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Yeast and mammals utilize similar cytosolic components to drive protein transport through the Golgi complex

(vesicular stomatitis virus glycoprotein/cell-free system/oligosaccharide processing)

WILLIAM G. DUNPHY, SUZANNE R. PFEFFER, DOUGLAS O. CLARY, BINKS W. WATTENBERG, BENJAMIN S. GLICK, AND JAMES E. ROTHMAN

Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305

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ABSTRACT **Vesicular transport between successive compartments of the mammalian Golgi apparatus has recently been reconstituted in a cell-free system. In addition to ATP, transport requires both membrane-bound and cytosolic proteins. Here we report that the cytosol fraction from yeast will efficiently substitute for mammalian cytosol. Mammalian cytosol contains several distinct transport factors, which we have distinguished on the basis of gel filtration and ion-exchange chromatography. Yeast cytosol appears to contain the same collection of transport factors. Resolved cytosol factors from yeast and mammals complement each other in a synergistic manner. These findings suggest that the molecular mechanisms of intracellular protein transport have been conserved throughout evolution. Moreover, this hybrid cell-free system will enable the application of yeast genetics to the identification and isolation of cytosolic proteins that sustain intracellular protein transport.**

Eukaryotic cells establish and maintain the identities of subcellular organelles by means of a series of precisely programmed transfers of transport vesicles. The biochemical events by which these transport vesicles selectively extract desired proteins from one membrane-bounded compartment and then accurately deliver transported proteins only to the appropriate cellular destination are unknown. The first step in a mechanistic dissection of any biological process is to reconstitute the process in a cell-free system. With this functional assay, the required biochemical components can be defined. We have recently reported an *in vitro* system for the transport of protein between the successive cisternal compartments of the Golgi apparatus from mammalian cells (1, 2).

Transport within the mammalian Golgi apparatus is particularly amenable to study *in vitro*, since the oligosaccharide chains on exported glycoproteins undergo well-characterized covalent modifications in the successive compartments of the Golgi complex (3). These processing steps can serve as chemical indicators that intercompartmental transport has occurred. For example, the attachment of terminal *N*-acetylglucosamine (GlcNAc) to a glycoprotein such as the vesicular stomatitis virus (VSV) G protein occurs when it arrives in the *medial* Golgi compartment (2, 4). To measure this transport *in vitro*, we routinely incubate together two types of Golgi stacks. One type of stack is from a VSV-infected mutant line of Chinese hamster ovary (CHO) cells that lacks GlcNAc transferase I and consequently cannot add terminal GlcNAc (5); the other is from uninfected wild-type CHO cells. Transfer of the G protein to the wild-type Golgi stacks is detected by the addition of tritiated GlcNAc. Transport in the cell-free system requires both cytosolic and membrane-bound proteins.

The elucidation of sorting mechanisms will require identification of the proteins that facilitate vesicular transport. The cell-free system will enable purification of the responsible proteins. This task would be aided by modern molecular genetic methods. However, with the mammalian cell-free system, a complementary genetic approach is not feasible. To obviate this technical limitation, we have developed a hybrid yeast–mammalian transport system. Secretion has been studied extensively in yeast, principally by Schekman and colleagues (6–10), who have defined the secretory pathway in yeast with a large collection of secretory (*sec*) mutants that are conditionally defective in particular steps in intracellular protein transport. They have found that the overall organization of intracellular transport in yeast is the same as in animal cells. Here we report biochemical experiments which demonstrate that the entire complement of cytosolic transport factors from yeast will replace their mammalian counterparts in our cell-free system.

MATERIALS AND METHODS

Materials. Bio-Gel A-1.5m (100–200 mesh) was from Bio-Rad Laboratories. UDP-[^{3}H]GlcNAc (specific activity, 24 Ci/mmol; 1 Ci = 37 GBq) was synthesized according to Lang and Kornfeld (11). [2-^{3}H]ATP (specific activity, 20 Ci/mmol) was from Amersham. Sources of other materials were described previously (2).

Growth of Yeast Cells. We used the *Saccharomyces cerevisiae* strain X2180-1A (kindly provided by Ronald Davis, Stanford University). Yeast were grown in YPD medium (7) at 25°C to mid-logarithmic phase (OD_{600} = 2–4).

