

Identification of a 25-kD Protein from Yeast Cytosol That Operates in a Prefusion Step of Vesicular Transport Between Compartments of the Golgi

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Abstract. We have identified a 25-kD cytosolic yeast protein that mediates a late, prefusion step in transport of proteins between compartments of the Golgi apparatus. Activity was followed using the previously described cell free assay for protein transport between Golgi compartments as modified to detect late acting cytosolic factors (Wattenberg, B. W., and J. E. Rothman. 1986. *J. Biol. Chem.* 263:2208–2213). In the reaction mediated by this protein, transport vesicles that have become attached to the target membrane during a preincubation are processed in preparation for fusion. The ultimate fusion event does not require

the addition of cytosolic proteins (Balch, W. E., W. G. Dunphy, W. A. Braell, and J. E. Rothman. 1984. *Cell.* 39:525–536). Although isolated from yeast, this protein has activity when assayed with mammalian membranes. This protein has been enriched over 150-fold from yeast cytosol, albeit not to complete homogeneity. The identity of a 25-kD polypeptide as the active component was confirmed by raising monoclonal antibodies to it. These antibodies were found to specifically inhibit transport activity. Because this is a protein operating in prefusion, it has been abbreviated POP.

THE understanding of how proteins are transported between compartments of the cell has been greatly enhanced by the introduction of cell free systems that measure these processes in assays where individual components can be manipulated and isolated (Blobel and Dobberstein, 1975; Balch et al., 1984; Gruenberg and Howell, 1986; Braell, 1987; Diaz et al., 1988; Goda and Pfeffer, 1988; Baker et al., 1988; Ruohola et al., 1988; Davey et al., 1985; Beckers et al., 1987). One of the first assays to be constructed measuring vesicular transport was for the movement of a membrane glycoprotein, the vesicular stomatitis virus (VSV)¹ G protein, from a *cis* to a medial compartment of the Golgi (Balch et al., 1984). In this assay, transport is measured from a “donor” to an “acceptor” Golgi fraction. The donor consists of membranes derived from VSV-infected cells, using a glycosylation mutant deficient in the addition of a distal *N*-acetylglucosamine (GlcNAc). Acceptor is derived from uninfected wild-type cells. Donor therefore contains underglycosylated G protein and acceptor has the luminal enzyme that adds GlcNAc to that G protein when it is transported from donor to acceptor. This transport is therefore conveniently marked by the addition of GlcNAc to G protein, which is experimentally accomplished by the measurement of [³H]GlcNAc incorporation into G protein im-

munoprecipitates. This assay has been used for an extensive characterization of the transport mechanism.

Intercompartmental Golgi transport requires energy in the form of nucleotide triphosphates, physiological pH, and osmolarity, and a number of proteins, both soluble and membrane associated (Balch et al., 1984; Balch and Rothman, 1985; Wattenberg and Rothman, 1986; Block et al., 1988). One of these proteins, the NEM sensitive factor (NSF) has been isolated (Block et al., 1988) and its gene cloned (Wilson et al., 1989). The cloned gene was shown to have strong homology to the yeast gene *SEC18*, whose product was known by genetic criteria to be involved in ER to Golgi transport. The two gene products were found to have functional homology as well, and it has subsequently been shown that NSF participates in ER to Golgi transport in a mammalian system (Beckers et al., 1989) and also has a role in endocytic fusion (Diaz et al., 1989). This substantiates early speculation that the components and mechanisms of transport vesicle production, targeting, and fusion used in movement of proteins through the Golgi would be shared with other transport pathways.

Because donor and acceptor can be separately manipulated in this system, it has been possible to perform a kinetic analysis that has defined substeps in the transport reaction (Balch et al., 1984; Wattenberg et al., 1986). These appear to correspond to events in the formation, attachment, and fusion of transport vesicles. In one of these kinetically defined subreactions, transport vesicles attach to the acceptor membrane to form a prefusion intermediate termed the low

1. *Abbreviations used in this paper:* GlcNAc, *N*-acetylglucosamine; LCRI, low cytosol requiring intermediate; NEM, *N*-ethylmaleimide; NSF, NEM-sensitive factor; POP, protein operating in prefusion; SNAP, soluble NSF attachment protein; VSV, vesicular stomatitis virus.

cytosol requiring intermediate (LCRI) (Wattenberg et al., 1986). It was so named because the steps leading to the formation of the LCRI require much higher levels of cytosol than the subsequent steps that process the intermediate to fuse transport vesicles into the acceptor membrane. This phenomenon simply reflects that the factors required for the formation of the LCRI are less active in crude cytosol than those that are required in the ensuing reactions. A rather surprising result of the kinetic analysis of transport is that the process that leads to fusion after vesicle attachment is relatively slow. This is fortunate from an experimental standpoint as it allows the capture of a substantial portion of the transported G protein in the LCRI simply by a kinetic trap. The reactions that lead to the fusion of transport vesicles can then be studied separately from the reactions that form and target the vesicles.

Here we describe how we have used this procedure to detect, enrich for, and unambiguously identify a 25-kD protein that is apparently the only cytosol derived component required to complete the transport process after the formation of the LCRI. A preliminary characterization of this activity has been described using bovine brain cytosol as a starting material (Wattenberg and Rothman, 1986). The results presented here are for a protein from the yeast *Saccharomyces cerevisiae*. It has previously been shown that components in yeast cytosol can supply the cytosolic needs of mammalian Golgi (Dunphy et al., 1986). We have chosen to pursue the yeast protein to take advantage of the well developed yeast molecular genetics. We have termed this a protein operating in prefusion (POP).

