Sequential Intermediates in the Transport of Protein between the Endoplasmic Reticulum and the Golgi*

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Semi-intact cells, a cell population in which the plasma membrane is perforated to expose intact intracellular organelles (Beckers, C. J. M., Keller, D. S., and Balch, W. E. (1987) Cell 50, 523–534), efficiently reconstitute vesicular trafficking of protein from the endoplasmic reticulum (ER) to the cis Golgi compartment. We now extend these studies to biochemically dissect transport of protein between the ER and the Golgi into a series of sequential intermediate steps involved in the budding and fusion of carrier vesicles. At least two broad categories of transport intermediates can be detected, those that involve early steps in transport and those involved in late, fusion-related events. Early transport steps require the transport of protein through a novel intermediate compartment in which protein accumulates at reduced temperature (10 °C). We demonstrate that both entry and exit from this 10 °C compartment can be successfully reconstituted in vitro. A late step in delivery of protein to the cis Golgi compartment requires Ca2+ (pCa7) and is coincident with a step which is sensitive to a peptide analog which blocks interaction between the Rab family of small GTP-binding proteins and a downstream effector protein(s) (Plutner, H., Schwaninger, R., Pind, S., and Balch, W. E. (1990) EMBO J. 9, 2375–2384). The combined results suggest that a single round of vesicular transport between the ER and the Golgi involves a rapid transit through N-ethylmaleimide-sensitive, guanosine 5'-[3-O-thio]triphosphate-sensitive, ATP- and cytosol-dependent step(s) involved in vesicle formation or transport to a novel intermediate compartment, followed by a regulated fusion event triggered in the presence of Ca2+ and functional components interacting with member(s) of the Rab gene family.

Significant progress toward an understanding of the biochemical basis for the vesicular transport of protein through the secretory pathway of eukaryotic cells has been afforded by the development of novel in vitro assays which reconstitute most stages of transport from the endoplasmic reticulum (ER) to the cell surface (Balch et al., 1984a; Beckers et al., 1987; Rothman, 1987; Baker et al., 1986; Ruohola et al., 1988; de Curtis and Simons, 1988, 1989). Transport between each stage involves the cyclical budding and fusion of transport vesicles and is likely to have both common biochemical components as well as unique components which assure specificity to different stages of intercompartmental transport. Yeast genetics have now defined at least 26 proteins including the SEC (Novick et al., 1980), BET (Newman and Ferro-Novick, 1987), YPT (Segev et al., 1988; Schmitt et al., 1988; Bacon et al., 1989), SAR 1 (Nakano and Muramatsu, 1989) and ARF (Stearns et al., 1990) gene products which are required for transport of protein from the ER to the cell surface. Of these, 11 SEC proteins (Novick et al., 1981), RPT1p, SAR1p, YPTp, and ARFp are required for transport of protein between the ER and the Golgi. When temperature-sensitive mutants defective in each of these gene products are incubated at the restrictive temperature they accumulate secretory protein in the ER, elaborate ER, and Golgi membranes, and, in some cases, accumulate populations of intermediate carrier vesicles (Novick et al., 1981; Segev et al., 1988; Kaiser and Schekman, 1990). These ER to Golgi-specific proteins include both soluble and membrane-associated components. In addition, genetic interactions between the various SEC gene products involved in transport between the ER and the Golgi (Kaiser and Schekman, 1990), and between the trans Golgi and cell surface in yeast (Salminen and Novick, 1987, 1989; Walworth et al., 1989) suggest that these components function in the context of a complex biochemical machine which coordinates vesicular transport of protein.

To focus on transport between early compartments of the secretory pathway, we have developed an in vitro assay which measures the transport of vesicular stomatitis virus (VSV) G protein between the ER and the Golgi of mammalian cells (Beckers et al., 1987; Beckers and Balch, 1989). This assay involves the use of semi-intact cells, a population of cells in which the plasma membrane has been perforated to reveal intact, intracellular organelles (Beckers et al., 1987). Transport is detected by following the processing in vitro of the high mannose (mana) asparagine-linked core-oligosaccharide of the vesicular stomatitis virus G (VSV-G) protein acquired in the ER to a form containing only 5 mannose residues, a reaction diagnostic of the cis Golgi enzyme α-1,2-mannosidase I (Tabas and Kornfeld, 1979; Balch et al., 1986; Beckers et al., 1987). Transport of VSV-G protein between the cis and medial (Balch et al., 1984a) and the medial and trans Golgi compartments (Rothman, 1987) has also been successfully reconsti-
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EXPERIMENTAL PROCEDURES

Materials—Semi-intact cells used for the analysis of ER to Golgi transport were prepared from clone 15B cells infected with the wild-type or tsO45 strain of VSV using the swelling method as described previously (Beckers et al., 1987). Trans-[35S]-label [32P]methylamine and [35S]cysteine (>1000 Ci/mmole) was purchased from ICN Biomedicals, Inc. (Irvine, CA). Cytosol used in transport assays was prepared from uninfected 15B cells as described previously (Beckers et al., 1987; Beckers and Balch, 1989). GTPyS was obtained from Boehringer Mannheim. Endoglycosidase D (endo D) was obtained from Boehringer Mannheim or prepared from the culture supernatant of Diplococcus pneumoniae (Glasgow et al., 1977). Stock solutions of N-ethylmaleimide (NEM) (100 mM in H2O) or glutathione (GSH) (100 mM in 50 mM Hepes-KOH, pH 7) were prepared immediately prior to use.