Preparation of Cytosol. Fresh calf brain was frozen in liquid N_2 at a local slaughterhouse. Thawed brain tissue was homogenized in 2 vol of homogenization buffer per g of tissue by eight strokes at 1500 rpm with a motor-driven Teflon pestle. The homogenization buffer contained 25 mM Hepes/KOH at pH 7.0, 0.5 M KCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium metabisulfite, 1 μM pepstatin, 0.5 mM 1,10-phenanthroline, and leupeptin at 10 μg/ml. For preparation of yeast cytosol, yeast cells (washed by centrifugation at 5000 × *g* in 10 mM potassium phosphate, pH 7.0) were suspended in 2 vol of homogenization buffer per g (wet weight) of yeast cells. The homogenization buffer was the same as for brain tissue except that the Hepes concentration was increased to 100 mM. Twenty milliliters of this suspension was mixed with 30 g of glass beads (0.5-mm diameter) and homogenized on ice in six 15-sec bursts (separated by 20-sec cooling intervals) with a Bead-Beater (Biospec Products).

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Abbreviation: VSV, vesicular stomatitis virus.

Brain or yeast homogenates were centrifuged at 15,000 rpm for 15 min in the JA-17 rotor (Beckman). The resulting supernatant was centrifuged for 60 min at 45,000 rpm in an SW 50.1 rotor (Beckman). The supernatant was dialyzed for 8 hr (with one change of buffer) against 100 vol of 25 mM Hepes/KOH at pH 7.0 containing 50 mM KCl, 1 mM $MgCl_2$, and 1 mM dithiothreitol (dialysis buffer). After dialysis, the cytosol was centrifuged again at 45,000 rpm for 60 min in the SW 50.1 rotor. Cytosol was routinely frozen in liquid N_2 and stored at −70°C. Prior to assay, yeast cytosol was additionally filtered through a Sephadex G-25 gel column (see ref. 2), equilibrated in dialysis buffer, to remove low-molecular-weight inhibitors that dialyzed poorly. Cytosol from CHO cells was prepared exactly as described previously (2).

Ion-Exchange Chromatography of Yeast and Bovine Cytosols. Yeast cytosol was prepared as described above and desalted into 25 mM Hepes/KOH, pH 7.0/1 mM $MgCl_2$ (desalting buffer) on a 10-ml column of Sephadex G-25. The protein peak (1.2 ml) was loaded onto 2 ml of DE-52 DEAE-cellulose (Whatman, 0.8 × 3.3 cm) equilibrated with desalting buffer. The column was washed with 4 ml of desalting buffer and then eluted with a linear gradient (total volume, 20 ml) of 0–400 mM KCl in desalting buffer. The flow-rate was 4 ml/hr, and the fraction size was 0.7 ml. For ion-exchange chromatography, brain cytosol was prepared essentially as described above, except that the homogenization buffer contained 0.25 M sucrose, lacked $MgCl_2$, and was pH 8.0. Ion-exchange chromatography was carried out essentially as described above for yeast cytosol with the exception that the buffer lacked $MgCl_2$ and was pH 8.0.

Preparation of Donor and Acceptor Golgi Membranes on Sucrose and Maltose Gradients. Donor Golgi membranes from VSV-infected CHO clone 15B cells and acceptor Golgi membranes from wild-type CHO cells were purified on sucrose gradients as described by Balch *et al.* (2) with the modifications of Wattenberg *et al.* (12). For purification on maltose gradients, the protocol was identical except that the 35% and 29% (all wt/wt) sucrose layers in the gradients were replaced with 35% and 29% maltose.

Assay of G Protein Transport in a Cell-Free System. The assay conditions were similar to those reported earlier (2). In addition to donor and acceptor Golgi membranes (2.5 μg of protein each) and the indicated cytosolic fractions, assay mixtures contained (in a final volume of 50 μl) 25 mM Hepes/KOH at pH 7.0, 15 mM KCl, 25 mM magnesium acetate, 50 μM ATP, 250 μM UTP, 2 mM creatine phosphate, 7.3 international units of rabbit muscle creatine kinase, and 0.15 μCi of UDP-[^{3}H]GlcNAc. Incubations were for 60 min at 37°C.

For assay of yeast cytosol, we made the following changes. Donor and acceptor Golgi membranes (2.5 μg of protein each per assay) from maltose gradients were used (see above). The concentrations of magnesium acetate and UTP were changed to 1.5 mM and 50 μM, respectively. Finally, incubations were carried out at 32°C for 1–2 hr.