Materials and Methods

Materials

Glycerol and ammonium sulfate were of highest available purity from Bethesda Research Laboratories (Gaithersburg, MD). DE-52 was from Whatman Inc. (Clifton, NJ). Creatine phosphokinase, Hepes, propyl-agarose and Sephadexes A-25 and G-75 were purchased from Sigma Chemical Co. (St. Louis, MO). YM-10 membranes were from Amicon Corp. (Danvers, MA). A prepacked Mono-Q HR 5/5 column was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). SDS-PAGE molecular weight standards were from Bio-Rad Laboratories (Rockville Center, NY). Proteins used as gel filtration standards were from Sigma Chemical Co. Yeast culture materials were from Difco Laboratories Inc. (Detroit, MI). UDP-(6-³H)GlcNAc (26.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA). ATP and UTP were purchased from Pharmacia Fine Chemicals. Ascites fluid containing a monoclonal antibody to VSV G protein (clone 8G5) (Lefrancois and Lyles, 1982) was a gift from Dr. Leo Lefrancois of the Upjohn Company (Kalamazoo, MI). Polyclonal rabbit anti-mouse serum was obtained from Cappel Laboratories (Malvern, PA). All other chemicals were of reagent grade and were purchased from commercial sources.

Cells and Culture

Wild-type CHO-K1 cells were from the laboratory of Dr. J. Rothman (Princeton University) and CHO mutant clone 15B (Gottlieb et al., 1975) was obtained from Dr. Stuart Kornfeld (Washington University, St. Louis, MO). Alternatively, lecl cells (Stanley et al., 1975), a line with a genetically identical lesion to 15B cells were used (American Type Tissue Culture Collection, Rockville, MD). Results from the two were indistinguishable. Cells were maintained in suspension (wild type) or monolayer culture (15B or lecl) in alpha-MEM containing 10% FCS. Stocks of VSV were grown in monolayer BHK cells as described by Balch et al. (1984a). The infection of 15B cells with VSV was done as described in that report as was the preparation of homogenates from wild type and VSV-infected 15B cells, the isolation of Golgi fractions from those homogenates, and the preparation of cytosol from either wild-type or mutant cells. The protein content of the

membrane fractions was generally between 0.5 and 1 mg/ml, and of cytosol was ~5 mg/ml.

The mouse myeloma fusion partner SP2/0 was obtained from Dr. Ann Berger of the Upjohn Company. Hybridoma lines were grown in DME/high glucose containing 4 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 20% FBS. For HAT selection, this medium was supplemented with 10 µg/ml hypoxanthine, 400 nM aminopterin, 16 µM thymidine. For large scale purification of monoclonal antibodies, clonal lines were grown to confluency in complete medium, then split 1:2 in DME containing 1% Nutridoma-SP (Boehringer, Indianapolis, IN), and allowed to grow for 2 d. mAb was then purified from culture supernatants by precipitation with 50% ammonium sulfate and subsequent dialysis against PBS.

The Assay for Cytosol Components Acting at a Late Stage in Intra-Golgi Transport

This assay is essentially the same as that previously reported (Wattenberg and Rothman, 1986). A preincubation mix is assembled containing (for each tube subsequently to be assayed) 5 µl of donor membranes, 5 µl of acceptor membranes, and 2.5 µl of cytosol in a 50-µl volume containing 25 mM Hepes, pH 7.5, 25 mM KCl, 2.5 mM Mg(C₂H₃O₂)₂, 50 µM ATP, 250 µM UTP, 2 mM creatine phosphate, and 7.3 U/ml creatine phosphokinase. This mix is incubated for 25 min at 37°C. This results in the formation of a prefusion complex termed the LCRI. Membranes were then pelleted at 12,000 g for 3 min through a cushion of 50 µl of 0.3 M sucrose in a 1.5-ml microfuge tube (~600 µl of preincubation/tube). Membranes were resuspended in a buffer containing 25 mM Hepes (pH 7.5), 15 mM NaCl, 1.5 mM Mg(C₂H₃O₂)₂, 50 µM ATP, 250 µM UTP, 2 mM creatine phosphate, 7.3 U/ml creatine phosphokinase, 0.25 M sucrose, and 2.5 µCi/ml (87 nM) UDP-(³H-GlcNAc). Cytosol fractions were added and incubations were continued for an additional 90 min. The assay was terminated by addition of 50 µl of detergent solution containing 50 mM Tris (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 1% Na-cholate. VSV G protein was precipitated by addition of 0.6 µl of monoclonal antibody 8G5 in ascites fluid, and 7 µl of rabbit anti-mouse serum. After incubation at 37°C for 15 min the immunoprecipitate was collected by retention on type HA, 0.45 µM filters (Millipore Continental Water Systems, Bedford, MA). After washing, filters were dried and counted using Ready-Safe (Beckman Instruments, Inc., Palo Alto, CA).

"Standard" Transport Assay for Soluble Components Required at All Steps of Transport

These assays were carried out essentially as previously described (Balch et al., 1984a). Briefly incubations containing 5 µl of donor and 5 µl of acceptor were incubated with cytosol in buffer of the same composition as that in the first stage of the assay for POP activity except that [³H]UDP-GlcNAc (2.5 µCi/ml) was included. Incubations were terminated and immunoprecipitated after 60 min of incubation at 37°C.

Preparation of Yeast Cytosol

The yeast used for these preparations is UC7558 (a, leu2, pep4::URA3, ura3, gal2, bar1, suc2), from the Upjohn collection. The important features of this strain are that it is deleted for Pep4, a vacuolar protease that activates other proteases, and Suc2, invertase, which is inhibitory in this assay because of its production of hexoses from sucrose contained in the added membrane fractions. This has been shown to deplete the ATP required for transport (Dunphy et al., 1986).