Incubation Conditions and Analysis of Transport—The ER to Golgi transport assays using semi-intact cells were performed as described previously (Beckers et al., 1987; Beckers and Balch, 1989). Briefly, transport incubations contained in a final total volume of 40 µl (final concentration): 25 mM Hepes-KOH, pH 7.2, 90 mM KCl, 2.5 MtpAc, 5 mM EDTA, 1.8 mM CaCl2, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU of rabbit muscle creatine phosphokinase, 25 µg of cytosol, and 5 µl (25–30 µg of protein; 1–2 x 106) of semi-intact cells. Transport was initiated by transfer to 30 °C. Assays were supplemented with additional reagents as indicated under “Results.” After termination of transport by transfer to ice, the membranes were pelleted by a brief (30 s) centrifugation in a microcentrifuge at top speed. For endo D treatment, the pellet was subsequently solubilized in an endo D digestion buffer and digested with endo D as described previously (Beckers et al., 1987). For endoglycosidase H digestion, samples were pelleted and the material solubilized by the addition of 50 µl of 0.1% sodium deoxycholate in 100 mM NaOAc, pH 5.5, and bound glycoproteins were digested overnight at 37 °C in the presence of 1 milliunit of endoglycosidase H. Endo D and endoglycosidase H digestions were terminated by adding a 5 x concentrated gel sample buffer (Laemmli, 1970) and boiling for 5 min. The samples were analyzed by sodium dodecyl sulfate-polycrylamide gel electrophoresis using 7.5% acrylamide gels (Beckers et al., 1987), autoradiographed, and the fraction of VSV-G protein processed to the endo D-sensitive or endo H-resistant forms was determined by densitometry (Beckers et al., 1987; Beckers and Balch, 1989).

RESULTS

Cytosol Is Required for an Early Step in Transport—We have previously shown that the appearance of the processed form of VSV-G protein occurs only after a 10–20 min lag period during incubation of semi-intact cells in the presence of cytosol (a high speed supernatant of crude cell homogenate) and ATP, and is followed by a linear period of processing in which 40–70% of the total VSV-G protein is trimmed to the man5 oligosaccharide form after a 90 min incubation (Beckers et al., 1987; Beckers and Balch, 1989). The lag period is likely to comprise events which are required for the formation (budding) of carrier vesicles from the ER and their delivery (fusion) to the cis Golgi compartment. If soluble components found in the cytosol are required only for an early step in transport, then incubation of cells for a short period of time followed by pelleting and reincubation in a cytosol-free mixture would result in the continued transport of VSV-G protein to the cis Golgi. This would result in the appearance of a significantly larger fraction of processed (man5) VSV-G protein than that found at the time of cytosol depletion. Alternatively, if components in the cytosolic fraction are required at a late step, such as fusion of a carrier vesicle to the cis Golgi compartment, then processing would stop immediately upon the removal of cytosol since processing by α-1,2-mannosidase I per se is not rate limiting (Beckers and Balch, 1989; see below). In this case, the amount of VSV-G protein processed after additional incubation in a cytosol-free mixture would be expected to be identical to that observed at the time of cytosol depletion.

To test these two possibilities, semi-intact cells were incubated for increasing time at 30 °C in the presence of cytosol prior to pelleting and reincubation in its absence for a total time of 90 min at 30 °C. As shown in Fig. 1, after a 10 min incubation in the presence of cytosol, a time at which less than 10–15% of the total VSV-G protein transported had been processed to the man5-oligosaccharide form (Fig. 1, open circles), greater than 60–70% of the total VSV-G protein transported was processed upon further incubation in a cytosol-free mixture (Fig. 1, closed circles). These results suggest that soluble components essential for vesicular transport either complete their function early, or rapidly associate with and thereby prime the ER and Golgi for use during a later step.

A Membrane-associated Factor Required Early in Transport Is Rapidly Dissociated upon Incubation in Vitro—Semi-intact cells define a unique environment in which the ER, the Golgi, and all of the transported VSV-G protein are efficiently retained within the perforated cell boundary for up to 90 min of incubation in vitro (Beckers et al., 1987; Beckers and Balch, 1989). These results suggest that both the rate and extent of transport of VSV-G protein between the ER and the Golgi should be largely independent of the concentration of semi-intact cells present in the transport reaction. Alternatively, since pretreatment of membranes on ice in the presence of

trypsin efficiently inactivates transport (data not shown), membrane-associated factors likely to be involved in the budding and fusion of carrier vesicles may also undergo cyclical dissociation and reassociation with membranes. In this case, the concentration of semi-intact cells used in the assay may substantially effect the observed kinetics of transport.

To investigate the role of membrane-associated components, semi-intact cells were incubated in an increasing volume of the complete incubation mixture containing a constant concentration of cytosol and ATP. A striking dependence was observed between the rate of transport and the final membrane concentration (Fig. 2, inset, open circles). In contrast, when semi-intact cells containing VSV-G protein were incubated in an increasing volume of the complete incubation mixture which was additionally supplemented with uninfected semi-intact cells to insure a constant total membrane concentration, no dilution effect was observed in the assay (Fig. 2, inset, closed circles). The results suggest that a diffusible, rate-limiting factor dissociates from semi-intact cells after initiation of transport.