Determination of ATP Levels. The level of ATP in the transport assay was monitored with tracer [^{3}H]ATP as described (13).

RESULTS

Yeast and Mammalian Cytosol Interchangeably Drive Protein Transport. In preliminary experiments, we investigated the ability of unfractionated yeast cytosol to substitute for mammalian cytosol (from either bovine brain or CHO cells) in the cell-free system for the transport of the VSV G protein. Under our standard assay conditions, the cytosol fraction from yeast was completely inert (not shown). However, in mixing experiments, yeast cytosol completely inhibited the ability of bovine brain cytosol to stimulate the incorporation of [^{3}H]GlcNAc into the VSV G protein. Since yeast contain high levels of carbohydrate-metabolizing enzymes such as invertase and hexokinase, the inhibition might have been due to depletion of ATP induced by the sucrose present in the enriched Golgi preparations that we employ in the assay. Indeed, in the presence of 0.2 M sucrose (the routine concentration in the assay), yeast cytosol completely eliminated ATP, despite the inclusion of an ATP-regenerating system (data not shown). To circumvent this difficulty, we purified the donor and acceptor Golgi membranes for the assay in density gradients composed of an alternate disaccharide—maltose (which is not a substrate for invertase). In the presence of Golgi membranes from maltose gradients, yeast cytosol efficiently sustained *in vitro* transport of G protein (Fig. 1) and no longer inhibited the transport activity of bovine brain cytosol (not shown). As anticipated, ATP levels now remained constant throughout the course of the incubation period. In optimization experiments, transport in the presence of yeast cytosol proceeded up to 2-fold more efficiently at 32°C than at 37°C (the optimum for bovine brain cytosol). This lower temperature is closer to the optimum for yeast growth (≈30°C).

The optimal buffer conditions differed only slightly from those previously established (see *Materials and Methods*). In control experiments, yeast cytosol-driven transport displayed the same set of specificities that we earlier demonstrated for mammalian cytosol (Fig. 2). Transport required both donor and acceptor Golgi membranes, as well as ATP. Also, the transported G protein resided in sealed Golgi membranes impermeable to trypsin. In addition, boiled yeast cytosol did not sustain transport (not shown), as would be expected if the active components in cytosol were proteins.

In comparison with bovine brain and CHO cytosol, yeast cytosol was approximately 20% as efficient in sustaining transport (see legend to Fig. 1). Yeast cytosol was less potent (on a per μg of protein basis) and also stimulated transport to a lower plateau level (compare Figs. 1 and 3). The lower efficacy of yeast cytosol may be attributable to less efficient interaction of yeast cytosolic proteins with mammalian proteins on the Golgi membranes.

Yeast and Mammalian Cytosols Contain Similar Complements of Transport Factors. Transport of the G protein *in vitro*

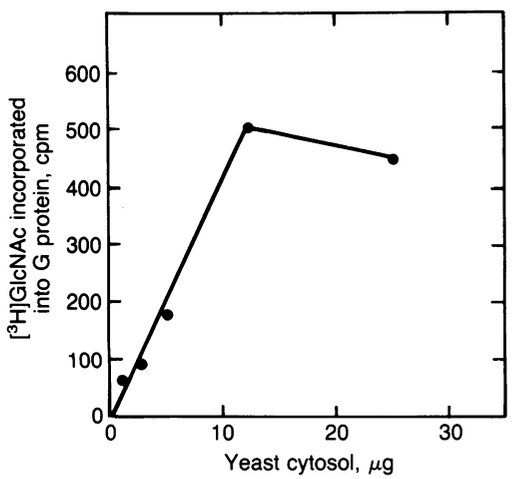

FIG. 1. Yeast cytosol sustains intercompartmental protein transport in the mammalian Golgi apparatus. The incorporation of [^{3}H]GlcNAc into G protein in the presence of increasing amounts of yeast cytosol was determined; the incorporation in the absence of cytosol was 92 cpm. Assays were performed at 32°C with maltose-containing Golgi fractions. Yeast cytosol was 21% as active (on a per μg of protein basis) as CHO cytosol in the same assay, and 16% as active as bovine brain cytosol (see Fig. 4).

