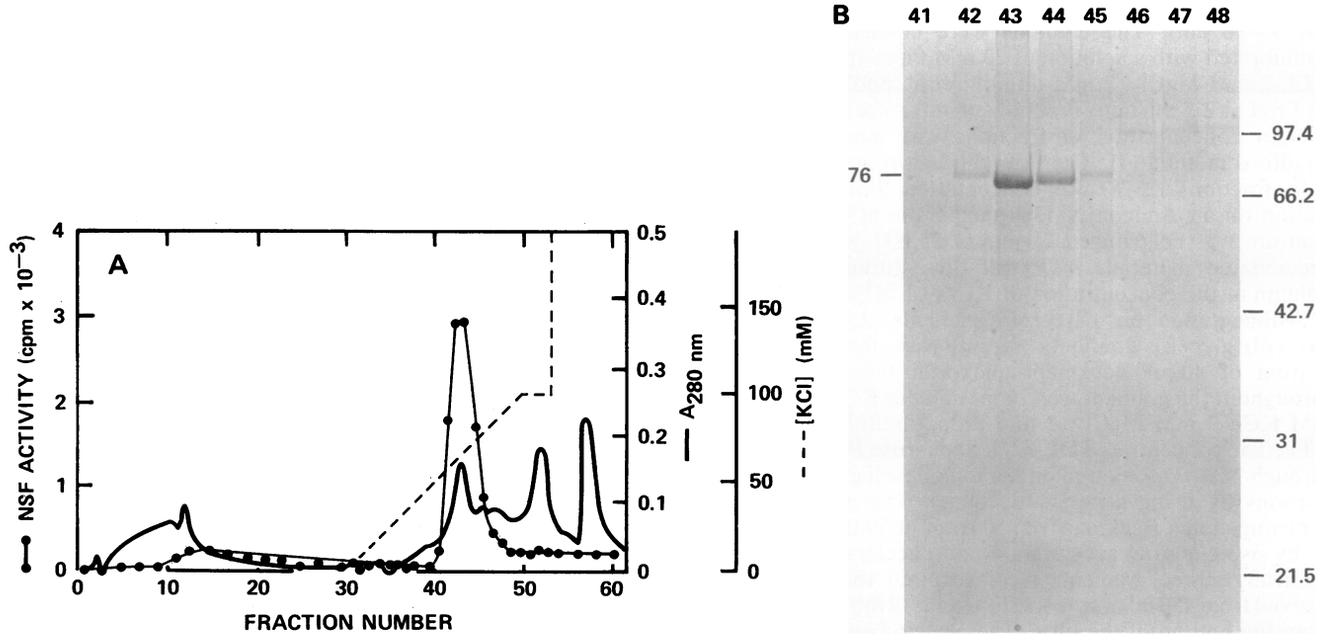

FIG. 3. Chromatography of NSF on a Mono S FPLC column. (*A*) Elution profile. ●, NSF activity in 0.01-μl samples. Note that the assay was saturated in fractions 43 and 44. Solid line, absorbance at 280 nm reflecting protein concentration. Dashed line, concentration of KCl. The 0–100 mM KCl gradient began with fraction 30. Note that at fraction 53 the concentration of KCl was increased to 0.2 M. (*B*) SDS/polyacrylamide (10%) gel electrophoresis (Coomassie blue staining) of the proteins present in the fractions containing NSF activity. The molecular mass markers (in kDa) at right were electrophoresed in a parallel lane and consisted of phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa). The molecular mass of NSF (76 kDa) was determined from its mobility relative to these standards.

and to a lesser extent in buffer containing sulfate ion, but in the absence of these ions NSF is stable over a broad pH range (pH 6.5–8).