For small scale preparations, 1.5 liters of yeast were grown in YEPD medium (2% bacto-yeast extract, 1% bacto-peptone, 2% glucose) and harvested in late log phase. The cells were pelleted by a 10-min centrifugation at 6,000 rpm in a rotor (GSA; Sorvall, Wilmington, DE) for 10 min, resuspended in 10 mM KPO₄ pH 7.0, and recentrifuged. This was repeated once, the resulting pellets were weighed, and then resuspended in lysis buffer (100 mM Hepes, 500 mM KCl, 5 mM MgCl₂, 1 µM pepstatin, 1 mM PMSF, 1 mM nabetabisulfite, 10 µg/ml leupeptin, 0.5 mM 1,10 phenanthroline, pH 7.0) at 2 ml of buffer/g of pellet. The resuspended pellet was lysed in a Bead Beater (Biospec Products, Bartlesville, OK) using equal volumes of yeast suspension and 0.5-mm glass beads with six 20 s bursts interspersed by 30-s cooling periods. All operations were performed at 4°C. The lysate was cleared with a low speed centrifugation (26,900 g in an SS34 rotor, 15 min, Sorvall) and a high speed centrifugation (201,800 g, type 60 rotor, 60 min, Beckman Instruments, Inc.). The cleared lysate was then dialyzed overnight into column buffer (25 mM Hepes, 1 mM MgCl₂, pH 7.0).

An inhibitor of the transport assay was removed by gel filtration over a Sephadex G-25 column equilibrated with column buffer. The G-25 column was always at least 10-fold greater volume than the load of yeast extract. The resulting desalted extract is termed "yeast cytosol."

For large scale preparations, 250 liter of yeast were grown in YEPD to late log or early stationary phase. Densities at harvest were typically 8–14 OD units. The cell pellet was collected in a centrifuge (Sharples-Stokes Div., Warminster, PA), resuspended in ~100 liters of 10 mM KPi (pH 7.0; 4°C) and repelleted. This pellet was frozen overnight at -20°C. Pellets typically weighed 2–4 kg. The pellet was thawed by agitation in a twofold (vol/wt) volume of cold lysis buffer. The suspension was lysed by passage through a glass bead homogenizer (Dynamil) at a rate of 6 liters/h. The lysate was centrifuged at 5,000 g in a H4000 rotor (Sorvall) for 30 min. The resulting cloudy supernatant was concentrated approximately fourfold and buffer was exchanged to column buffer using an Amicon Corp. (Danvers, MA) DC10L hollow fiber filter (molecular weight cut off, 10,000). This lysate was stored overnight at 4°C and then centrifuged for 1 h at 235,000 g in a 45Ti rotor (Beckman Instruments Inc., Palo Alto, CA), yielding a somewhat cloudy, reddish supernatant.

Removal of a Transport Inhibitor by A-25 Chromatography

3.2 liters of cleared lysate were applied to a 10-cm by 40-cm column of Sephadex A-25 equilibrated with column buffer at a rate of 4.8 liters/h. The column was eluted with column buffer at the same rate, and the major protein peak was collected and pooled in a total volume of 4 liters. This material is referred to as yeast cytosol.

Chromatographic Enrichment of POP

DEAE Cellulose Chromatography of Yeast Cytosol. 4 liters of yeast cytosol were applied to a 10-cm × 20-cm column of DE-52 (equilibrated with column buffer), washed with 8 liters of column buffer and eluted with a 0–400 mM NaCl gradient (in column buffer) in a total gradient volume of 16 liters. Flow rate was 800 ml/h and fractions were 600 ml.

Propyl-agarose Chromatography. Active fractions from DE-52 chromatography were pooled. The pool was then dialyzed extensively against 2.6 M ammonium sulfate/20% glycerol (vol/vol) in 25 mM Hepes/1 mM EDTA, pH 7.0. An inactive protein precipitate was removed by centrifugation (30 min at 70,000 g). A propyl-agarose column of 5 cm by 20 cm was equilibrated with the dialysis buffer and half of the pooled fractions were loaded. The column was washed with 100 ml of dialysis buffer and then eluted with 2.6–1.4 M ammonium sulfate gradient in column buffer containing 20% (vol/vol) with glycerol in a gradient volume of 4 liters. Flow rate was 400 ml/h and fractions were 50 ml. A portion of each fraction was extensively dialyzed against column buffer before each assay utilizing a multichannel dialyzing apparatus (Bethesda Research Laboratories).

Gel Filtration Chromatography. The active pool from hydrophobic chromatography was dialyzed against column buffer containing 200 mM NaCl and 20% glycerol. This pool was divided in half. Each half was concentrated ~15-fold on a YM-10 membrane (Amicon Corp.) and then was separately loaded onto a 5-cm × 88-cm column of Sephadex G-75 (medium) equilibrated with the same buffer. The load volume was ~45 ml. The load and elution rate was 100 ml/h. Fractions were 35 ml. The column was calibrated with blue dextran (V_0), ovalbumin (44 kD), superoxide dismutase (35 kD), and cytochrome C (12.5 kD).

Mono-Q Ion-Exchange Chromatography. Active fractions from gel filtration chromatography were pooled and dialyzed extensively against 25 mM Tris/1 mM MgCl₂, 15% glycerol (vol/vol), pH 8.0. 5.4 ml of this pool was loaded onto a Mono-Q column (HR 5/5) at a rate of 0.4 ml/min. This was washed with the dialysis buffer (3 ml) and eluted with a gradient of 0–100 mM NaCl in the dialysis buffer. Total gradient volume was 42 ml, elution rate was 0.4 ml/min, and fraction size was 1 ml.

Gel Electrophoresis, Electrophoretic Transfer, Immunoblotting, and Purification of p25 from Polyacrylamide Gels

PAGE was carried out essentially according to Laemmli (1970) where the running gel contained 15% acrylamide, using the mini-protean gel system (Bio-Rad Laboratories).

Western blotting. Proteins separated by SDS-PAGE were electroblotted to nitrocellulose membranes at 100 V for 60 min in a buffer containing

14.4 g glycine, 3 g Tris base, and 200 ml methanol/liter. Membranes were blocked by incubation in PBS, containing 0.05% Tween-20 (PBS + T) with 5% nonfat dry milk for at least 60 min. Hybridoma supernatants or purified IgG fractions were diluted in PBS + T and incubated with blocked membranes for at least 60 min. Membranes were then washed three times with PBS + T and a 1:1,000 dilution of peroxidase-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in PBS + T was added. After a 60-min incubation, membranes were washed three times with PBS + T and once with PBS. Peroxidase activity was detected by addition of the substrate 4-chloro-1-naphthol.