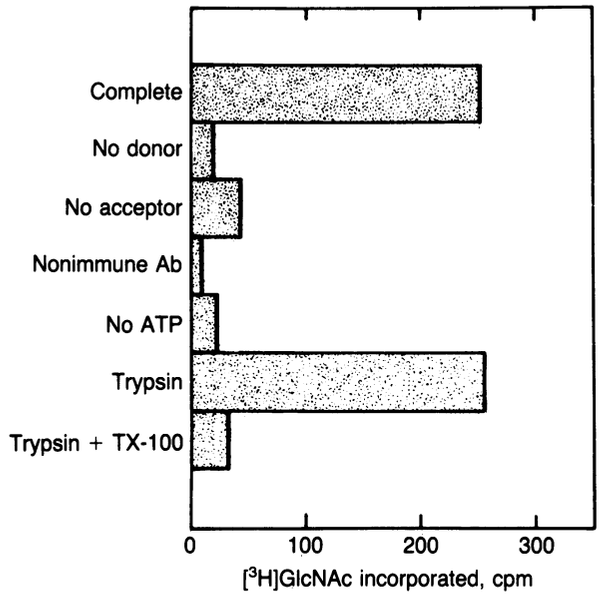

FIG. 2. Specificity of protein transport driven by yeast cytosol. Yeast cytosol (20 μg of protein) was assayed in the cell-free transport system as described for Fig. 1. The [3H]GlcNAc incorporation in the absence of cytosol was 50 cpm. As indicated, donor or acceptor Golgi membranes or ATP (and its regenerating system) were omitted from the assay. To show that it was G protein that received [3H]GlcNAc, we used nonimmune serum for immunoprecipitation. To demonstrate that the transported G protein resided in sealed Golgi membranes, we treated (immediately after the transport incubation) with trypsin (1 mg/ml) in the absence or presence of 0.1% Triton X-100. After a 30-min incubation at 37°C, we added soybean trypsin inhibitor (1 mg/ml) and proceeded with determination of [3H]GlcNAc incorporation as usual.

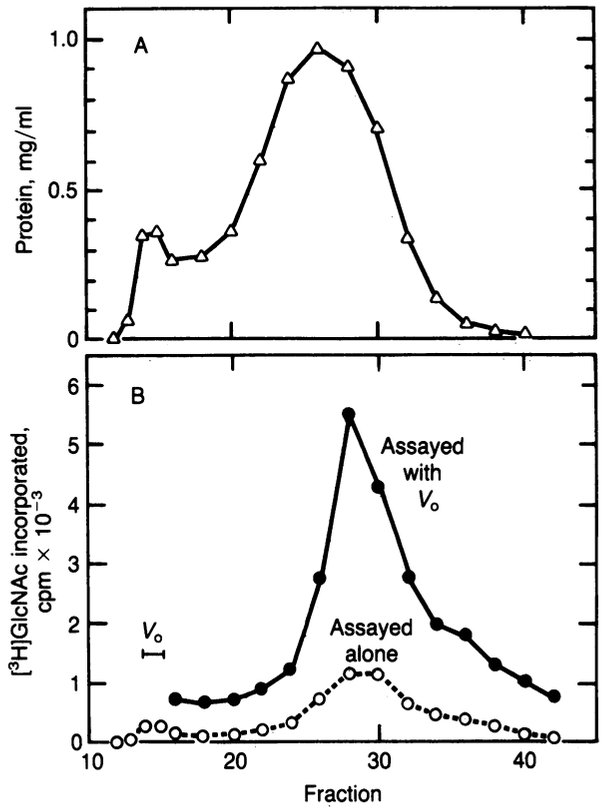

FIG. 3. Bio-Gel A-1.5m chromatography reveals a large transport factor in bovine brain cytosol. One milliliter of brain cytosol (7 mg of protein) was chromatographed on a 20-ml (1 × 25 cm) Bio-Gel A-1.5m column equilibrated in 25 mM Hepes/KOH at pH 7.0 containing 50 mM KCl, 1 mM $MgCl_2$, and 1 mM dithiothreitol. The volume of the collected fractions was 0.5 ml. (*A*) Fractionation pattern of total brain cytosol proteins. Protein concentration was determined by the Bradford method (15) with bovine serum albumin as standard. The yield of protein was 77%. (*B*) Fractionation of cytosolic factors that sustain protein transport. Aliquots (10 μl) of each fraction were assayed in the cell-free transport assay either in the absence (○) or in the presence (●) of 20 μl of the pooled V_o fraction (corresponding to fractions 14 and 15). The yield of transport activity was 55%.

