Vesicle Fusion in Protein Transport through the Golgi in Vitro Does Not Involve Long-Lived Prefusion Intermediates. A Reassessment of the Kinetics of Transport As Measured by Glycosylation

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ABSTRACT: The well-characterized cell-free assay measuring protein transport between compartments of the Golgi [Balch, W. E., Dunphy, W. G., Braell, W. A., & Rothman, J. E. (1984) *Cell 39*, 405–416] utilizes glycosylation of a glycoprotein to mark movement of that protein from one Golgi compartment to the next. Glycosylation had been thought to occur immediately after vesicles carrying the glycoprotein fuse with their transport target. Therefore, the kinetics of glycosylation were taken to reflect the kinetics of vesicle fusion. We previously isolated and raised monoclonal antibodies against a protein (the prefusion operating protein, POP) which is required in this assay at a step after vesicles have apparently been formed and interacted with the target membranes, but long before glycosylation takes place. This was therefore presumed to be a reaction involving targeted but unfused vesicles. Here we report that POP is identical to uridine monophosphokinase, as revealed by molecular cloning. We show that POP is not active in transport per se but instead enhances the glycosylation used to mark transport. This indicated that, contrary to previous assumptions, glycosylation might lag significantly behind vesicle fusion. We directly show this to be true. This alters the interpretation of several earlier studies. In particular, the previously reported existence of a late, prefusion intermediate, the "NEM-resistant intermediate", can be seen to be due to effects on glycosylation and not indicative of true fusion events.

Utilization of cell-free assay systems which reconstitute the vesicular transport of secreted proteins through the eukaryotic secretion pathway is beginning to unfold the mechanisms underlying these complex processes. One of the first of these assays to be constructed (Balch et al., 1984a) reconstitutes the transport of the vesicular stomatitis virus (VSV)[1] membrane glycoprotein (G protein) from the cis to the medial Golgi. This assay has been utilized to elucidate the vesicular nature of the transport process and to identify several kinetic intermediates in the reaction (Balch et al., 1984b; Malhotra et al., 1988; Melançon et al., 1987; Wattenberg et al., 1986; Orci et al., 1986).

The assay measures the transport of the VSV G protein between two populations of Golgi membranes. G protein originates in donor membranes prepared from VSV-infected CHO cells. The cells used are lacking *N*-acetylglucosamine (GlcNAc) transferase I activity. Therefore, the G protein oligosaccharide is deficient in that sugar. The target, acceptor, Golgi are prepared from wild-type CHO cells, which therefore contain the transferase. Transport of G protein between donor and acceptor is marked by the acquisition of GlcNAc by G protein upon its arrival in the acceptor membranes and exposure to the transferase.

Kinetic and biochemical analyses of the reconstituted Golgi transport reaction have been used to construct a model consisting of a series of transport intermediates through which G protein progresses during the transport process. According to this model, transport vesicles are formed bearing a proteinaceous coat, are released, and then attached to their target with the coat intact (Melançon et al., 1987). The uncoating of transport vesicles is sensitive to inhibition by nonhydrolyzable GTP analogues. This implicates the involvement of a GTP-binding protein in a step just preceding uncoating. After vesicle uncoating, an intermediate, known as the low cytosol requiring intermediate (LCRI), is formed (Wattenberg et al., 1986). The LCRI was presumed to consist of uncoated transport vesicles attached, but as yet unfused, to their target membrane. The formation of the LCRI is dependent on at least three proteins which act after vesicle uncoating. The *N*-ethylmaleimide sensitive factor (NSF) is a homotetramer of 76-kDa subunits (Block et al., 1988). Mutation of the yeast homologue of NSF, sec18p, leads to the accumulation of unfused transport vesicles (Kaiser & Schekman, 1990). This confirms the function of NSF in the steps which lead to vesicle fusion, although it is as yet unresolved whether NSF acts in fusion itself or in preceding steps such as vesicle attachment. The binding of NSF to Golgi membranes is mediated by three soluble proteins, the soluble NSF attachment proteins (SNAPs) (Weidman et al., 1989; Clary et al., 1990) as well as by an as yet unidentified membrane receptor. Similar to what is observed for mutation of the NSF gene, the mutation of a yeast SNAP homologue (sec17) leads to the accumulation of unfused transport vesicles (Kaiser & Schekman, 1990).

The LCRI is so named because once it has formed, only low levels of cytosol are required to complete the glycosylation of G protein. In contrast, relatively high levels of cytosol are required to form the LCRI. The observation that donor and acceptor must be coincubated to produce the LCRI led to the conclusion that the LCRI represents a transport intermediate consisting of unfused transport vesicles. Surprisingly, the LCRI intermediate is formed relatively early in each round

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[1] Abbreviations: VSV, vesicular stomatitis virus; POP, prefusion operating protein; GlcNAc, *N*-acetylglucosamine; LCRI, low cytosol requiring intermediate; NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide sensitive factor; SNAP, soluble NSF attachment protein; UMPK, uridine monophosphokinase.

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of transport (Wattenberg et al., 1986). All G protein has reached the LCRI prefusion intermediate after approximately 20–25 min, whereas the complete glycosylation of all G protein takes upwards of 60 min. In this model, there is therefore a lengthy period in which attached but unfused vesicles are processed to allow fusion to proceed. This was though to reflect a tight control placed on the fusion process [review by Wattenberg (1990)].

Operationally, the kinetic hesitation at the level of the LCRI can be used to measure the cytosolic requirements of the post-LCRI processes. Donor and acceptor membranes are preincubated with high levels of cytosol for 25 min to allow formation and attachment of transport vesicles and formation of the LCRI intermediate. These membranes are then pelleted to remove the original cytosol, and cytosol fractions are added, along with buffer components required for the final steps of the assay. Using this assay, it was found that there was a single, 25-kDa polypeptide which was sufficient to supply all of the post-LCRI cytosolic requirements (Wattenberg et al., 1989). We named this protein POP (prefusion operating protein). Although mammalian cytosol was first used to identify POP activity, we also found the activity in yeast cytosol and used yeast cytosol as a source from which to isolate POP. Monoclonal antibodies were raised against the putative POP protein and used to confirm that this polypeptide was in fact responsible for POP activity.