Gel Purification of p25. Mono-Q fractions containing p25 as ~10% of the total protein were run on 15% SDS-PAGE. After electrophoresis, the gel was briefly stained with Coomassie G-250 (Holbrook and Leaver, 1976) until the band pattern was apparent. The band migrating at 25 kD was excised with a razor blade and minced into 1-mm² pieces. Protein was electroeluted in 50 mM ammonium bicarbonate at 8 mA for 4 h using an electroelution device (Mini Protean II; Bio-Rad Laboratories) as per manufacturer's recommendations. The eluant was concentrated by lyophilization. Protein purity and recovery were estimated by rerunning on SDS-PAGE and staining with Coomassie blue R-250.

Determination of p25 Content in Crude Fractions by Densitometry of Immunoblots

The amount of P25 in a Mono-Q fraction was estimated by densitometric scanning of a Coomassie blue-stained SDS acrylamide gel using a CS-930 dual wave length scanning densitometer (Shimadzu Scientific Instruments Inc., Kyoto, Japan). Knowing the protein concentration of the Mono-Q fraction, an estimate of p25 concentration could be made. Mono-Q fractions containing a known amount of p25 were then blotted to nitrocellulose and reacted with POPI antibody as described. Staining intensity was determined by densitometry and a standard curve prepared. Similar blots of crude DE-52 fractions of known protein concentration allowed estimation of p25 content of these fractions.

Production of Monoclonal Antibodies against p25

Mouse Immunization and Fusion. Two female CFA-1 mice were injected subcutaneously with a Mono-Q fraction similar to that shown in Fig. 2, containing 50 µg of protein (5–10 µg of p25) in complete Freund's adjuvant. Mice were boosted three times at 3-wk intervals with similar 50-µg subcutaneous injections in incomplete Freund's adjuvant. 2 d before the fusion mice were injected intravenously with 5 µg of pure p25, as isolated from SDS-PAGE. One mouse was killed, the spleen removed and minced in PBS. Spleen cells were isolated and fused to the mouse myeloma fusion partner SP2/0 using PEG. Hybridomas were selected by growth in DME medium containing HAT. Lines secreting antibodies to p25 were detected by ELISA and were cloned through two rounds of limiting dilution.

ELISA. ELISA plates were prepared by adding 50 µl of carbonate buffer, pH 9.6, containing 3 ng of gel purified p25 to each well of a 96-well half area ELISA plate (Costar, Cambridge, MA) and incubating for 24 h at 4°C. Unadsorbed antigen was removed and plates washed three times with PBS + T. Nonspecific binding sites were blocked by addition of 100 µl of PBS + T containing 0.1% gelatin (Sigma Chemical Co.) for 60 minutes. Wells were washed three times with PBS + T and 50 µl of hybridoma supernatant was added. After incubation at room temperature for 60 min, wells were washed three times with PBS + T. 50 µl of a 1:1,000 dilution of HRP-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories) in PBS + T was added to each well and incubated for 60 min at room temperature. Wells were again washed four times with PBS + T, and the peroxidase activity was detected using the substrate 2,2'-azino-di(3-ethylbenzthiazoline sulfonate). Reactions were stopped by adding SDS to 2.5% and the color read at 405 nm using a multi-scan plate reader (Flow Laboratories, Inc., VA).

Results

Initial studies have shown that there is a specific proteinaceous factor in mammalian cytosol that drives a late step in the intercompartmental transport of protein through the Golgi (Wattenberg and Rothman, 1986), although this factor was not identified. In this late step, a prefusion complex consisting of transport vesicles attached to the acceptor Golgi compartment is acted on to fuse the transport vesicles into

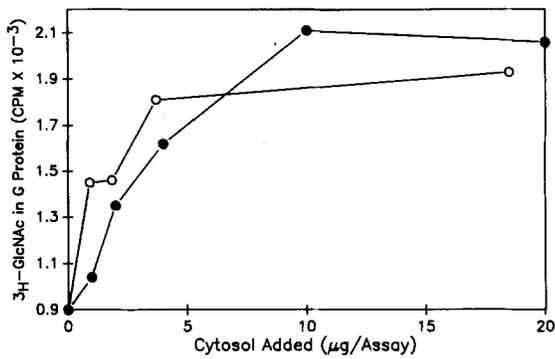


Figure 1. Activity of yeast and CHO cytosols in the assay for late acting transport factors. Yeast (closed circles) and CHO cell (open circles) cytosols were prepared as described in Materials and Methods, and activity was titered in the assay for late acting factors.

the acceptor. Recently yeast cytosol was found to supply all of the required soluble factors for transport to proceed (Dunphy et al., 1986), presumably including POP. We have pursued the purification and identification of POP in yeast to take advantage of the powerful tools of molecular genetics available in this organism.

POP activity is measured in a two step assay. In the first incubation VSV G protein is driven into the prefusion intermediate (called the LCRI) by the action of an excess of unfractionated mammalian cytosol for 25 min at 37°C. Because

the ensuing steps are slow, most G protein is kinetically trapped in the LCRI after 25 min of incubation (Wattenberg et al., 1986). The membranes from this incubation are then pelleted through a sucrose cushion to remove the unfractionated cytosol and resuspended with buffer, an ATP regenerating system, [^3H]UDP-GlcNAc, and the cytosol fraction to be tested for POP activity. This mixture is then further incubated for 90 min before immunoprecipitation of G protein and the determination of [^3H]GlcNAc incorporation into the immunoprecipitates.

A comparison of the POP activity in cytosols derived from CHO levels and yeast is shown in Fig. 1. As expected, yeast cytosol did indeed contain POP activity. Surprisingly, yeast and CHO cytosols had comparable levels of activity. When tested in the standard assay, where it must provide the soluble factors needed for early steps of transport as well as POP, yeast cytosol is only 10–20% as effective as mammalian cytosol (Dunphy et al., 1986).