involves at least three kinetic intermediates (12, 14). These intermediates seem to represent distinct stages in the budding and fusion of transport vesicles carrying G protein. It is reasonable to assume that a distinct set of proteins would be responsible for steps in budding and fusion. Cytosol would then provide multiple active constituents that only in combination could drive the entire transport process. We have recently obtained evidence by gel filtration and ion-exchange chromatography that mammalian cytosol contains multiple transport factors. We examined whether functionally equivalent factors are present in yeast.

Bovine brain cytosol was fractionated on Bio-Gel A-1.5m, and proteins chromatographing in both the excluded (V_o) and included (V_i) regions of the column were assayed for their ability to stimulate the *in vitro* transport of G protein (Fig. 3). By itself the V_o fraction was only slightly active. However, upon addition of this fraction to fractions from the included region of the column we observed a 2- to 4-fold greater level of [3H]GlcNAc incorporation than if the activities of the two fractions were simply additive (Fig. 4). The synergistic stimulation by the V_o fraction suggests that brain cytosol contains at least one transport-facilitating factor with native molecular mass greater than 1.5 megadaltons. We have observed a similar factor in cytosol from CHO cells, mouse myeloma cells, and rat liver (not shown). The activity of the V_i fraction by itself was substantial, approaching 50% that of complete cytosol at saturating levels of V_i fraction. Explanations for this apparent lack of complete dependence of transport on the high molecular weight fraction are (*i*) the Golgi membrane fraction may already contain bound V_o factor; (*ii*) the V_i fraction may contain unassembled "subunits" of the V_o factor; or (*iii*) the V_o factor may enhance transport but may not be absolutely required.

To investigate whether yeast cytosol also contains this large transport factor, we chromatographed yeast cytosol on Bio-Gel A-1.5m under the same conditions. As with brain cytosol, the yeast V_o fraction alone had little activity, but it did cause a 2- to 3-fold stimulation in the activity of the bovine brain V_i fraction (Fig. 5). This shows that the yeast V_o factor will substitute for the bovine factor in complementing the V_i fraction to promote transport. Unlike total yeast cytosol, the yeast V_o factor could be assayed in the presence of Golgi membrane fractions containing either sucrose or maltose. This is due to the fact that the inhibitory activities in yeast cytosol (presumably including invertase and hexokinase) chromatograph solely in the included region of the Bio-Gel A-1.5m column. At equivalent protein concentrations of the bovine brain V_o fraction, the yeast V_o fraction was approximately 20% as active in facilitating transport (see legend to Fig. 5). The active component(s) of the V_o fraction showed the thermolability characteristic of a protein. Half-inactivation of the activity occurred upon incubation at 50°C for 30 min (data not shown).

We have also obtained evidence for distinct transport factors in mammalian cytosol by ion-exchange chromatography. Four peaks in transport-sustaining activity can be resolved by chromatography of bovine brain cytosol on DEAE-cellulose (17). Peak A corresponds to the flow-