Here we have used those monoclonal antibodies to clone the gene encoding POP. Surprisingly, sequence analysis indicated that POP was identical to uridine monophosphokinase (UMPK). It could be shown that the apparent activity of UMPK in the transport assay was due to its ability to enhance the uptake of UDP-GlcNAc, the substrate for the glycosylation reaction which marks transport. This finding suggested that the glycosylation of G protein was limiting in the transport assay. Since the identity of the proposed transport intermediates is based on the kinetics of glycosylation of G protein, these results prompted a direct test of whether the kinetics of glycosylation mirrored the kinetics of vesicle fusion.

Materials and Methods

Materials. The yeast genomic library in λ gt11 was purchased from Clonetech (Palo Alto, CA). Bacterial culture medium components were purchased from Difco (Detroit, MI). Poly(ethylene glycol), nucleoside monophosphokinase, adenine monophosphokinase, creatine phosphokinase, and Sephadex G-75 were purchased from Sigma Chemical Co. (St. Louis, MO). UDP-[6-^{3}H]GlcNAc was purchased from New England Nuclear Labs (Boston, MA). [^{32}P]ATP and [^{125}I]protein A were purchased from Amersham Corp. (Arlington Heights, IL). All DNA-modifying enzymes and restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). Sequenase kit was purchased from U.S. Biochemical Corp. (Cleveland, OH). Monoclonal antibodies against VSV G protein were a gift of Dr. Leo Lefrancois of the Upjohn Co. (LeFrancois, 1984). Rabbit anti-mouse IgG was purchased from Kirkegaard and Perry Labs (Gaithersberg, MD). X-OMAT-AR X-ray film was purchased from Eastman Kodak (Rochester, NY).

Cells and Culture. CHO-K1 cells were from the laboratory of Dr. James Rothman (Sloan-Kettering Institute, NY). CHO Lec-1 cells, a glycosylation defective mutant (Stanley et al., 1975), were obtained from ATCC. Cells were maintained in suspension culture in α-MEM medium containing 10% fetal calf serum. VSV stocks were prepared, cells were infected, and Golgi fractions and CHO cytosol fractions were prepared as described (Balch et al., 1984a). Hybridoma cultures producing monoclonal antibodies to yeast POP were grown in DME/high glucose medium containing either 10% fetal calf serum or 1% Nutridoma-SP (Boehringer Mannheim, Indianapolis, IN).

Assay for POP Activity. The assay for POP activity was performed as described (Wattenberg et al., 1989). In a preincubation step, 5 μL each of donor and acceptor Golgi membranes were incubated at 37 °C for 25 min with 2.5 μL of unfractionated CHO cell cytosol in a final volume of 50 μL in a solution containing 25 mM HEPES, pH 7.5, 25 mM KCl, 2.5 mM magnesium acetate, 50 μM ATP, 250 μM UTP, 2 mM creatine phosphate, and 7.3 units/mL creatine phosphokinase. Membrane were pelleted at 13000 rpm for 3 min through a cushion of 0.3 M sucrose and then resuspended in 40 μL of a solution containing 25 mM HEPES, pH 7.5, 15 mM NaCl, 2.5 mM magnesium acetate, 50 μM ATP, 250 μM UTP, 2 mM creatine phosphate, 7.3 units/mL creatine phosphokinase, and 2.5 μCi/mL UDP[6-^{3}H]GlcNAc. Up to 10 μL of putative POP fractions were added, and incubation was continued for 90 min at 37 °C. Reactions were terminated by the addition of 50 μL of a solution containing 50 mM Tris, pH 7.5, 250 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1% sodium 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 (Kirkegaard and Perry Labs, Gaithersburg, MD). After further incubation at 37 °C for 15 min, the immunoprecipitate was collected by filtration through type HA 0.45-μm filters (Millipore, Bedford, MA). After being washed, filters were dried and counted using Ready-Safe scintillant (Beckman, Palo Alto, CA).

SDS–PAGE and Western Blotting. SDS–PAGE was carried out as described (Laemmli, 1970). Running gels contained 15% acrylamide. Proteins separated by SDS–PAGE were electroblotted to nitrocellulose membranes for 60 min at 100 volts in a solution containing 14.4 g of glycine, 3 g of Trizma base, and 200 mL of methanol per liter. Filters were blocked by incubation for 60 min in PBS containing 0.05% Tween-20 and 10% (w/v) Carnation nonfat dry milk. Proteins reacting with monoclonal antibodies to POP were detected by incubation of blocked filters with hybridoma supernatants diluted 1:25 in PBS-Tween for 60 min. After being washed, filters were then incubated with alkaline phosphatase labeled Goat anti-mouse IgG (ProMega Biotech, Madison, WI), and alkaline phosphatase activity was detected with 5-bromo-4-chloro-3-indoline phosphate and nitroblue tetrazolium.

Expression Library Screening. The yeast genomic expression library was screened for clones expressing POP-related sequences essentially as described (Young & Davis, 1983). Approximately 1×10^5 plaque-forming units were plated per 150-mm petri dish; 2×10^6 plaques were screened in all. Plaque lifts to nitrocellulose paper (Schleicher & Schuel, Keene, NH) were made essentially as described (Sambrook et al., 1989). Filters were blocked in a solution of phosphate-buffered saline containing 0.05% Tween-20 (PBST) and 10% (w/v) nonfat dried milk. Filters were then incubated with PBST containing a 1:25 dilution of tissue culture supernatants from hybridoma cell lines producing monoclonal antibodies to the POP protein. After incubation, filters were washed with PBST and further incubated with PBST containing a 1:5000 dilution of rabbit anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD). Filters were washed again and positive plaques identified by incubation with PBST containing [^{125}I]protein A and autoradiography. Positive plaques were picked and taken through three cycles of rescreening until pure clones were obtained.

DNA Preparation and Subcloning. CsCl-banded phage DNA and plasmid DNA were prepared as described (Sambrook et al., 1989). All DNA digestions and ligations were performed as recommended by suppliers' instructions. Ca^{2+} competent *Escherichia coli* (strain DH5 alpha) were prepared and transformed as described (Morrison, 1979). Transformed bacteria were grown in LB supplemented with 75 μg/mL ampicillin.