Yeast cytosol was fractionated through ion exchange, hydrophobic, gel filtration, and high performance ion-exchange chromatographies (Fig. 2). The elution position of yeast POP activity on DEAE-cellulose, between 30 and 50 mM salt, was similar to that previously reported for POP activity from calf brain cytosol. In addition gel permeation chromatography revealed that POP behaved as a protein of approximately 25 kD, also the same as that shown previously for the bovine form. A summary of this enrichment procedure when performed on a large scale is presented in Table I. In general,

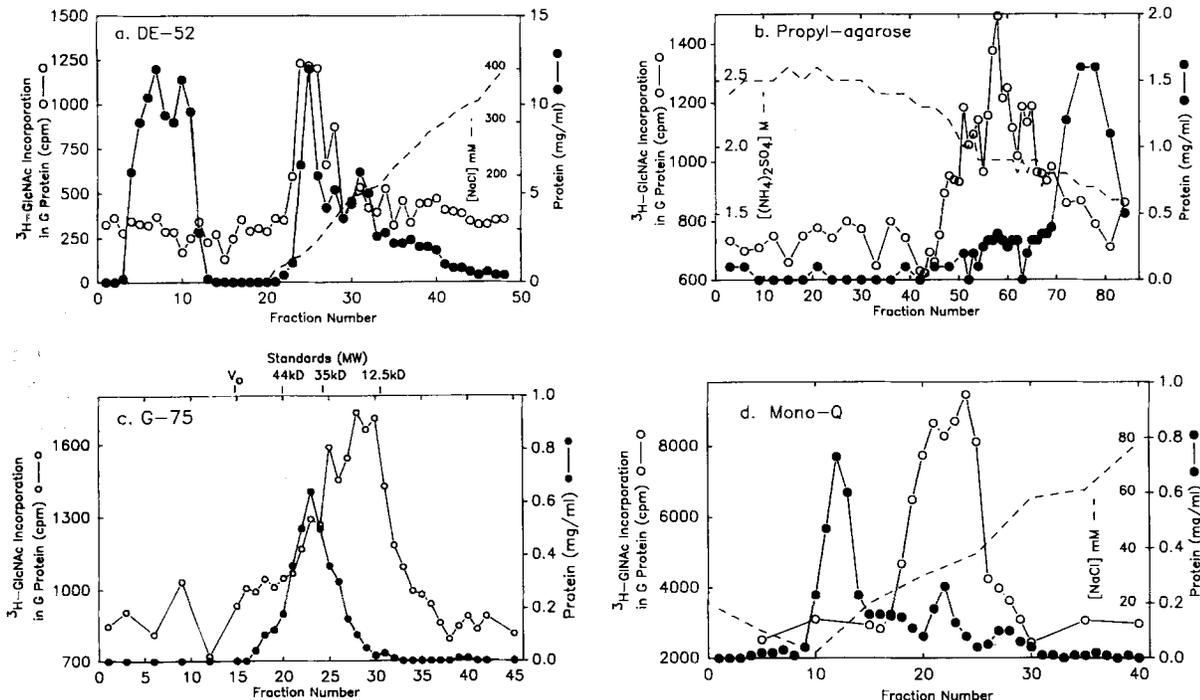


Figure 2. Enrichment of POP through ion exchange, hydrophobic, and gel permeation chromatographies. Details of the chromatography are described in Materials and Methods. In each panel are shown activity in the assay for late acting factors (open circles), protein concentration (filled circles), and salt concentration when applicable (dashed lines). *a*, Ion exchange chromatography on DEAE-cellulose. Yeast cytosol was fractionated on DE-52. *b*, Hydrophobic chromatography on propyl-agarose. Active DE-52 fractions (fractions 24–26) were dialyzed in 1.8 M ammonium sulfate, loaded, and chromatographed on propyl-agarose. *c*, Gel permeation chromatography on Sephadex G-75. Propyl-agarose fractions 48–68 were dialyzed and loaded onto Sephadex G-75. Molecular mass standards from calibration runs are indicated at the top of the panel. *d*, Ion exchange chromatography on Mono-Q. G-75 fractions 26–32 were dialyzed and applied to Mono-Q as described.

Table I. Summary of the Chromatographic Enrichment of POP

Pool	Volume (ml)	Total protein (mg)	Total activity (cpm)	Recovery of activity (percentage of A-25)	Specific activity (cpm/ μ g, fold enrichment over A-25)
A25	4,000	1.5×10^5	3.9×10^9	100	2.7×10^4 (1)
DE52	1,600	1.5×10^4	1.4×10^9	36	9.7×10^4 (3.6)
Propylagarose	182	1310	4.1×10^8	10	3.1×10^5 (11.5)
G-75	103	35.0	6.2×10^7	1.6	1.8×10^6 (67)
Mono-Q	23	2.1	9.7×10^6	.25	4.7×10^6 (174)

Shown are the results for pooled fractions resulting from the chromatography steps illustrated in Fig. 2. After the DE-52 step several identical smaller pools were generated and subject to parallel purifications as described in Materials and Methods. To estimate the figures shown above, the pool of one representative run for each chromatographic step was chosen, and the resulting numbers were multiplied by the number of runs required to completely process the material from the preceding step. Frozen pools were titered in a single assay. These figures also reflect losses due to preparative steps for each column such as dialysis and concentration.

the recovery of activity was quite variable. In pilot studies almost quantitative recovery of activity could at times be demonstrated at each chromatographic step; however, this was not consistent. The reasons for the variable loss of activity are not clear. Because recovery of activity was limited, we regard the enrichment of 170-fold over the starting material to be a lower limit for the actual purification of POP, and consider it likely that a considerable portion of the purified protein in these fractions is inactive.

When an active pool from the purification procedure was re-run over Mono-Q, a further purification was found (Fig. 3). When the resulting fractions were analyzed by PAGE (Fig. 3, *b*) a polypeptide of 25 kD (p25) was found to coelute with activity (Fig. 3, *a*). The band at 20 kD corresponds to copper/zinc superoxide dismutase as judged by enzymatic activity and Western blotting (data not shown). The identity of the bands of 15 and 30 kD are unknown.