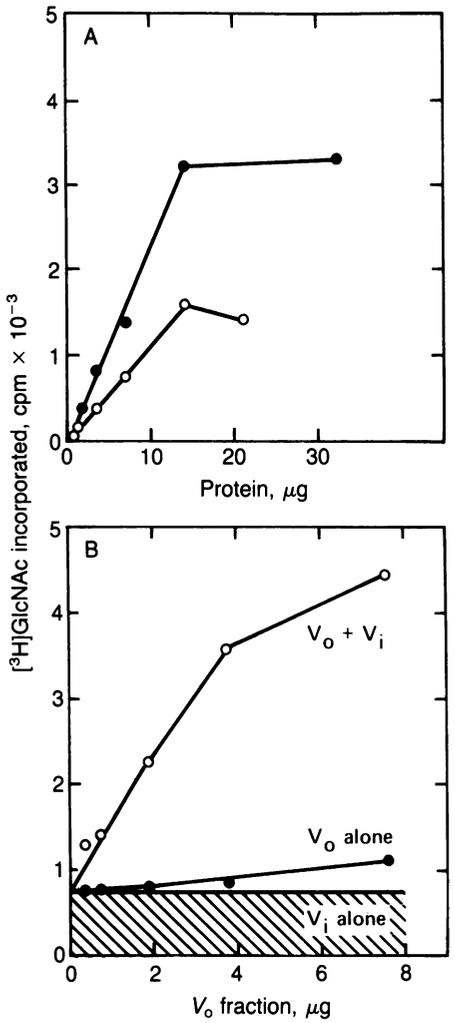

FIG. 4. Linear dependence of protein transport on the resolved V_o and V_i fractions from bovine brain cytosol. Excluded (V_o) and included (V_i) proteins separated by the Bio-Gel A-1.5m column chromatography depicted in Fig. 3 were tested either separately or in combination for their ability to facilitate *in vitro* transport of G protein. Fractions 28–30 were pooled for the V_i fraction (protein concentration, 0.68 mg/ml). The protein concentration of the pooled V_o fraction (fractions 14 and 15) was 0.38 mg/ml. (*A*) The indicated amounts of unfractionated bovine brain cytosol (●) or its corresponding V_i fraction (○) were assayed in the cell-free transport system. (*B*) Synergistic stimulation of G protein transport in the presence of the combined V_o and V_i fractions from bovine brain cytosol. The shaded area depicts the level of [^{3}H]GlcNAc incorporation stimulated by a fixed amount of V_i fraction (6.8 μg of protein). The V_o fraction was assayed in the absence (●) or presence (○) of the V_i fraction. For incubations containing the V_o fraction alone, the level of [^{3}H]GlcNAc incorporation has been added to the level of incorporation in the presence of the V_i fraction alone. The resulting line (through the closed circles) therefore indicates the expected levels of incorporation if the activities of the V_o and V_i fractions were simply additive.

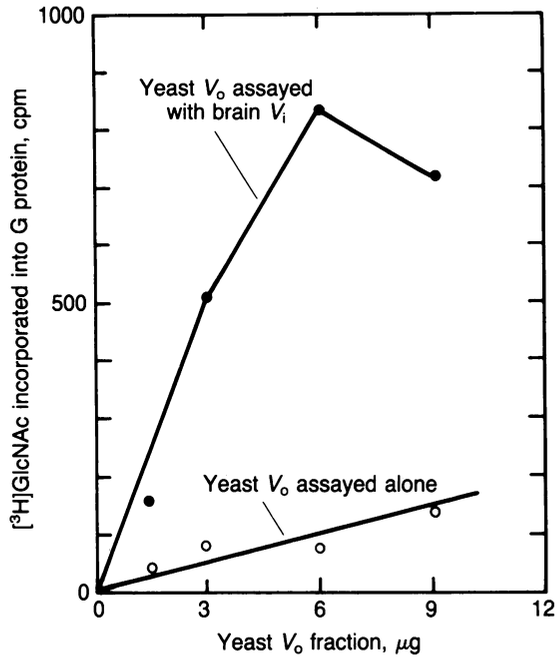

FIG. 5. Yeast cytosol also contains a high molecular weight transport factor. One milliliter of yeast cytosol (9.3 mg of protein) was fractionated on a 20-ml Bio-Gel A-1.5m column as described in the legend to Fig. 1. The pooled V_o fraction (1 ml containing 0.6 mg of protein) was assayed in the absence (○) or the presence (●) of bovine brain V_i fraction (5 μg of protein; an amount that alone sustained the incorporation of 422 cpm of [^{3}H]GlcNAc in the cell-free transport assay). The level of incorporation of [^{3}H]GlcNAc resulting from the brain V_i fraction alone has been subtracted. For comparison, the yeast V_o fraction stimulated the incorporation of [^{3}H]GlcNAc 23% as efficiently (on a per μg of protein basis) as the bovine brain V_o fraction (the same preparation assayed in Figs. 3 and 4). Assays were performed at 37°C with sucrose-containing Golgi fractions.

through fraction under the employed ionic conditions (see legend to Fig. 6). Peaks B, C, and D elute successively at KCl concentrations from approximately 20 mM to 200 mM. The activities in these four peak fractions are additive and do not complement each other in a synergistic manner. Yeast cytosol was chromatographed on DEAE-cellulose under similar conditions (Fig. 6). There were likewise four distinct peaks in transport activity—peak A (flow-through fraction) and peaks B, C, and D (which all eluted at KCl concentrations of 200 mM or lower). Although the elution positions of the putative peaks B, C, and D from yeast do not correspond precisely to their bovine brain counterparts, the overall similarity of the elution profiles of yeast and brain cytosol is nonetheless striking.