Since the dilution-sensitive factor defines a rate-limiting step, we investigated the temporal requirement for this factor(s) in transport. For this purpose, a complete incubation containing semi-intact cells, cytosol, and ATP was incubated for increasing time at 30 °C prior to dilution into a 10-fold excess mixture, containing ATP and cytosol. Subsequently, membranes were incubated for a total time of 90 min. If the dissociable factor was required at an early step in transport, we would expect that sensitivity of the membrane fraction to dilution would be rapidly lost during initial periods of incubation at 30 °C. Alternatively, if the factor was required late in transport, we would expect an abrupt inhibition of processing of VSV-G protein upon dilution at all times of transport. As shown in Fig. 2 (closed circles), after incubation for 10–15 min, a time point at which less than 10–20% of the total VSV-G protein has been processed to the manα form, greater than 80% of the total VSV-G protein transported had passed through the dilution-sensitive step. These results suggest that the dilution-sensitive component functions early in the transport of VSV-G protein between the ER and the Golgi.

**NEM-sensitive Factor(s) Are Required during an Early Step in Transport**—In order to dissect the role of components in vesicular transport, we analyzed the temporal effect of NEM on ER to Golgi transport. When Golgi membranes are pretreated with NEM, their ability to transport protein between Golgi compartments is lost (Balch et al., 1984b, Malhotra et al., 1988). Transport activity can be restored to NEM-treated Golgi membranes by the addition of a soluble cytosolic factor, or the supernatant fraction prepared by preincubation of Golgi membranes in the presence of ATP (Malhotra et al., 1988). This restorative factor, referred to as NSF (Block et al., 1988), is a homotetramer of a 76-kDa protein which also requires both an integral membrane protein receptor and low molecular mass soluble factors for function (Weidman et al., 1989). Morphological and biochemical studies of intra-Golgi transport have shown that uncoated transport vesicles accumulate in the absence of NSF, indicating that formation of transport vesicles, but not their consumption is independent of NSF (Malhotra et al., 1988; Orci et al., 1989). As such, NSF is believed to function as a component of a prefusion molecular complex which promotes delivery of carrier vesicle content to the target Golgi compartment (Malhotra et al., 1988; Wattenberg and Rothman, 1986, Orci et al., 1989; Wattenberg et al., 1986, 1990). In addition, NSF is 48% homologous to the SEC18 gene product (Wilson et al., 1989), an essential protein involved in ER to Golgi transport in yeast (Novick et al., 1980). Incubation of the temperature-sensitive SEC18 mutant at the restrictive temperature results in the aberrant accumulation of transport vesicles between the ER and the Golgi compartment (Kaiser and Schekman, 1990). In mammalian cells, we have shown that an antibody to NSF, which inhibits intra-Golgi transport in vitro (Block et al., 1989), also inhibits transport of protein from the ER to the cis Golgi compartment in vitro (Beckers et al., 1989).

To explore the temporal requirements for NSF and other NEM-sensitive components in VSV-G protein transport, we took advantage of the exceptionally rapid inactivation of ER to Golgi transport by 1 mM NEM at 30 °C (<1 min) (Beckers et al., 1989). A complete incubation mixture containing semi-intact cells, cytosol, and ATP was incubated for increasing time at 30 °C prior to the addition of 1 mM NEM. Subsequently, cells were incubated in the presence of NEM for a total of 90 min to allow any VSV-G protein which had progressed beyond the NEM-sensitive step(s) at the time of addition of NEM to be processed to the manα-oligosaccharide form. If the NEM-sensitive step is a late event in transport (or if α-1,2-mannosidase I is NEM sensitive), then addition of NEM will result in an abrupt inhibition of processing yielding a level of trimmed VSV-G protein identical to that observed for a parallel control incubation terminated by transfer to ice at the time of NEM addition. In contrast, if the NEM-sensitive step is an early event in transport then VSV-G protein will be transported through this step after a brief period of incubation. In this case, the amount of processed VSV-G protein observed after an additional 90 min of incubation in the presence of NEM will exceed that of the control incubation.

As shown in Fig. 3 (open squares), after only 10–15 min of incubation, a time period in which less than 10–20% of the total VSV-G protein transported could be detected in the manα-processed form, nearly 70–80% of the total VSV-G protein observed after an additional 90 min of incubation in the presence of NEM will exceed that of the control incubation.

![Fig. 2. The dilution-sensitive component is required during an early step in transport.](http://www.jbc.org/fig.png)
protein transported was processed in the presence of NEM. These results suggest that a large fraction of VSV-G protein is rapidly mobilized to a NEM-resistant intermediate step during the typical 10–15 min "lag period" in transport. Since these results were similar to the mobilization of VSV-G protein to a GTPyS-resistant transport intermediate (Beckers and Balch, 1989), we tested in parallel the effect of NEM and GTPyS on the same preparation of semi-intact cells. As shown in Fig. 3 (open triangles), addition of GTPyS after increasing time of incubation gave nearly identical results to those observed for NEM.