To confirm that p25 was responsible for POP activity, we developed cell lines producing monoclonal antibodies to the protein. Mice were immunized with Mono-Q column fractions in which p25 constituted from 5–10% of total protein (see Materials and Methods). To screen for the desired hybridomas p25 was purified in a denatured form from SDS polyacrylamide gels. This antigen was then used in ELISA assays. Five stable lines have been established which produce antibodies reactive against p25. All five lines produce antibodies of the subclass IgG1. One of these, POP1, is further characterized below.

POP1 was used to probe for p25 in immunoblots. Shown in Fig. 4 is a Western blot using POP1 to stain a Mono-Q fraction (lane 5), a relatively crude yeast fraction from DE-52 chromatography of yeast cytosol (lane 6), and cytosols derived from bovine brain and CHO cells (lanes 7 and 8). POP1 strongly and specifically recognizes a protein of 25 kD in both crude and purified yeast fractions. No other proteins are bound by this antibody in the yeast fractions. There is no reaction against either mammalian cytosol. When p25 was measured in cytosol and Mono-Q fractions by quantitating immunoblots densitometrically (see Materials and Methods), it was determined that p25 constitutes \sim .008% of the protein in the DE-52 fraction.

The correlation between POP activity and p25 was tested by preincubating cytosol fractions with antibody and testing for transport activity. A relatively crude cytosol fraction, the

pool resulting from DE-52 chromatography, was treated with either control IgG1 or POP1 antibodies for 4 h at 37°C. The activity of these fractions, as well as a fraction without any added antibody, is shown in Fig. 5 *A*. The 37°C incubation itself only slightly decreased the activity of this fraction (data not shown). Preincubation of the fraction with POP1 resulted in a profound inhibition of activity whereas the control antibody had only a slight effect. The level of inhibition by POP1 was \sim 80% when compared to the control antibody. The remaining activity plateaued at low levels of added cytosol, and did not increase with additional added protein. The reason for this behavior is not clear. Inhibition of activity occurred with 4°C pretreatment of cytosol fractions with antibody; however, the inhibition was not as complete (data not shown.)

POP1 only very weakly immunoprecipitates p25 (data not shown). This is not uncommon for monoclonal antibodies and presumably accounts for the relatively high amounts of antibody required to inactivate POP activity. To rule out the possibility that the observed inhibition was because of a non-specific effect of this particular antibody preparation on the cytosol fraction or on the assay itself, the antibodies were preincubated with cytosol from CHO cells. The immunoblot depicted in Fig. 4 indicates that POP1 will not react with mammalian POP. This preincubation had no effect on the POP activity of CHO cytosol (Fig. 5 *b*.) Therefore, the inhibition we observed by POP1 on yeast cytosol is a specific effect of the interaction of antibody with p25.

Although there is considerable indirect evidence that the LCRI is a transport intermediate, this has not been directly shown. To substantiate that POP participates in the transport reaction as a whole, antibody-treated cytosol fractions were tested in the standard transport assay, which requires the components involved in early as well as late substeps (Fig. 6). In contrast to ion-exchange fractionations of mammalian cytosol (Wattenberg and Rothman, 1986), the DE-52 pool from yeast cytosol contains all required transport factors. When this fraction is treated with POP1, the standard activity is clearly reduced, although not as strongly as activity measured in the assay for POP activity (Fig. 5). This was not unexpected as the standard activity of yeast cytosol is low when compared to CHO cytosol (Dunphy et al., 1986, and Fig. 6). Since POP activity of yeast and CHO cytosols are similar (Fig. 1), the ratio of the activity of factors required

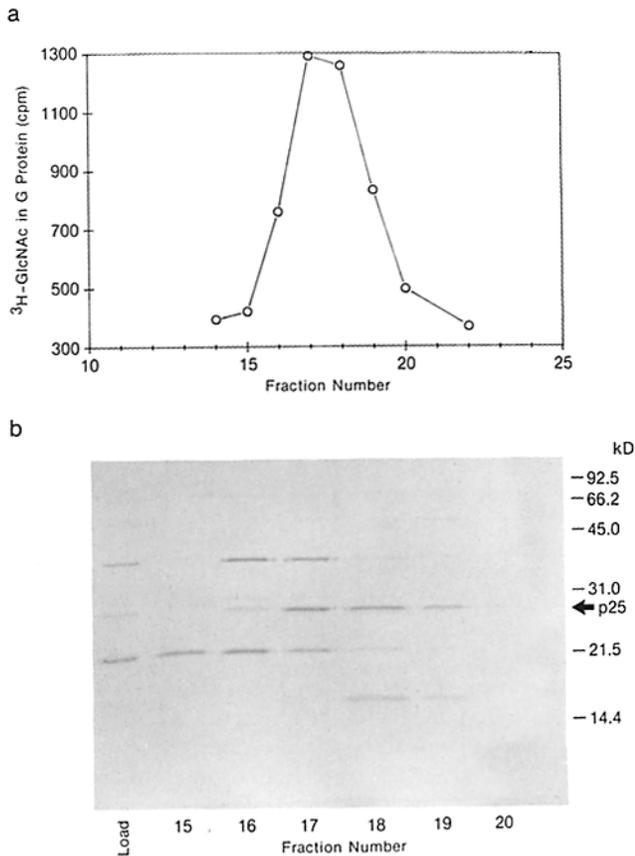


Figure 3. A 25-kD protein co-purifies with POP activity on rechromatography of Mono-Q fractions. Active fractions from a Mono-Q fractionation (66 μ g in 3 ml) similar to that shown in Fig. 2, *d*, were pooled and dialyzed into 25 mM Tris/1 mM EDTA/15% glycerol (pH 8.0), and then rechromatographed on Mono-Q under the same conditions as originally used. Protein content was too low to be accurately measured. *a*, Transport activity. *b*, PAGE (15% acylamide) of fractions from Mono-Q fractionation. Shown are the fraction loaded onto the column (*Load*, 10 μ l), and samples of fractions from the column (25 μ l/lane) stained with Coomassie blue. At right are indicated the migration of molecular mass standards. The position of p25 is indicated by an arrowhead.

in early steps to POP activity is low when assayed in the heterologous mammalian system. Therefore, only a minor amount of POP activity is required to complement the limited levels of early factors.