Purification of NSF. Cytosol prepared from a 120-liter culture of CHO cells was precipitated with PEG, and then passed through successive DE-52 and S Sepharose Fast Flow columns, to which NSF does not bind. Then, NSF (sedimenting at 12 S) was effectively separated from bulk protein by velocity sedimentation through a glycerol gradient (Fig. 2). Mono S FPLC (Pharmacia) (Fig. 3) was used as the final step. NSF was retained on the Mono S column at the low-salt concentration employed. NSF activity eluted in a sharp peak in a KCl gradient at ≈60 mM KCl (Fig. 3*A*). The peak of activity coincided with a peak of absorbance at 280 nm. The peak fractions (fractions 43 and 44 in Fig. 3*A*) were pooled and contained NSF purified 1000-fold relative to cytosol, with an overall yield of 12% in activity. It is very likely that this degree of purification is an underestimate due to inactivation of NSF during cation-exchange steps (steps 4 and 6). About 140 μg of NSF were obtained from 120 ml of CHO cytosol (600 mg of protein). Based on the yields in the purification, we estimate that NSF accounts for between 0.02% and 0.2% of the cytosolic protein (the exact number depending on the extent of inactivation during purification).

NSF Is Most Likely a Tetramer of 76-kDa Polypeptide Chains. A single polypeptide of ≈76 kDa coelutes with NSF activity from the FPLC Mono S column (Fig. 3*B*). The 76-kDa polypeptide precisely cosedimented with NSF activity at 12 S in analytical glycerol velocity gradients (data not shown). Based on the elution volume of NSF activity relative to a standard series of markers during FPLC gel filtration with Superose 12, we calculated the Stoke's radius of NSF to be 63 Å (data not shown). Combining the Stoke's radius with the sedimentation constant, we estimate that the native molecular mass of NSF is 307 kDa. Thus, it would appear that NSF is a tetramer of 76-kDa polypeptide chains.

The excellent correlation of NSF activity with the presence of the 76-kDa subunit provides strong evidence that this polypeptide chain is indeed NSF. The activity of the purified NSF fraction is completely eliminated by treatment with 1 mM NEM at 0°C, and under these same conditions the 76-kDa polypeptide is labeled with [^{14}C]NEM (data not shown). The activity of purified NSF (data not shown) is stimulated by long-chain acyl-CoA in a fashion similar to that of crude NSF (2).

Monoclonal Antibodies to NSF. To establish definitively that the 76-kDa polypeptide chain is the active subunit of NSF, we prepared murine monoclonal antibodies against purified NSF. An IgM (from hybridoma clone 4A6) was obtained that reacted with purified NSF in an RIA but not in immunoblots. We could confirm that the IgM antibody was directed at the 76-kDa polypeptide (putatively NSF) by immunopurification of this band from cytosol under native conditions (Fig. 4). The 76-kDa chain was bound by the IgM 4A6 (lane 4) but not the control IgM (lane 3). This 76-kDa antigen and purified NSF had identical partial proteolysis (17) maps (data not shown). We conclude that the IgM 4A6 is directed against the major 76-kDa chain in the NSF preparation.

The anti-76-kDa chain IgM strongly inhibited the transport reaction, whereas the control IgM did not (Fig. 5). This inhibition could be overcome by purified NSF. Adding 0.25 μg of purified NSF led to a requirement for an additional 0.8 μg of IgM 4A6 for 50% inhibition (Fig. 5), corresponding to ≈0.96 mol of IgM pentamer per mol of NSF tetramer.

We conclude that the 76-kDa chain contains NSF activity and that an IgM directed against this polypeptide inhibits the ability of NSF to promote transport.