Although the kinetic studies could not directly resolve a temporal difference between the function of NEM and GTPyS-sensitive components in transport, a different approach could be used to order these two steps. A transport reaction containing NEM-inactivated membranes and cytosol was incubated in the presence of GTPyS for 20 min at 30 °C, a time period sufficient for complete inactivation by GTPyS (Beckers and Balch, 1989). If the GTPyS and NEM-sensitive steps are sequential and the NEM-sensitive step precedes the GTPyS-sensitive step, then no inactivation of transport is likely to occur by pretreatment of cells for 20 min with GTPyS. On the other hand, if the GTPyS step precedes the NEM-sensitive step, or inactivates transport in a fashion independent of NSF activity, transport will be inhibited when cells are pelleted and resuspended in a mixture containing 1 mM GTP and fresh cytosol. As shown in Fig. 4, preincubation of NEM-treated semi-intact cells and cytosol with GTPyS inhibited subsequent transport in the presence of GTP and fresh cytosol. Since GTPyS inactivates transport with a t½ of 2–3 min (Beckers and Balch, 1989) in contrast to the more rapid inactivation by NEM (t½ of 15 s (Beckers et al., 1989)), the data favor the interpretation that the GTPyS step precedes the NEM-sensitive step in transport.

ATP Is Required for an Early Step in ER to Golgi Transport—Transport requires energy in the form of ATP (Beckers et al., 1987). To define the temporal importance of ATP in transport, semi-intact cells and cytosol were incubated in the presence of ATP prior to rapid depletion (less than 30 s) by addition of hexokinase/glucose to the transport reaction (Balch and Rothman, 1985). Subsequently, cells were incubated in the absence of ATP for a total time of 90 min. Using this experimental strategy, if the ATP-dependent step is an early event, any VSV-G protein which is mobilized through the ATP-free period will continue to be transported to the cis Golgi compartment, and if ATP is required immediately prior to fusion of a transport vesicle with the cis Golgi, then ATP depletion will result in an abrupt inhibition of transport and processing. As shown in Fig. 5, after a 10–15 min incubation in the presence of ATP, a time point at which only 20–25% of the total VSV-G protein transported was processed by cis Golgi compartments (Fig. 5, open circles), nearly 70–80% of the total VSV-G protein transported did not require ATP for subsequent processing (Fig. 5, closed circles). These results suggest that during the first 10–15 min of incubation in vitro a large fraction of the VSV-G has been transported through an "activated" intermediate. Transport steps from this intermediate culminating in fusion with the cis Golgi compartment appear to be independent of the presence of ATP in the assay buffer. These results do not preclude the possibility that ATP bound in an early step in transport is utilized at a late step.

VSV-G Protein Accumulates in a Late Transport Intermediate in the Presence of EGTA—We have recently provided direct evidence for a role of Ca²⁺ in the regulation of vesicular transport of protein between the ER and the Golgi (Beckers and Balch, 1989). Transport was shown to be strongly inhibited by incubation in the presence of EGTA (Beckers and
Balch, 1989) and is optimal when the free Ca\(^{2+}\) is adjusted between 0.03–0.3 \(\mu\)M (Beckers and Balch, 1989), values similar to the physiological levels of intracellular Ca\(^{2+}\) (Carafoli, 1987; Berridge and Irvine, 1989). Previous studies using the Ca\(^{2+}\) ionophore A23187 have also alluded to the possible importance of Ca\(^{2+}\) in vesicular transport. Incubation of plasma cells in the presence of A23187 and EGTA inhibits the processing and transport of immunoglobulin to the cell surface (Tartakoff and Vassalli, 1977), as well as the transport of procollagen and fibronectin from fibroblasts (Uchida et al., 1979). At least two yeast gene products have been implicated in Ca\(^{2+}\)-related secretory function in yeast. A temperature-sensitive mutation in YPT1 (ypt1\(^{ts}\)), a gene product essential for ER to Golgi transport, causes growth arrest and a defect in secretion when the mutant is grown at the restrictive temperature (Schmitt et al., 1988). Inhibition of secretion can be partially reversed by growing cells in the presence of elevated extracellular Ca\(^{2+}\), leading to speculation that YPT1p may be involved in Ca\(^{2+}\) regulation (Schmitt et al., 1988). A defect in a second gene, PMR1 which encodes an ER-associated P-type Ca\(^{2+}\)-ATPase, also results in the abnormal transport of secreted protein (Smith et al., 1989; Rudolph et al., 1989). Deletion of PMR1 results in yeast which grow poorly on media containing low concentrations of Ca\(^{2+}\) are hypersensitive to EGTA and secrete proteins normally retained or degraded by the ER (Rudolph et al., 1989). Deletion of PMR1 also suppresses a ypt1-1 mutant defective in ER to Golgi transport (Rudolph et al., 1989), suggesting an interaction between PMR1p and YPT1p. In addition, an in vitro assay which reconstitutes the transport of protein between yeast ER and Golgi membranes requires Ca\(^{2+}\) (Baker et al., 1990).

We have previously demonstrated that inhibition of transport by EGTA in vitro is fully reversible (Beckers and Balch, 1989). Transport from the EGTA-sensitive step requires cytosolic components including NSF (Beckers and Balch, 1989) and ATP, but is insensitive to the non-hydrolyzable analog of GTP, GTPyS (Beckers et al., 1989). To begin to dissect the possible role(s) of Ca\(^{2+}\) in vesicle formation, delivery, or fusion, the kinetics of transport of VSV-G protein from the EGTA-sensitive step were examined. Semi-intact cells, cytosol, and ATP were incubated for 60 min at 30 °C in the presence of 5 mM EGTA to allow VSV-G to be transported to the step inhibited in the absence of Ca\(^{2+}\). Semi-intact cells were subsequently pelleted and resuspended in the presence of the EGTA/Ca\(^{2+}\) buffer (pCa7). As shown in Fig. 6, panel A, after a 60-min incubation in the presence of EGTA, no VSV-G protein was transported to the cis Golgi compartment as indicated by the absence of the endo D-sensitive form of VSV-G (Fig. 6, closed circles, \(\Delta t = 0\) min). Release of VSV-G protein from the EGTA block resulted in rapid transport to the cis Golgi compartment (Fig. 6, panel A, closed circles) without the characteristic lag period observed for ER to Golgi transport (Fig. 6, panel A, open circles). The initial rate of transport from the EGTA-sensitive step is nearly five times the rate of transport of VSV-G protein from the ER under identical conditions (Fig. 6, panel A, compare closed with open circles). These results tentatively suggest that it is unlikely that EGTA blocks an early step involved in vesicle formation, but rather, that the absence of Ca\(^{2+}\) inhibits a late, prefusion step allowing VSV-G protein to exit the ER and accumulate at the face of the cis Golgi.