Discussion

We have identified a 25-kD yeast protein (POP) that acts in a late prefusion step of vesicular transport between Golgi compartments. The yeast activity was assayed in a heterologous system using Golgi membranes prepared from CHO cells. Although we do not as yet have a homogeneous preparation of this protein, monoclonal antibody technology allowed us to make an unambiguous identification of the protein responsible for POP activity. The antibody (designated POP1) recognizes only a single 25-kD band on immunoblots of yeast cytosol fractions. It inhibited the late activity of yeast cytosol fractions when preincubated with them, but did not inhibit the activity of CHO cytosol, in which there are

no immunoreactive proteins. This substantiates POP1 is inhibiting by a specific interaction with the active component.

A preliminary characterization of POP activity from bovine brain cytosol (Wattenberg and Rothman, 1986) demonstrated that the mammalian protein elutes from DEAE-cellulose at \sim 30–50 mM salt and sizes as a globular protein of 25 kD. These characteristics are very closely matched by yeast POP. This physical similarity should not be surprising as the two sources yield functionally homologous activities. However, this homology is not so exact that there is a strong cross reaction with the monoclonal antibody used here. The production of a polyclonal serum using pure POP may reveal more of the structural similarity between yeast and mammalian POP.

The variability in recovery of activity during the manipulation of yeast POP is of concern and interest. The intrinsic stability of the protein seems high. Loss of activity is minimal even with incubation at 37°C for several hours in crude fractions or for several days at 4°C in both crude and purified preparations (data not shown). One possibility is that there is a second protein component being supplied by the yeast cytosol which is required in the consumption of the LCRI. We consider this unlikely since each chromatographic step has resulted in quantitative recovery during pilot studies. However, until we can test the transport activity of homogeneous POP this explanation cannot be entirely excluded. Alternatively, there may be a small molecular weight cofactor bound to POP that is released if the protein is slightly denatured (for instance, by interaction with a chromatographic matrix).

The only other protein which has been isolated using the cell free transport assay is the NSF. This is an ATP binding protein that appears to cycle on and off the Golgi membranes (Glick and Rothman, 1987). The attachment of NSF to the

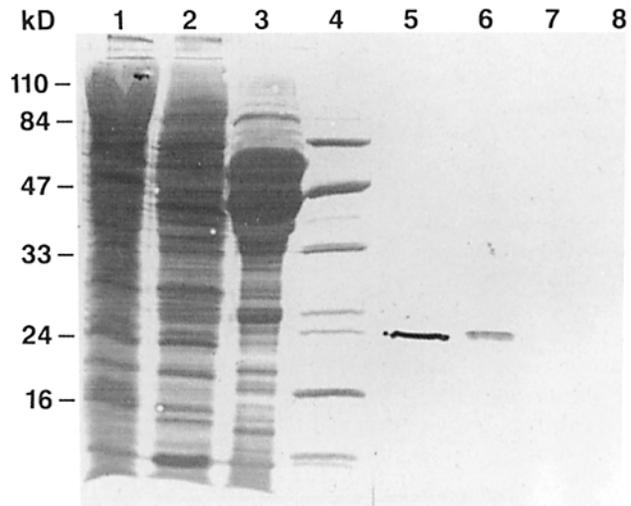


Figure 4. Characterization of anti-p25 monoclonal antibody POP1 by immunoblotting. Gel electrophoresis was carried out as described in Materials and Methods on CHO cell cytosol (lanes 1 and 8, 100 μ g), bovine brain cytosol (lanes 2 and 7, 100 μ g), an active pool from DE-52 chromatography of yeast cytosol (lanes 3 and 6, 70 μ g), and a p25 enriched Mono-Q fraction (lane 4, 5 μ g, and lane 5, 1 μ g). Lanes 1–4, protein staining with fast green. Lanes 5–8, immunoblotting with antibody POP1 using a 1:30 dilution of cell culture supernatant.