To define the smallest possible fragment encoding POP, subclones were prepared utilizing a unique *Pst*1 site which divided the 6.0 kb *Eco*R1 insert into fragments of 2.0 and 4.0 kb. SDS–PAGE and Western blotting was performed on extracts from bacterial clones expressing these constructions, and it was found that the coding sequence for POP was on the 4.0 kb fragment. A series of mung bean nuclease deletions from the *Eco*R1 site distal to the *Pst*1 site was done, and the remaining fragments were examined by SDS–PAGE and Western blotting for their ability to express the POP protein. After a deletion of 1700 bp, the remaining 4.3 kb fragment would still encode a full size POP protein. Taken together, these results indicated that the coding sequence for the POP protein as well as the promoter which directed its expression was contained on the 2.3 kb fragment bounded by the mung bean deletion and the *Pst*1 site.

DNA Sequence Determination. CsCl-purified double-stranded plasmid DNA was the substrate for dideoxy chain termination sequencing reactions utilizing a Sequenase Kit (U.S. Biochemical Corp., Cleveland, OH) and [^{32}P]dATP (Amersham Corp., Arlington Heights, IL). DNA denaturation, primer annealing, and chain extension reactions were carried out as recommended by the manufacturer. M13 forward and reverse primers (U.S. Biochemical Corp.) were utilized for initial sequencing reactions; subsequent reactions were primed utilizing oligonucleotides synthesized by the laboratory of Dr. N. Hatzenbuhler (Upjohn unit 7240) and based upon previously determined 5′ sequences.

Measurement of UDP-GlcNAc Uptake. For each sample, a preincubation mix was prepared containing 5 μg of acceptor membranes, 5 μL of CHO cytosol, buffer, and an ATP-regenerating system in a final volume of 50 μL as described for the assay for POP activity. This was incubated for 25 min at 37 °C and then pelleted for 3 min at 12000 rpm in a Beckman microfuge through a cushion of 50 μL of 0.3 M sucrose. The pellet was resuspended in 45 μL of a mix containing 25 mM Tris, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.5, and the ATP-regeneration system described above. The resuspended membranes were further incubated for 45 min at 37 °C, and then 5 μL of a mixture containing 0.625 μCi each of [^{14}C]inulin (Amersham, Arlington Heights, IL; 7.2 mCi/mmol) and UDP-[^{3}H]GlcNAc (New England Nuclear, Boston, MA; 16.9 Ci/mmol) was added for 30 s, and the membranes were pelleted through 200 μL of 0.3 M sucrose. Pellets were then resuspended for scintillation counting. The level of UDP-GlcNAc transported into the lumen of the Golgi was calculated by subtracting from the ^{3}H counts the amount of UDP-GlcNAc external to the Golgi based on the level of [^{14}C]inulin (a nontransportable standard) associated with the Golgi pellets according to the following formula: $^{3}H_i = {}^{3}H_t - ({}^{14}C_t \times {}^{3}H_s/{}^{14}C_s)$, where $^{3}H_i$ is the calculated lumenal UDP[6-^{3}H]GlcNAc, $^{3}H_t$ is the total ^{3}H in the Golgi pellet, $^{14}C_t$ is the total ^{14}C in the Golgi pellet, and $^{3}H_s/^{14}C_s$ is the ratio of ^{3}H to ^{14}C in the incubation mix.

Results

Cloning of the Gene Encoding POP Reveals It To Be URA6, the Gene for Uridine Monophosphokinase. Putative clones encoding POP were identified on the basis of immunoreactivity of protein expressed from a well-characterized phage expression system (Young & Davis, 1983) using a monoclonal antibody to POP (Wattenberg et al., 1989). Restriction digestion of phage DNA from each of six positive clones showed a common 6.0 kb *Eco*RI fragment (not shown). These clones were expressed as lysogens, and the proteins were examined by SDS–PAGE followed by Western blotting. Each of the clones produced a single immunoreactive protein of 25 kDa which comigrated with authentic yeast POP (data not shown). We had therefore isolated a DNA fragment encoding a protein cross-reactive with the anti-POP antibodies and of the same molecular weight as authentic POP. Curiously, these data suggested that the protein was not being made as a β-galactosidase fusion protein, as expected, but rather as a free protein being driven from its own promoter.

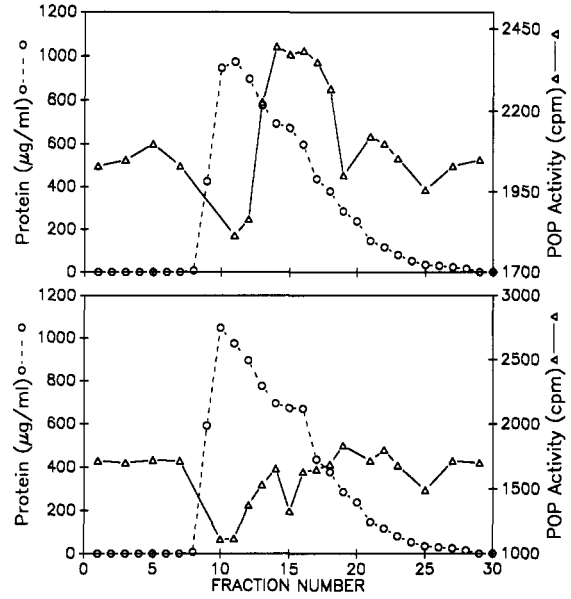

FIGURE 1: *E. coli* carrying a plasmid encoding POP produce POP activity. *E. coli* transformed with a PUC19 plasmid containing the putative POP coding sequence (top panel) or with PUC19 alone (bottom panel) were grown overnight at 30 °C in L-broth containing 75 μg/mL ampicillin. Cells were harvested by centrifugation at 13000 rpm, resuspended in one-tenth volume of 50 mM Tris, pH 7.5, and 1 mM $MgCl_2$ (TM), and recentrifuged. Cells were resuspended in 1.100 volume of TM and lysed by sonication. Cell lysates were centrifuged for 60 min at 100000*g*. Supernatants were chromatographed on Sephadex G-75 equilibrated in TM buffer. Column fractions were assayed for POP activity as described under Materials and Methods (triangles). Protein content is indicated by open circles.