To define the temporal location of the block imposed by Ca\(^{2+}\) depletion, semi-intact cells, cytosol, and ATP were incubated for increasing time at 30 °C prior to the addition of EGTA. After addition of EGTA, cells were incubated for an additional total time of 90 min to allow any VSV-G protein which had been transported past the Ca\(^{2+}\) requiring step prior to the addition of EGTA to be delivered to the cis Golgi. As shown in Fig. 7, addition of EGTA to the assay resulted in an abrupt inhibition of transport of VSV-G protein. In this case, the amount of VSV-G protein processed to the man-,oligosaccharide form after an additional 90 min of incubation in the presence of EGTA (Fig. 7, closed circles) was identical to that observed in a parallel control incubation which was terminated by transfer to ice at the time of EGTA addition (Fig. 7, open circles). These results are in direct contrast to the NEM and GTPyS-resistant intermediates in which the rate of mobilization into these intermediates is nearly 2–3-fold the rate of fusion of vesicles with the cis Golgi compartment. It is apparent that Ca\(^{2+}\) is required at late step, presumably immediately preceding fusion to the cis Golgi compartment.

In contrast to the reversibility of inhibition of transport by EGTA, preincubation of semi intact cells in the presence of elevated Ca\(^{2+}\) results in an irreversible inactivation of transport.
port. As shown in Fig. 6 (panel B), when semi-intact cells were incubated in the presence of 10 μM Ca2+ transport is inhibited by nearly 80% (Fig. 6, panel B, lane c). Transport from the site of Ca2+ inhibition could not be reinitiated by reincubation in the presence of the EGTA/Ca2+ buffer (pCa7) (Fig. 6, inset, lane e). Analysis of the temporal effect of elevated Ca2+ (10 μM) suggests that an early step, possibly export from the ER, is sensitive to high Ca2+ (data not shown).

Calcium Ionophore A23187 Inhibits Transport of VSV-G Protein in Intact Cells—To extend our observations that Ca2+ is an essential factor for transport of VSV-G protein between the ER and Golgi in vivo, we examined whether Ca2+-dependent transport of VSV-G protein could be detected using the Ca2+ ionophore A23187. For this purpose, 15B cells infected with ts045-VSV-G protein were treated with increasing concentrations of A23187 for 10 min at the restrictive temperature (40 °C). Cells were subsequently shifted to the permissive temperature (30 °C) and incubated for an additional total time of 60 min (closed circles).

To examine the time at which VSV-G protein transport between the ER and the Golgi in vivo was sensitive to inhibition by A23187, intact cells incubating at 40 °C were shifted to the permissive temperature (30 °C), and at various times after shift to 30 °C A23187 was added to the cells in the presence of 1 mM extracellular Ca2+ or in the presence of 1 mM extracellular EGTA (no Ca2+). Cells were subsequently incubated for an additional total time of 60 min to allow any VSV-G protein which had been transported past the step inhibited by nearly 80% (Fig. 6, panel B, closed circles). The latter results suggest that an early transport step immediately preceding fusion to the cis Golgi is sensitive to Ca2+ depletion. In contrast, addition of A23187 in the presence of 1 mM extracellular Ca2+ caused complete inhibition of transport only when added immediately prior to shifting to the permissive temperature, but not at later time points (Fig. 8, panel B, closed squares). The results suggest that an early transport step, like that observed in vitro (Fig. 8, panel B, closed circles), is sensitive to elevated Ca2+.

VSV-G Protein Accumulates in a Transport Intermediate at Reduced Temperature (15 °C)—Incubation of cells at reduced temperature has provided an exceptionally useful approach for accumulation of protein in subcellular compartments of both the endocytic and exocytic pathways. In the secretory pathway, prolonged incubation of cells at reduced temperature (15 °C) results in the accumulation of newly synthesized proteins in a pre-Golgi compartment which consists of a heterogeneous collection of tubular-vesicular membranes which are morphologically and biochemically distinct from the rough endoplasmic reticulum (RER) (Sara& Hed-
We have previously established conditions to study the role of the 15 °C intermediate in vitro by following the transport of vesicular stomatitis virus G protein (Balch et al., 1986). When VSV-G is radiolabeled with [35S]methionine and subsequently chased for up to 90 min at 15 °C, no protein can be detected in the manα1,2-oligosaccharide form, a modification indicative of delivery to the cis Golgi compartment (Balch et al., 1986). However, when these cells are shifted from 15 to 30 °C, VSV-G protein is transported to the cis Golgi compartment with kinetics which exceed the normal rate of transport from the RER, and which lack the characteristic 10–15 min lag period observed in vivo for transport from its site of synthesis in the RER (Balch et al., 1986).