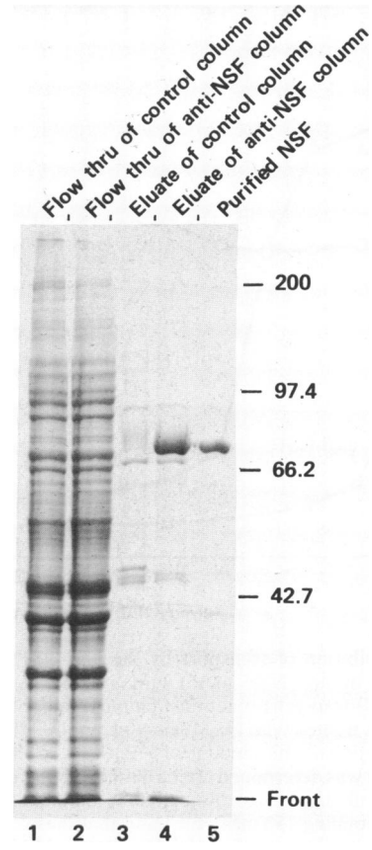

FIG. 4. Immunopurification of NSF. A total of 250 μl of IgM antibodies (4 mg/ml in 100 mM Hepes, pH 7.5/100 mM KCl) was coupled to 250 μl of Affi-Gel 10 (washed in water) by incubating for 16 hr at 4°C and then quenching with 0.2 M ethanolamine·HCl (pH 8) (1 hr at 4°C). The gels containing 3 mg of IgM per ml were washed before use with 20 vol of column buffer containing 10% (vol/vol) glycerol, 20 mM Hepes (pH 7), 100 mM KCl, 2 mM $MgCl_2$, and 0.5 mM ATP and then with 10 vol of 0.2 M glycine hydrochloride (pH 2) and then re-equilibrated in the column buffer at pH 7. Two IgM antibodies were employed, 4A6 that is directed against NSF and 1C2 that is a control IgM purified from ascites fluid and directed against an unrelated cytosolic antigen (provided by V. Malhotra and M. Paquet of this laboratory). Each resin (250 μl) was incubated with 2 ml of CHO cytosol (5 mg/ml, prepared with ATP and KCl) for 2 hr at 4°C with gentle stirring. Then, the suspension was poured into a small column and washed with 8 ml of column buffer. Specifically bound proteins were then eluted with 2 ml of 0.2 M glycine hydrochloride plus 10% (vol/vol) glycerol (pH 2). Samples of the unbound proteins (flow-through of the column) and specifically bound proteins (the pH 2 eluate) were then analyzed by SDS/gel electrophoresis (10% gel, stained with Coomassie blue). Samples of equivalent volume were electrophoresed for the control and anti-NSF columns. Samples were precipitated with 10% (wt/vol) trichloroacetic acid before electrophoresis. Lanes: 1 and 2, flow-through fractions of control (1C2) and anti-NSF (4A6) antibody columns, respectively; 3 and 4, proteins eluted at pH 2 from control and anti-NSF columns, respectively; 5, purified NSF (1.5 μg). About 3 μg of NSF was immunopurified from 2 ml of CHO cytosol by this procedure. Molecular mass markers (kDa) electrophoresed in a parallel lane consisted of myosin (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), and ovalbumin (42.7 kDa).

DISCUSSION

Vesicular transport requires numerous protein components acting in concert. The task of purifying transport components is greatly facilitated when one component can be eliminated at a time, transforming the overall reaction into an assay specific for the single missing component. Such a condition is created after selective inactivation with NEM. This ma-