DISCUSSION

We have demonstrated here that cytosolic proteins from yeast will substitute for their mammalian counterparts in facilitating intercompartmental protein transport. This remarkable conservation implies that yeast and animals construct their membrane-bounded organelles by the same mechanisms. The degree of conservation is further emphasized by the fact that individual resolved transport factors from yeast and bovine brain are similar in their chromatographic properties. Moreover, the yeast and mammalian proteins must be able to interact with one another to facilitate transport. Yeast cytosolic proteins must act upon Chinese hamster proteins on the Golgi membranes. Also, the complementation between the yeast V_o factor and the brain V_i fraction is consistent with efficient interaction between cytosolic proteins from the two species. Beyond this, we have recently found that cytosol and Golgi membranes from a wide variety of other species will also function in the cell-free system (18). Cytosols from wheat germ, slime molds, and fish all effectively drive transport.

For genetic analysis of eukaryotic processes, yeast cells have rapidly become the experimental system of choice. For many biochemical and morphological studies, on the other hand, higher eukaryotes still have experimental advantages. In the case of protein export, cell fractionation techniques for the purification of organelles involved in the export pathway are well established. In addition, the morphological route

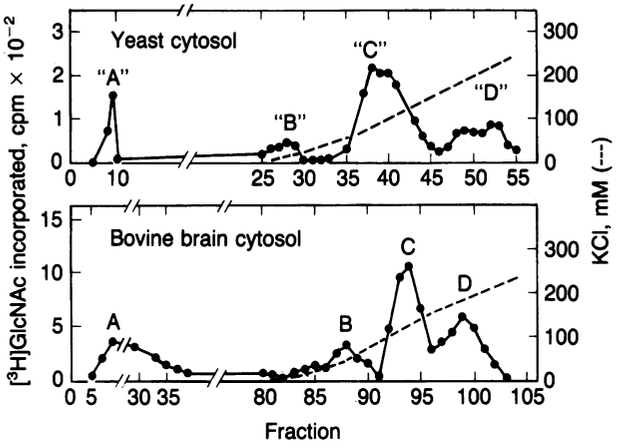

FIG. 6. Yeast and bovine brain cytosols show similar fractionation patterns upon ion-exchange chromatography. (*Upper*) Ion-exchange chromatography of yeast cytosol. Aliquots (5 μl) of fractions were assayed as described for total yeast cytosol (see *Materials and Methods*) except that the incubations were carried out at 25°C for 4 hr. Each incubation mixture was supplemented with 0.5 μg of unfractionated CHO cell cytosol. The pattern in the absence of added CHO cytosol was similar (not shown). The incorporation due to the CHO cytosol alone (200 cpm) has been subtracted from each point. (*Lower*) Ion-exchange chromatography of bovine brain cytosol. Aliquots (2.5 μl) were assayed as described in *Materials and Methods*. In addition to an aliquot from the indicated fraction, each incubation mixture was supplemented with 0.5 μg of unfractionated CHO cell cytosol. The activity profile without added CHO cytosol was similar (data not shown). The background incorporation (subtracted from each point) due to the CHO cell cytosol alone (a separate batch from that used in *Upper*) was 570 cpm.

traversed by exported proteins and the processing steps undergone by these proteins have been extensively characterized in higher cells (3, 16).

We have combined the respective advantages of yeast and mammalian cells for the study of protein transport by developing a hybrid yeast–mammalian cell-free transport system. As a result, the powerful tools of yeast genetics can now be used to identify, and to produce in quantity, the cytoplasmic proteins that catalyze protein transport. One approach would be to test if known yeast *sec* mutants are deficient in specific cytosolic factors. Preliminary attempts to correlate the *sec* mutants (7) with any of the soluble factors that drive G protein transport have been inconclusive (unpublished data). Another approach would be to purify one or more yeast cytosolic factors. Conditional mutants could then be generated *in vitro* to test the *in vivo* validity of the transport component. Identification of additional transport enzymes could be accomplished by the isolation of suppressor mutants.

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