To relate the biochemical requirements for transport of protein from the 15 °C intermediate to those involved in export from their site of synthesis in the RER, transport in vitro from the 15 °C intermediate was analyzed using two different experimental approaches. In the first approach, VSV-G protein was chased in vivo by following the transport of VSV-G protein from the RER (Fig. 9, open circles) is not observed. Instead, VSV-G processing by α-1,2-mannosidase I to manα1,2-oligosaccharide form is initiated at the onset of incubation (Fig. 9, closed circles). In addition, there is a 2-fold increase in the rate of delivery to the cis Golgi compartment compared with transport from the RER and a higher percentage of the total VSV-G protein transported compared with export from the RER. These results not only indicate that the kinetic properties of transport from the 15 °C intermediate observed in vivo have been successfully reconstituted in vitro, but also suggest that processing by α-1,2-mannosidase I is not a rate-limiting reaction in RER to Golgi transport.

Using a second approach, we tested the ability of semi-intact cells to reconstitute transport of VSV-G protein from the RER to the 15 °C intermediate in vitro. In this case, semi-intact cells containing VSV-G protein in the RER were prepared following our standard protocol for study of ER export. Subsequently, these cells were incubated at 15 °C (Fig. 10, closed circles) or 30 °C (Fig. 10, open circles) in the presence of cytosol and ATP. The control incubation at 30 °C showed the usual lag (15–20 min) in the appearance of the processed form of VSV-G protein (Fig. 10, open circles). In contrast, no processing was observed during incubation at 15 °C for 90 min (Fig. 10, closed circles). After 90 min of incubation at 15 °C, semi-intact cells were shifted to the permissive temperature (30 °C). In this case, (Fig. 10, closed circles) only a short lag (<5 min) was observed before initiation of processing by α-1,2-mannosidase I. These results suggest that VSV-G protein has been mobilized into a transport intermediate during incubation in vitro at 15 °C. It is apparent that semi-intact cells can efficiently reconstitute both the delivery to and exit from the 15 °C intermediate.

**Transport from the 15 °C Intermediate to the cis Golgi Compartment Is Insensitive to Homogenization**—Transport of VSV-G protein from its site of synthesis in the RER to the Golgi in vitro is inhibited when cells are homogenized prior to incubation (Beckers et al., 1987). Homogenization leads to extensive vesiculation of the ER and perhaps a loss of RER export function through physical separation of the site of VSV-G protein synthesis from putative transitional regions of the ER believed to be involved in protein export (Palade, 1975). If efficient transport from the 15 °C intermediate is a consequence of the accumulation of VSV-G protein in a transitional region of the ER, or transfer to a novel intermediate vesicular compartment, then transport from this site may be insensitive to homogenization. In support of this conclusion, transport of VSV-G protein in vitro between highly enriched Golgi compartments (Balch et al., 1984a), or from ER prepared from mitotic cells (Balch et al., 1987) has established that compact Golgi compartments, or physiologically disassembled ER can efficiently reconstitute transport using cell homogenates.

To examine the possibility that VSV-G proteins accumulate in a physically distinct compartment at 15 °C, semi-intact cells containing VSV-G protein in the 15 °C compartment were divided into two portions. One portion was preincubated at 15 °C for 60 min prior to homogenization; the remaining portion was homogenized after 60 min on ice. When these cell homogenates were incubated in the presence of cytosol and ATP at 30 °C, no transport was observed from homoge-
nates containing VSV-G protein in the RER (Fig. 11, lane b). In contrast, efficient transport (>40–60%) was routinely observed from homogenates prepared from cells which had been preincubated for 60 min at 15 °C (Fig. 11, lane d). Similar results were obtained when homogenates were prepared from intact cells containing VSV-G protein in the RER or the 15 °C intermediate, respectively (data not shown). These results suggest that the majority of VSV-G protein moves from a homogenization-sensitive site in the RER to a distinct homogenization-insensitive site during incubation at 15 °C.

Transport from the 15 °C Intermediate Requires ATP and Reduced Cytosol—As shown in Fig. 12 (inset), transport from the 15 °C compartment to the cis Golgi requires both ATP and cytosol. Since processing of oligosaccharides by \( \alpha-1,2- \) mannosidase I does not require ATP or additional cytosolic components (Tulsiani and Touster, 1988), it is reasonable to assume that the homogenization-resistant compartment is a pre-cis Golgi compartment. However, since the concentration of cytosol contributes significantly to the rate of RER to Golgi transport (Beckers et al., 1987; data not shown), we examined whether transport from the 15 °C intermediate is similarly sensitive to the concentration of cytosol present in the assay. For this purpose, semi-intact cells containing VSV-G protein in either the RER or the 15 °C intermediate were incubated in the presence of increasing concentrations of cytosol. The concentration of cytosol required for 50% transport from the 15 °C compartment (Fig. 12, closed circles) was always found to be 2-3-fold less than that required for export from the RER (Fig. 12, open circles). These results suggest that a component(s) which limits the rate or extent of transport of VSV-G protein from the RER is consumed during the transport of VSV-G protein to the 15 °C intermediate.