In the absence of protein sequence information, it was important to confirm that the cloned DNA sequence encoded authentic POP by illustrating that the sequence could produce POP activity in *E. coli* extracts. Cell lysates were prepared by sonication from overnight cultures of *E. coli* which had been transformed with either the vector PUC19 alone or the putative POP clone in the vector PUC19. After centrifugation of lysates at 100000*g*, the resulting supernatants were chromatographed in Sephadex G-75 to remove an unknown endogenous inhibitor of our assay, and the resulting column fractions were assayed for POP activity. As seen in Figure 1, the chromatography of the lysate from cells carrying the POP clone resulted in a large peak of POP activity which eluted from the column at a position near the elution position (25 kDa) of native yeast POP (top panel, triangles). Assays of column fractions of lysates prepared from PUC19 transformed cells showed no POP activity (lower panel, triangles). This substantiated that the clone isolated utilizing the monoclonal antibodies did indeed encode the protein responsible for POP

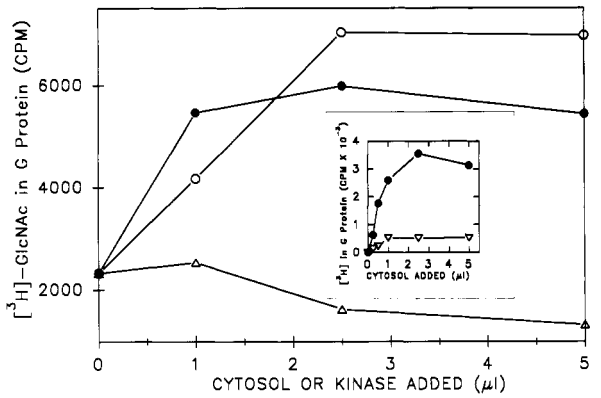

FIGURE 2: Commercially available UMPK supplies POP activity in a reconstituted transport assay. Bovine nucleoside monophosphokinase (UMPK, filled circles) and myokinase (adenosine monophosphokinase, triangles) were reconstituted at 2.0 mg/mL in 10 mM Tris, pH 7.5, 1 mM $MgCl_2$, and 10% glycerol. Both of these, as well as CHO cytosol (approximately 2 mg/mL, open circles) were tested for POP activity by titration under our assay conditions as described under Materials and Methods. (Inset) POP/UMPK activity in CHO cytosol is sensitive to alkylation by *N*-ethylmaleimide. CHO cytosol either untreated (closed circles) or treated with 1 mM NEM on ice for 15 min, and then 2 mM DTT to quench the NEM (open triangles) was titered in the late stage assay for POP activity. A minus cytosol background of 1200 cpm was subtracted from each point.

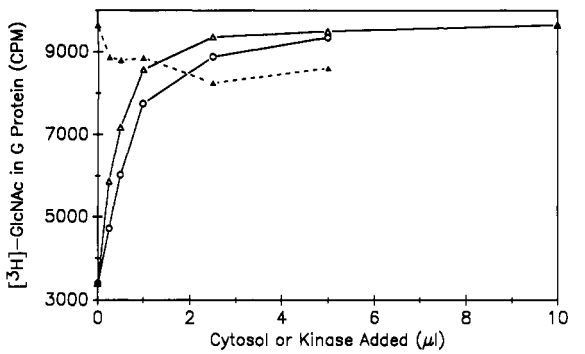

FIGURE 3: Comparison of POP activity as supplied by UMPK or CHO cytosol. Bovine nucleoside monophosphokinase (UMPK) was reconstituted at 5 mg/mL in 10 mM Tris, pH 7.5, 1 mM $MgCl_2$, and 10% glycerol. CHO cytosol was prepared as described under Materials and Methods. UMPK (open triangles) and CHO cytosol (approximately 2 mg/mL, open circles) were titered in the POP assay. CHO cytosol was also titered in the presence of a constant amount (10 μL) of UMPK (closed triangles).

activity.

The region of the 6 kb *Eco*RI fragment encoding the putative POP gene was determined by subcloning into the plasmid vector PUC19. Restriction and mung bean nuclease digestion of this subclone, combined with SDS–PAGE and Western blotting of the proteins from bacterial clones carrying the resulting plasmids, defined a 2.3 kb fragment encoding POP (see Materials and Methods). A single open reading frame was completely included within this fragment. This open reading frame was found to encode a protein of 204 amino acids with a predicted molecular weight of 22 933 Da, a size in close agreement with the observed molecular weight of POP of 25 000 Da on both SDS–PAGE and column chromatography. A search of GeneBank showed a close homology of the POP sequence to a number of nucleotide kinases. Review of the literature for sequences not yet in the database revealed that the POP sequence was identical to the reported sequence of the yeast *URA6* gene. *URA6* encodes uridine monophosphokinase (UMPK) as reported by Liljelund et al., (1989). *URA6* has also been demonstrated to be allelic to the *SOC8* gene (Liljelund & Lacroute, 1986).

Commercial Uridine Monophosphokinase Is Active in the Transport Assay for POP. To substantiate that POP was in fact UMPK, commercially available bovine UMPK as well as adenosine monophosphokinase (AMPK) were tested in the late stage transport assay. As seen in Figure 2, UMPK (filled circles), but not AMPK (triangles), could fully substitute for the POP activity in CHO cytosol (open circles) in our late stage transport assay. This strongly supported our conclusion that POP was in fact UMPK. It should be noted that the commercial preparation of UMPK has a relatively low specific activity. It has been found previously that purification of UMPK results in substantial inactivation of UMPK activity (Wattenberg et al., 1986). In the course of these experiments, it was noted that the POP activity in CHO cytosol was sensitive to the thiol alkylating reagent *N*-ethylmaleimide (NEM) (Figure 2, inset). This property is notable because NEM treatment has been used to define a late transport intermediate (Balch et al., 1984b) (see Discussion).

Are Cytosolic Proteins Required after the Formation of the LCRI Intermediate? It was important to determine whether there were additional cytosolic components in addition to UMP-kinase that might be involved at this step of transport. The activity of commercially available UMPK was therefore compared to the activity of unfractioned CHO cytosol in the assay for post-LCRI transport steps. As seen in Figure 3, UMPK (open triangles) could drive the transport reaction to a similar level as unfractionated CHO cytosol (open circles). In addition, the ability of CHO cytosol to increase the processing of the LCRI when titered in the presence of excess UMPK was tested (filled triangles). No increased activity was noted. These data strongly indicate that after the LCRI has formed no further cytosolic proteins are required.