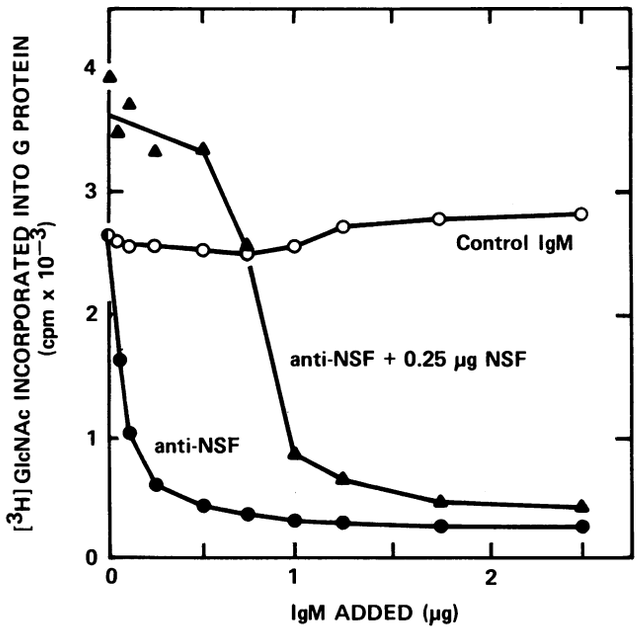

FIG. 5. Inhibition of transport by the anti-NSF IgM antibody. Control (1C2) or anti-NSF (4A6) IgM antibodies were added to standard (50 μl) transport reactions (employing donor and acceptor membranes not treated with NEM, cytosol, and ATP) at the indicated final concentrations. The amount of [^{3}H]GlcNAc incorporated into VSV-G protein was determined after a 60-min incubation at 37°C, after a 15-min preincubation on ice. Note that the antibodies were dissolved in a buffer containing 150 mM NaCl and that these transport assays had final concentrations of 15 mM KCl and 40 mM NaCl. These KCl and NaCl concentrations were maintained independent of the amount of antibody added, because it was found that higher salt greatly reduced nonspecific inhibition by control IgMs (presumably by preventing their precipitation at low salt during the incubation). ○, Control antibody; ●, anti-NSF antibody; ▲, anti-NSF antibody added to incubation mixtures that also contained 0.25 μg of purified NSF.

neuver enables the selective measurement of the activity of NSF in even the crudest fractions and has allowed the purification of a component of the transport machinery according to its function.

Native NSF is a homo-oligomer of 76-kDa polypeptide chains, almost certainly a tetramer. Other subunits do not seem to be present because there are no other polypeptide chains even close to a stoichiometric amount with the 76-kDa chain in NSF preparations or when NSF is immunopurified from crude cytosol under native conditions (Fig. 4). However, a very small polypeptide chain might have escaped notice. It remains a formal possibility that there is heterogeneity among the 76-kDa subunits. However, partial N-terminal amino acid sequence analysis of cyanogen bromide peptides (accounting for a total of 171 residues—20–25% of the polypeptide chain; data not shown) offers no evidence of heterogeneity. Also, none of these fragments have significant homology with any known protein sequences in the National Biomedical Research Foundation Databank (April 1988) including the *hsp70* gene family. Thus, it appears that the NSF polypeptide is a functional unit that has not been described to our knowledge.

NSF was purified on the basis of its ability to restore transport between the cis and medial (16) Golgi compartments in which the transport-coupled incorporation of [^{3}H]GlcNAc is measured (8) and partially purified NSF restores transport in a transport-coupled sialylation assay (3) that measures movement of the VSV-G protein into the trans-Golgi compartment. The 4A6 anti-NSF antibody also inhibits this latter movement (data not shown), which would suggest that NSF acts at multiple levels within the Golgi stack.

A simple calculation suggests that NSF acts catalytically rather than stoichiometrically in promoting transport and is thus recycled. About 0.2 pmol of VSV-G protein is transported into the medial Golgi compartment (15). A typical vesicle (70 nm in diameter) will contain an average of about 10 VSV-G protein molecules, based on available data (12, 18). Therefore, the transport of 0.2 pmol of VSV-G protein from cis to medial Golgi compartments will require 1.5×10^{10} vesicles. The total number of vesicles formed (at all levels of the stack) will clearly be several times greater. Saturating levels of transport are observed when 2.6 ng of pure NSF is added to standard (50 μl) transport assay mixtures (data not shown); this corresponds to 5×10^9 NSF tetramers, assuming all tetramers are active. In summary, one tetramer added to the assay mixture is sufficient to metabolize at least three transport vesicles en route from cis to medial Golgi compartments. This is undoubtedly a gross underestimate, because the extensive loss of activity during the purification implies that only a small fraction of the purified NSF is actually functional and because only a fraction of the NSF added is likely to serve in the cis medial step being considered.

We have described (19) a series of biochemical and morphological studies that reveal a role for NSF in catalyzing the fusion of transport vesicles with Golgi cisternae.

This paper is dedicated to Professor Arthur Kornberg on the occasion of his seventieth birthday. His fine example has greatly influenced the course of the laboratory's work over the years. We particularly thank Dr. Robert Arathoon for generously providing the large quantities of CHO cells needed to purify NSF. We also thank Dr. Fred Keeley for the NSF amino acid composition, Dr. William Henzel for the microsequence data, Lyne Paquet for expert technical assistance, and Connie Lopez-Schneider for preparation of the manuscript. This work was supported by a National Institutes of Health grant (DK 27044). M.R.B. was a Senior Fellow of the American Cancer Society (California Branch), Grant 5-4-87, and was supported by Institut National de la Santé et de la Recherche Médicale throughout most of the course of this work.

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