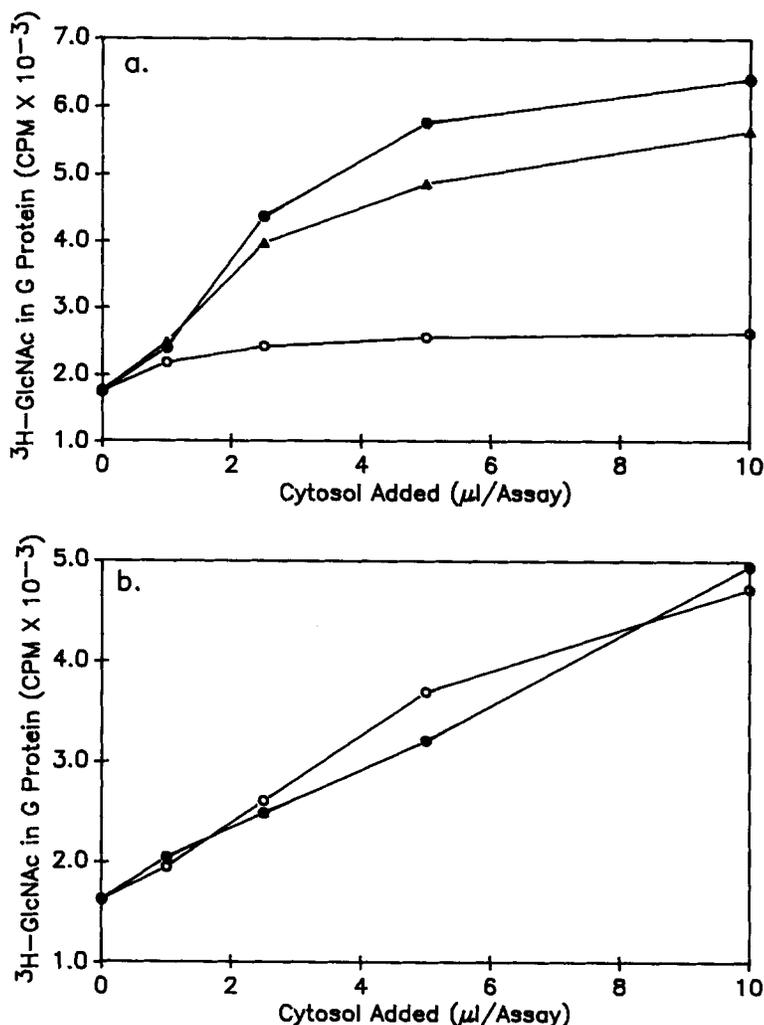


Figure 5. Monoclonal antibody POP1 inhibits transport activity of yeast but not CHO cytosol. To 50 μl of cytosol was added 7.5 μl (32 μg) of either POP1 or B-1 (a control IgG, monoclonal directed against IL-1 β), or 7.5 μl of PBS. The cytosol was then incubated for 4 h at 37°C, centrifuged for 3 min at 12,000 rpm, and the supernatant was titered for activity in the assay for late acting factors. In the absence of added antibody, both yeast and CHO cytosols were only slightly affected by the incubation at 37°C when compared to controls held on ice (not shown). *a*, Treatment of a pool resulting from DE-52 chromatography of yeast cytosol (9.2 mg/ml protein). PBS control (filled circles); anti-p25 POP1 (open circles); control antibody B-1 (closed triangles). *b*, Treatment of CHO cell cytosol (9 mg/ml protein). PBS control (filled circles); anti-p25 POP1 (open circles).

membrane is mediated by a soluble factor termed SNAP (soluble NSF binding protein; Weidman et al., 1989). Kinetic and morphological studies (Malhotra et al., 1988; Orci et al., 1989) indicate that NSF acts after coated transport vesicles attach to the acceptor membranes and lose their coat protein, but before the formation of the LCRI. Therefore, POP comes into play after the interaction of NSF with the acceptor membranes.

As the only cytosolic protein apparently required at the last stages of transport, it is possible that POP is itself a fusogen. However, previous studies have shown that the final fusion event proceeds in the absence of added cytosolic factors (Balch et al., 1984b). If POP is a fusogen, it must attach to the membrane and become activated before the fusion event itself occurs. Preliminary data on the action of POP indicates that it must be continuously present during the consumption of the LCRI and that it is not depleted from cytosol during the transport reaction (data not shown). So its action must be brief and it apparently can act catalytically. The amount of POP required to drive the reaction can be calculated from the abundance of POP in the DE-52 pool (0.008%), the protein content of that pool (9.2 mg/ml) and the amount of the pool that saturates the assay (5 μl in a 50 μl assay). This indicates that only 74 ng/ml, ~ 3 nmol, will sustain the reaction. This low level is also consistent with a catalytic action of the protein.

Rothman has suggested that POP is part of a transient fusion complex that includes NSF and SNAP (Wilson et al., 1989). The amount of POP required in the assay, as calculated above, is significantly below that of purified NSF (Weidman et al., 1989). However, this comparison is between purified NSF, which may be substantially reduced in activity, and POP in a crude fraction. The abundance of NSF has been calculated to be between 0.2 and 0.02% of mammalian cytosolic protein. This within an order of magnitude of the abundance of POP. There is as yet no experimental evidence for a direct interaction between NSF/SNAP and POP. However, this remains an intriguing possibility.

It is particularly interesting that the events that lead up to fusion after the attachment of the transport vesicles are so lengthy in this system. This stands in contrast to the fusion of secretory vesicles at the cell surface where exocytosis can be extremely rapid once the excitatory signal has been transduced (for review, see Tartakoff, 1987). To what extent the kinetics of transport have been altered in this in vitro system remains to be established. However, the Golgi system may have evolved to limit fusion to defined regions of the cisternae and/or to prevent fusion of vesicles that have been incorrectly targeted. In the Golgi it may be more of a problem to control fusion than to initiate it. In this context POP may act to direct transport vesicles to sites of fusion as previously suggested (Wattenberg and Rothman, 1986), or to unmask

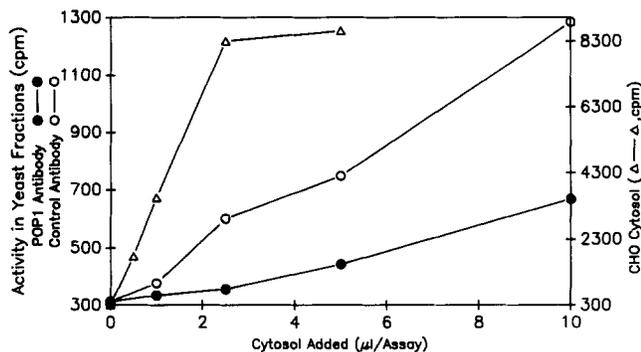


Figure 6. Yeast cytosol treated to inhibit POP activity is diminished in total transport activity. A yeast DE-52 pool was treated with either POP1 antibody or control antibody B-1 as described in the legend to Fig. 5. These cytosols were then titered in the standard assay which requires the activity of factors acting early in the transport assay as well as those acting at late steps. For comparison, the activity of CHO cytosol is also shown, note the large difference in activity between the CHO (open triangles, right ordinate) and yeast cytosols (left ordinate). Yeast DE-52 pool treated with control antibody B-1 (open circles); yeast DE-52 pool treated with POP1 (closed circles).

sites of fusion on the acceptor membrane in response to the arrival of transport vesicles that have passed a test of legitimacy.

The promise of a molecular analysis of vesicular transport in the cell is in the process of becoming realized. Now that POP has been identified and monoclonal antibodies have been produced against it we can begin a detailed analysis of how it acts to promote fusion, how it interacts with other known constituents, and what other components are involved in protein transport.

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