Are There ATP-Requiring Reactions after the Formation of the LCRI in Addition to That Catalyzed by UMPK? The glycosylation of G protein after the formation of the LCRI has been shown to require ATP (Wattenberg et al., 1986). UMPK would require ATP for its action, but are there other reactions, possibly related to vesicle fusion, which also require ATP? Although the incorporation of [^{3}H]GlcNAc into G protein in the absence of UMPK is low, it is measurable. The UMPK-independent glycosylation occurs because UMPK appears to enhance the transport signal rather than being absolutely required for it (see below). It was found that this UMPK-independent glycosylation could be enhanced by the inclusion of the phosphatase inhibitors sodium fluoride and sodium vanadate (data not shown), presumably by inhibiting the formation of UMP from UTP and UDP. The LCRI was formed as before by preincubation of donor and acceptor with cytosol and ATP for 25 min. The membranes were pelleted and then resuspended either with or without ATP (and an ATP-regenerating system), with and without UMPK, and UDP-[^{3}H]GlcNAc, and the reaction was allowed to continue for 90 min (Figure 4). The addition of ATP in the absence of UMPK resulted in a minimal enhancement of G protein glycosylation. In the presence of UMPK, ATP strongly stimulated the reaction, as expected. This illustrates that there are apparently no ATP-requiring reactions, other than that catalyzed by UMPK, following formation of the LCRI. The time course of formation of the ATP independent state was then measured and compared to the time course of entry into the LCRI and of glycosylation of G protein (Figure 5). The ATP-independent state (filled triangles) forms with kinetics indistinguishable from those of formation of the LCRI (open triangles). Both of these are well in advance of the glycosylation of G protein (filled circles), as previously found (Wattenberg et al., 1986).

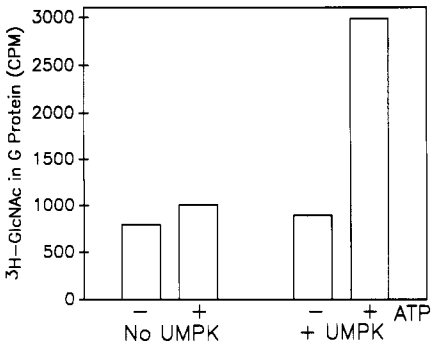

FIGURE 4: ATP requirement for post-LCRI reactions is solely due to UMP-kinase. Donor and acceptor membranes, cytosol, and an ATP-regenerating system were incubated at 37 °C for 25 min to produce the LCRI. The membranes were then centrifuged and resuspended as described under Materials and Methods in buffer containing UDP-[^{3}H]GlcNAc either with or without the ATP-regenerating system and with or without 25 μg of commercial bovine UMP-kinase. The reactions were then continued for 90 min before termination and immunoprecipitation.

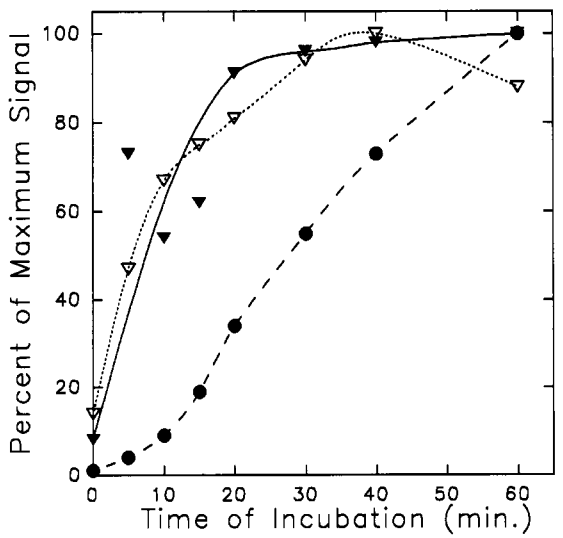

FIGURE 5: Time course of achieving ATP-independent glycosylation is similar to that of entry into the LCRI. The time course of glycosylation is shown by the closed circles, dashed line. Donor and acceptor membranes, cytosol, an ATP-regenerating system, and UDP-[^{3}H]GlcNAc were incubated as described under Materials and Methods for the times shown before immunoprecipitation. The time course of entry into the LCRI is shown by the open triangles, short dashed line. Donor and acceptor membranes, cytosol, and an ATP-regenerating system were incubated for the times shown and then diluted 10-fold into a mix containing the ATP-regenerating system, 2.5 μg/mL of commercial bovine UMP-kinase, and UDP-[^{3}H]-GlcNAc. After dilution, the incubations were continued for 60 min before immunoprecipitation. The time course of achieving ATP-independent glycosylation is shown by the closed triangles, solid line. Donor and acceptor membranes, cytosol, and an ATP-regenerating system were incubated for the times shown, pelleted as described under Materials and Methods, and then resuspended in buffer containing UDP-[^{3}H]GlcNAc, 25 mM KF, and 100 μM Na_3VO_4 and incubated for an additional 60 min before immunoprecipitation. For comparison, results are expressed as the percentage of the maximum amount of counts achieved in each time course. These maxima were 2428 cpm for the time course of glycosylation, 4196 cpm for the LCRI time course, and 1022 cpm for the ATP-independence time course.

POP Affects UDP-Glucosamine Uptake into the Golgi. Originally it had been thought that POP was acting directly in the process of fusing transport vesicles with their target membranes. However, the finding that UMPK activity could substitute for purified POP in the transport assay suggested that POP might be exhibiting apparent transport activity by enhancing the level of glycosylation used as a marker for transport. One possible mechanism for this effect would be

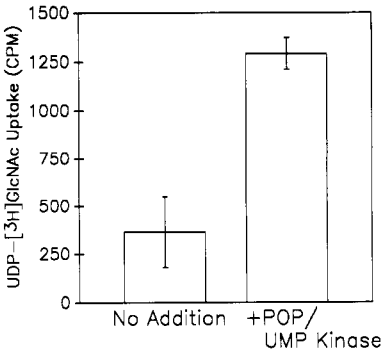

FIGURE 6: POP/UMPK enhances UDP-GlcNAc uptake into Golgi membranes under assay conditions. Acceptor membranes were incubated for 25 min at 37 °C in a mix which generates the LCRI (see Materials and Methods), pelleted, resuspended, and further incubated under the same conditions used to assay POP activity, with the exception that UDP-[6-^{3}H]GlcNAc was not included in the incubation. In this second incubation, one aliquot was incubated without any further additions, and a second was incubated with approximately 90 μg/mL (final concentration) of a partially purified POP fraction [purified through Mono-Q; see Figure 3 of Wattenberg et al. (1989)]. UDP-[6-^{3}H]GlcNAc uptake into Golgi membranes was determined as described under Materials and Methods. UDP-[6-^{3}H]GlcNAc uptake was completely blocked by the addition of 1 mM unlabeled UDP-GlcNAc (not shown). Shown is the mean plus and minus standard deviation of triplicate assays.