Transport from the 15 °C Intermediate Is Sensitive to NEM and EGTA—ER to Golgi transport is sensitive to NEM and requires the intra-Golgi NEM-sensitive factor NSF (Beckers and Balch, 1989). During export from the RER, VSV-G protein is rapidly mobilized into a pre-Golgi intermediate step which is NEM-resistant (Fig. 3). In order to determine whether transport from the 15 °C intermediate was NEM-sensitive, semi-intact cells (containing VSV-G protein in the 15 °C intermediate) and cytosol were treated with 1 mM NEM for 10 min on ice. As shown in Fig. 13 (inset, lane c) transport was strongly inhibited. To determine if NSF was required at this step, the transport reaction was supplemented with a monoclonal antibody to NSF which inhibits transport from the RER to the Golgi (Block et al., 1988). Addition of anti-NSF antibody inhibited the transport reaction by almost 70–80% (Fig. 13, inset, lane d). It is apparent from these results that NSF is still required for transport from the 15 °C compartment to the cis Golgi.

To analyze the time point at which the NEM-sensitive factor(s) are required for transport of VSV-G from the 15 °C intermediate to the cis Golgi, semi-intact cells containing VSV-G in the 15 °C intermediate were incubated for increasing time at 30 °C prior to the addition of NEM, and subse-
Sequential Intermediates in ER to Golgi Transport

Interestingly incubated for 90 min at 30 °C. As shown in Fig. 13, VSV-G protein was rapidly transported past the NEM-sensitive step(s). By 10 min of incubation, nearly 80% of the VSV-G protein transported is NEM-resistant (Fig. 13, closed circles). In contrast, transport from the RER to the cis Golgi in semi-intact cells generally requires a 20-min incubation to obtain a similar level of NEM-resistant transport (Fig. 3). These results are consistent with the observed accelerated rate of transport from the 15 °C intermediate in the cis Golgi when compared with the kinetics of transport from the RER.

In contrast to the early effects of NEM on transport, Ca"+ is required at a very late step in transport, one immediately preceding fusion to the cis Golgi. This step is preceded by a step which is sensitive to GTPyS (Beckers and Balch, 1989). Since transport from the 15 °C intermediate is also sensitive to GTPyS (Beckers and Balch, 1989), it was important to confirm the accumulation of VSV-G protein in a transport intermediate by prolonged incubation at 15 °C still requires Ca"+ for delivery to the cis Golgi. When semi-intact cells containing VSV-G protein in the 15 °C compartment were incubated in the presence of EGTA, no transport was observed (Fig. 13, inset, lane e). Upon removal of EGTA and addition of Ca"+, transport was restored as observed previously for RER to Golgi transport (Beckers and Balch, 1989; data not shown).


discussion

Ca"+ Requirement for ER to Golgi Transport—We have provided evidence for a step in transport sensitive to the absence of free cytoplasmic Ca"+ in vitro. Preincubation of semi-intact cells in the presence of EGTA results in the accumulation of VSV-G protein in a transport intermediate, which, when released from the block by addition of Ca"+ (pCa7), rapidly delivers VSV-G protein to the cis Golgi compartment. In this case, the normal lag (10–15 min) required for export from the ER to the cis Golgi is reduced to only 1–2 min. A second in vitro experiment provided direct evidence that VSV-G protein is trapped at a late transport step in the absence of Ca"+ addition of EGTA after increasing time of incubation abruptly inhibited processing of VSV-G protein. These results are consistent with the observed accelerated rate of transport from the 15 °C intermediate to the cis Golgi. This step is preceded by a step which is sensitive to GTPyS (Beckers and Balch, 1989; Baker et al., 1989), and is therefore likely to be the YPT1 compartment containing cells with the drug resulted in a general disassembly of the ER leading to the secretion of resident ER proteins (Booth and Koch, 1989). The nonspecific loss of resident ER proteins was observed only after a 4–8 h pretreatment of cells with the drug in the presence of 1 mM extracellular Ca"+ and is reminiscent of a yeast secretion mutant defective in an ER P-type Ca"+-ATPase (see below) (Rudolph et al., 1989). In addition, the effect of A23187 was limited to a few cell types and, interestingly, was ameliorated over the long term by the induction of heat-shock proteins (Booth and Koch, 1989). The effects of A23187 on VSV-G protein transport reported here were immediate, were characteristic of all cell lines tested, and occurred in the presence of extracellular Ca"+ (1 mM) Ca"+. While our results are highly correlative to those observed in vitro (which require only the addition of EGTA and are likely to measure the specific contribution of a cytosolic free Ca"+ pool to transport), any interpretation of the role of Ca"+ in transport based on the use of A23187 in vitro must be viewed with reservation. A23187 will alter the Ca"+ content of both the cytoplasmic and the luminal pools of Ca"+-sequestering organelles such as the ER, providing no information with respect to the functional Ca"+ pool involved in transport. In addition, A23187 has been shown to adversely affect a large range of metabolic pathways. Indeed, it is likely that the secondary effects of A23187 on cellular metabolism over both the short and long term may be strikingly different.