the scavenging of UMP in the cytosol at the expense of ATP. UMP is a an inhibitor of sugar nucleotide uptake into the Golgi (Perez & Hirschberg, 1985). The effect of UMPK on Golgi uptake of UDP-[^{3}H]GlcNAc was therefore directly tested. Golgi were incubated with UDP-[^{3}H]glucosamine in the presence or absence of purified yeast POP and assayed for UDP-GlcNAc uptake (Figure 6). This experiment demonstrated that the uptake of the UDP-GlcNAc was increased more than 3-fold by the presence of POP/UMPK activity. This suggests POP was detected as a required protein in the transport assay by virtue of its enhancement of the uptake of marker for glycosylation (UDP-[^{3}H]GlcNAc) and not by any effect on the fusion or processing of vesicles into acceptor Golgi.

Glycosylation Is Rate Limiting after Formation of the LCRI. It had been previously reported that glycosylation occurred essentially instantaneously after the transport of G protein to the acceptor compartment (Balch et al., 1984b). This result has been used to support the contention that the rate of G protein glycosylation in this assay is a direct measure of the rate of vesicle fusion. In contrast, as noted above, increasing the rate of sugar nucleotide uptake enhances the signal in the assay measuring the consumption of the LCRI, indicating that glycosylation itself might be rate limiting for the acquisition of [^{3}H]GlcNAc by G protein. To test this, the LCRI was formed as before, and the second incubation, in which the LCRI is consumed, was initiated (Figure 7). UDP-[^{3}H]GlcNAc was added either immediately when the second incubation was stated (open circles) or 20 min (filled circles) or 40 min (triangles) afterward. The time course of [^{3}H]GlcNAc incorporation into G protein was then measured. If vesicle fusion is the rate-limiting step, then adding sugar nucleotide after 20 or 40 min should lead to a burst of glycosylation of G protein, rapidly reaching the level of glycosylation seen when sugar nucleotide is present from the beginning of the reaction. Instead, however, it was observed that the rate of glycosylation was virtually identical whether sugar nucleotide was present at the beginning of the second incubation or after 20 or 40 min. This indicates that it is the glycosylation reaction itself, and not vesicle fusion, that is the

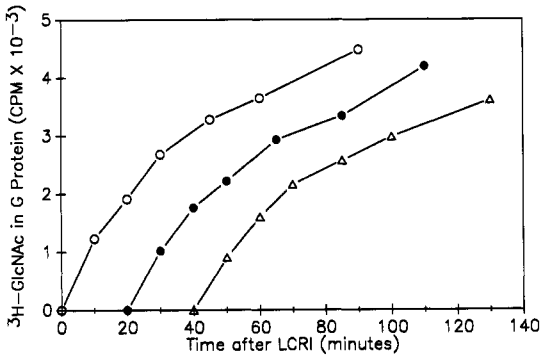

FIGURE 7: Glycosylation is rate limiting for incorporation of [^{3}H]-GlcNAc into G protein after the formation of the LCRI. To test whether the time course of G protein glycosylation reflects the time course of fusion, the LCRI was formed as described under Materials and Methods by preincubation of donor and acceptor membranes with CHO cytosol for 25 min at 37 °C, and then the membranes were pelleted and resuspended in buffer containing the ATP-regenerating system and 250 μg/mL bovine UMP-kinase. The time course of incorporation of [^{3}H]GlcNAc into VSV G protein was then measured for identical incubations where UDP-[^{3}H]GlcNAc (0.4 μM) was added after 0 (open circles), 20 (filled circles), and 40 (open triangles) min of initiation of the second incubation. Aliquots (50 μL) were taken at each time point and frozen until all points were collected and were then thawed and immunoprecipitated as described (Materials and Methods).

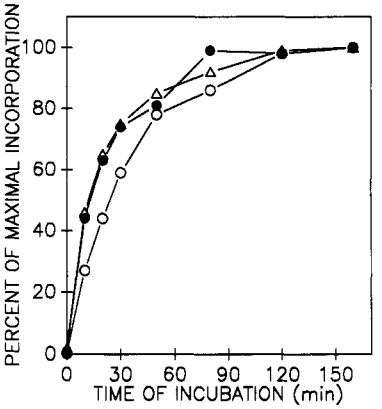

FIGURE 8: Increased concentrations of UDP-[^{3}H]GlcNAc increases the rate of incorporation of label into G protein after the formation of the LCRI. A time course of incorporation of [^{3}H]GlcNAc into G protein was measured after the formation of LCRI as described in the legend to Figure 7. UDP-[^{3}H]GlcNAc was added at either 0.4 (open circles), 4.0 (open triangles), or 20 μM (filled circles) immediately after resuspension of the preincubated donor and acceptor membranes, and aliquots were removed at the indicated times after initiation of the second incubation. For ease of comparison, [^{3}H]-GlcNAc incorporation in each time course is expressed as the percentage of incorporation at t = 160 min. That incorporation was 8465 cpm for 0.4 μM UDP-[^{3}H]GlcNAc, 15 613 cpm for 4.0 μM UDP-[^{3}H]GlcNAc, and 18 336 cpm for 20 μM UDP-[^{3}H]GlcNAc.

rate-limiting step in incorporation of [^{3}H]GlcNAc into G protein in this assay.

Since UMP-kinase appeared to increase [^{3}H]GlcNAc incorporation into G protein by enhancing the uptake of UDP-GlcNAc into the Golgi, it seemed reasonable that sugar nucleotide levels in the Golgi lumen might be rate limiting for the glycosylation reaction. To test this, the effect of sugar nucleotide concentration was measured on the rate of G protein glycosylation. It had previously been reported that the UDP-GlcNAc transporter in rat liver Golgi approaches saturation at 10–15 μM UDP-GlcNAc (Perez & Hirschberg, 1985) whereas only 0.4 μM [^{3}H]UDP is routinely used in the Golgi protein transport assay. Therefore, increasing the sugar nucleotide concentration in the assay mix should lead to increased levels in the Golgi lumen. The rate of [^{3}H]GlcNAc addition to G protein was measured after the formation of the LCRI in the presence of 0.4, 4.0, and 20 μM UDP-[^{3}H]-GlcNAc (Figure 8, open circles, open triangles, and filled circles, respectively). The higher levels of sugar nucleotide strongly accelerated the rate of glycosylation of G protein. The half-time of glycosylation at 0.4 μM sugar nucleotide was approximately 24 min, while the half-time at 4.0 and 20 μM sugar nucleotide was only 12–13 min. It is very unlikely that the levels of sugar nucleotide would affect the rate of vesicle fusion. This result therefore directly confirms that it is glycosylation, and not vesicle fusion, that is rate limiting in this reaction.

To test whether vesicle fusion might become rate limiting in the presence of high levels of sugar nucleotide, the rate of glycosylation was measured when sugar nucleotide was added either immediately or 20 or 40 min after the initiation of the post-LCRI reaction as described in the experiment depicted in Figure 7, but with near saturating (4.0 μM) levels of UDP-[^{3}H]GlcNAc (Figure 9). Even at the reduced half-time of glycosylation driven by this higher level of sugar nucleotide, there was no observable burst of glycosylation when sugar nucleotide was added at later times. This places an upper limit on the half-time of vesicle fusion after the formation of the LCRI at 12–15 min. The rate fusion could, however, be substantially faster.

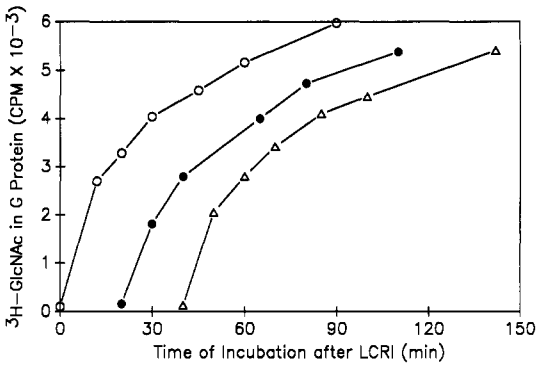

FIGURE 9: Glycosylation is rate limiting for incorporation of [^{3}H]-GlcNAc into G protein at a near saturating concentration of UDP-[^{3}H]GlcNAc. The kinetics of incorporation of [^{3}H]GlcNAc into G protein was measured when UDP-[^{3}H]GlcNAc was added either at t = 0 (open circles), t = 20 (filled circles), or t = 40 (open triangles) min after initiation of the post-LCRI reaction exactly as described in the legend to Figure 7 with the exception that 4.0 μM UDP-[^{3}H]GlcNAc was added.

Discussion

Cloning of POP Reveals It To Be a Protein Involved in Enhancing Glycosylation in the Transport Assay. Using a kinetically based assay, we previously identified a cytosolic protein, POP, which appeared to be required in the steps just preceding the fusion of Golgi transport vesicles with their target. We have utilized monoclonal antibodies against the yeast POP protein to screen an expression library of yeast genomic DNA in λ gt11. We unambiguously confirmed that our cloned fragment encoded POP by demonstrating that expression of the fragment in *E. coli* conferred POP activity onto lysates made from transformed cells. The identity of the appropriate reading frame in the original 6.0 kb fragment was determined by restriction analysis. This reading frame predicted a protein of 22 300 Da. An initial screening of the sequence database indicated a close homology to a number of nucleotide kinases. Closer analysis revealed an exact homology to yeast uridine monophosphokinase (UMPK).

The catalytic activity of uridine monophosphokinase is the exchange of phosphate between ATP and UMP to produce

UDP and ADP. The prospect that UMP-kinase might be exhibiting activity in our assay by reducing UMP levels was immediately apparent. Uridine nucleotides are present in our incubations both from UTP that is directly added, as well as endogenous nucleotides present in the Golgi preparations. There are vigorous phosphatases associated with the Golgi membranes as indicated by the requirement that an ATP-regenerating system, and not simply ATP alone, be used for transport to proceed. Therefore, there are certainly substantial levels of UMP under the assay conditions employed here. UMP is a predicted inhibitor of the Golgi transport assay because the assay depends on glycosylation as a marker for transport. The Golgi uptake of UDP-*N*-acetylglucosamine, the substrate for the glycosyltransferase used in the assay, has been shown to be mediated by an antiport mechanism (Perez & Hirschberg, 1985). This transporter exchanges UDP-GlcNAc for UMP. Therefore UMP on the cytoplasmic side of the Golgi will act as an inhibitor of UDP-GlcNAc uptake. A likely reason that UMP-kinase exhibits activity in our assay is that it scavenges UMP (utilizing the ATP supplied by the ATP-regenerating system) and thus increases UDP-GlcNAc transport, leading to an increased glycosylation signal. We directly confirmed this view by showing that UMP kinase increases UDP-GlcNAc uptake into the Golgi membranes used in the assay.

Glycosylation and Not Vesicle Fusion Is Rate Limiting in the LCRI Reaction. In an initial characterization of the Golgi transport assay, it was reported that the glycosylation reaction that adds GlcNAc to G protein was very rapid in comparison to the transport steps which deliver G protein from donor to acceptor (Balch et al., 1984b). It was thereafter assumed that the glycosylation reaction itself was not rate limiting in the transport assay and therefore that the rate of G protein labeling by [^{3}H]GlcNAc reflected the rate of vesicular transport and fusion. Thus it was surprising to find in this study that enhancing the rate of sugar nucleotide uptake into the Golgi strongly increased labeling in an assay designed to measure fusion steps selectively. This result indirectly suggested that glycosylation might be rate limiting. The finding that the rate of G protein glycosylation was independent of when sugar nucleotide label was added after initiation of the post-LCRI transport reaction illustrates that glycosylation is indeed slow relative to fusion. Finally, it was demonstrated that increasing the concentration of sugar nucleotide increased the rate of G protein glycosylation 2-fold in the transport assay. This directly shows that it is the rate of glycosylation, not the rate of vesicle fusion, that is being followed in this assay.

A Reassessment of Transport Intermediates in Golgi Transport; the LCRI, the "NEM-Resistant Intermediate", and the Involvement of Acyl-CoA's in Fusion. In order to consider the impact of these data on the current model of transport through the Golgi, it is necessary to review that model and the data that support it. The initial event in this model is the formation of nascent transport vesicles on donor membranes in a reaction known as "priming". The biochemical evidence for this event is that preincubation of donor membranes with cytosol and ATP before the addition of acceptor, reduces the 7–10-min lag time that is normally observed before [^{3}H]-GlcNAc begins to be incorporated into G protein at a linear rate (Balch et al., 1984b). This effect is selectively inhibited by primaquine. Preincubation of donor membranes results in a portion of the G protein in the donor to become resistant to the effects of primaquine (Hiebsch et al., 1991). A morphological transformation takes place under identical conditions. Structures resembling coated buds and vesicles form on the membrane surface (Balch et al., 1984b; Orci et al., 1986; Hiebsch et al., 1991). Biochemically and morphologically detected priming both require ATP, cytosol, and elevated temperature (Orci et al., 1986) and are sensitive to primaquine (Hiebsch et al., 1991). The interpretation of the priming reaction is unaffected by the results presented here for two reasons. First, the priming event occurs by preincubation of donor membranes and so is unrelated to glycosylation. Secondly, it has been directly shown that the initial lag time before glycosylation of G protein becomes linear is not due to slow sugar nucleotide uptake or glycosylation reactions (Balch et al., 1984b). Therefore, the lag time and conditions which affect it, such as donor priming, are related to transport reactions and not glycosylation.

It was previously thought that after vesicles attached to their target a series of prefusion intermediates were formed [for review, see Wattenberg (1990)]. The first of these is the LCRI. Its existence was inferred on the basis of the observation that, only 25 min into a reaction that continues for 60–90 min, an intermediate was formed whose consumption was solely dependent on POP (Wattenberg & Rothman, 1986; Wattenberg et al., 1989). The formation of the LCRI is dependent on coincubation of donor and acceptor, leading to the interpretation that the LCRI represented a prefusion intermediate. After the LCRI is consumed, it was proposed that a second prefusion intermediate is formed (Balch et al., 1984b). When the alkylating agent NEM is added to incubations, glycosylation will briefly continue and then subside. Glycosylation during this period is ATP dependent. The small amount of glycosylation occurring after NEM treatment was interpreted to represent G protein in a final prefusion state. The ultimate steps in fusion were therefore thought to be NEM resistant. The existence of this so called "NEM-resistant intermediate" can now be seen to be a misinterpretation of the data. UMPK is sensitive to NEM (Figure 2). The brief period of glycosylation occurring after NEM treatment most probably results from the time required to inhibit UMPK and build UMP levels. As shown in Figures 4 and 5, the ATP dependence is due to the action of UMPK.

Does the LCRI represent a prefusion intermediate? The half-time of glycosylation in the assay for post-LCRI reactions is approximately 12 min when using nearly saturating levels of sugar nucleotide. This is therefore the upper limit on the half-time of vesicle fusion after the formation of the LCRI. Without an independent measurement of vesicle fusion, it is not possible to determine if the actual fusion rate is substantially faster than this. We think it likely that the formation of the LCRI reflects fusion itself. In that case the LCRI would represent a state where all G protein oligosaccharides are exposed to the lumen of the acceptor Golgi and are simply awaiting glycosylation. This would explain why after the formation of the LCRI there is no longer a requirement for cytosolic factors or ATP.

A requirement for acyl-CoA's in transport through the Golgi at both vesicle budding and vesicle fusion steps has been suggested in several studies (Glick & Rothman, 1987; Pfanner et al., 1989; Pfanner et al., 1990). In probing the involvement of acyl-CoA's in vesicle fusion, Pfanner et al. (1990) concluded that acyl-CoA's were required after the formation of the LCRI. Our results might indicate that the action of acyl-CoA's is not on transport itself, but on glycosylation. We have not detected, however, an effect of acyl-CoA's on glycosylation in direct tests (data not shown). The acyl-CoA's may be required for maintaining or enhancing the integrity of the Golgi membranes. This explanation is supported by the finding

that the requirement for acyl-CoA's is amplified by the presence of ethanol or detergent. This would also account for the apparent acyl-CoA requirement in both vesicle formation and fusion; the stabilizing effect would be equally operative for donor and acceptor membranes. However, a direct role for acyl-CoA's in transport is not ruled out by our data.

The existing model of vesicular traffic in the cell-free Golgi transport system must be altered to accommodate these new findings. Previously it was thought that, after the formation of transport vesicles, the action of NSF and the SNAPs brought about the formation of the a prefusion intermediate, LCRI. Consumption of the LCRI was supposed to involve some lengthy, uncharacterized processing of vesicle and/or target membranes requiring acyl CoA's, to allow fusion to occur, and that fusion was preceded by the formation of final intermediate whose consumption was resistant to NEM. It is now clear that fusion occurs rapidly after, if not coincident with, the formation of the LCRI and that it is likely that there is no measurable NEM-resistant prefusion intermediate.

This reinterpretation is satisfying in that there is no longer a need to invoke a mysteriously lengthy processing of vesicles or target membranes before fusion occurs. It appears likely that vesicles fuse very quickly after contact with the acceptor membranes. In addition, it is evident that the entire transport reaction is much more rapid than previously thought. Assuming that the formation of the LCRI represents vesicle fusion, the half-time of the transport reaction is approximately 12 min (Wattenberg et al., 1986). The time course of transport measured in the assay is therefore on a time scale consistent with the transit of proteins through the Golgi as measured in vivo.

When a complex cellular process is reconstituted in vitro, the investigator must be aware that constraints have been placed on the process that would not exist in vivo. Similarly, some controls placed on a process in vivo may be lifted in an in vitro setting. Many of the assays for intercompartmental transport rely on glycosylation as an indicator of transport (Brandli, 1991). The findings reported here are an important reminder that biochemical approaches must optimize not only the transport process itself but also the glycosylation apparatus used as a reporter system. In addition, the influence of the rate of glycosylation on kinetic measurements must be kept in mind when interpreting those studies. Our success in defining the mechanism by which POP/UMP-kinase exhibits activity demonstrates that a combined biochemical, molecular biological approach can distinguish those components involved in protein transport reactions from those that optimize other aspects of an in vitro assay.

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