It is now recognized that the ER serves as the major Ca"+ reservoir in the cell (Berridge and Irvine, 1989) and contains both inositol triphosphate and GTP-responsive pools (Gish et al., 1989). At least two yeast proteins, YPT1, a small GTP-binding protein required for ER to Golgi transport (Segev et al., 1988; Schmitt et al., 1988) and PMR1, an ER-associated P-type Ca"+-ATPase (Rudolph et al., 1989) have been implicated in the regulation of transport of protein between the ER and Golgi in yeast. In mammalian cells the rab1 gene product (Gallwitz et al., 1983; Haubruck et al., 1983; Touchot et al., 1987; Zahroui et al., 1987) is 76% homologous to yeast YPT1, will substitute for YPT1 in yeast deletion mutants (Haubruck et al., 1989), and is therefore likely to be the YPT1 mammalian homologue. While we and others have been unable to document any direct relationship between the GTPyS inhibitable step in ER to Golgi transport and Ca"+ regulation in vitro (Beckers and Balch, 1989; Baker et al., 1989), it is tempting to speculate that an indirect-coupling does indeed exist between Rab1 function and the Ca"+-dependent step in transport. We have recently developed peptide analogs which block the interaction of the rab family of small GTP-binding proteins with a putative downstream effector protein required for fusion with the cis Golgi compartment (Plutner et al., 1990). The activity of this effector is coincident with the requirement for Ca"+ in vitro (Plutner et al., 1990). One possible explanation for the apparent rescue of the ypt1" phenotype by elevated extracellular Ca"+ (Schmitt et al., 1988) is that Ca"+ is an essential cofactor in a late-acting fusion

complex involving Rab1 and its effector. In this interpretation, elevated Ca\(^{2+}\) could promote a more efficient coupling of the mutant protein to this complex. The interpretation that the YPT1 response to elevated Ca\(^{2+}\) is indirect is also supported by the observations that PMR1 mutants suppress the lethality of a ypt1-1 mutant (Rudolph et al., 1989). PMR1 is an ER-associated \(\alpha\)-type Ca\(^{2+}\)-ATPase. Yeast cells expressing mutant pmr1 have a 5-50-fold increase in the level of foreign protein secretion, but secrete normal levels of a native protein such as invertase (Smith et al., 1985). Increased cytoplasmic Ca\(^{2+}\) in this case would lead to a more efficient association of ypt1-1p with the late-acting fusion complex. Alternatively, PMR channel activity may be regulated by a GTP-binding protein and provide an as yet unrecognized role in carrier vesicle formation or fusion.

The 15 \(^{\circ}\)C Intermediate, a Novel Compartment between the ER and the Golgi—We have provided evidence that both transport from the RER to the 15 \(^{\circ}\)C intermediate, and transport from the 15 \(^{\circ}\)C intermediate to the cis Golgi can be reconstituted in vitro. Analysis of the biochemical properties of transport of VSV-G protein from the 15 \(^{\circ}\)C compartment to the cis Golgi indicated that it was still sensitive to the inhibitors GTP\(_{\gamma}\)S, EGTA, and NEM, and required both ATP and additional cytosolic factors. Since \(\alpha\)-mannosidase I is insensitive to these conditions, it is apparent that VSV-G protein resides in a pre-cis Golgi compartment after prolonged incubation of cells in vivo or in vitro at 15 \(^{\circ}\)C. While the degree of cytosol-independent transport of VSV-G protein from the RER in semi-intact cells can be variable between preparations (5-15\% of total VSV-G protein transported), the concentration of cytosol-independent transport and the 15 \(^{\circ}\)C compartment was always reduced by 2-3-fold, suggesting that at least one limiting component required for export from the RER is consumed by preincubation in vivo or in vitro at low temperature. In addition, transport from the 15 \(^{\circ}\)C intermediate to the cis Golgi occurred at a rate which was nearly 2-fold that observed for transport from the RER suggesting that VSV-G protein had accumulated in a downstream transport intermediate, lacked a lag period, and was strikingly insensitive to cell homogenization, arguing for the redistribution of VSV-G protein into a physically unique intermediate step in transport. This redistribution of VSV-G protein to an active transport compartment less susceptible to inactivation by the shear forces imposed by homogenization was also supported by the observation that the total yield of transported VSV-G protein from the 15 \(^{\circ}\)C compartment is consistently larger (10-20\% of total VSV-G transported) than that observed for export from the RER.

Fig. 14 (panel B) illustrates two potential morphological correlates to the 15 \(^{\circ}\)C compartment. One possibility is that VSV-G protein accumulates in the transitional ER, a specialized region of the ER believed to be the site of export (Palade, 1975) (Fig. 14, panel B, lower pathway), during prolonged incubation at reduced temperature. In this model, the reduced cytosol concentration required for subsequent transport from the transitional ER to the cis Golgi would presumably reflect consumption of a cytosolic factor required for migration of VSV-G protein to the export site, or priming events leading to the formation of transport vesicles. These events could readily account for the striking homogenization resistance of transport from this intermediate step in transport to the cis Golgi. In addition, the loss of the lag period and the 2-fold increase in the rate of transport suggests that migration of VSV-G through the ER cisternae (and possibly incorporation into transport vesicles) accounts, at least in part, for the observed lag in ER to Golgi transport. A second possibility is that the 15 \(^{\circ}\)C compartment is biochemically distinct from either the RER or transitional ER (Fig. 14, panel B, upper pathway). The existence of a novel compartment between the ER and the Golgi has been the basis for speculation during the last several years. The discovery that resident ER proteins contain retention signals (Munro and Pelham, 1987; Nilsson et al., 1989) led to the suggestion that they are continuously retrieved from an intermediate "salvage" compartment en route to the cis Golgi compartment (Warren, 1987; Pelham, 1989). At least four lines of evidence now provide evidence for such a compartment: 1) the E1 glycoprotein of Semliki forest virus accumulates in a tubular-vesicular compartment which is morphologically distinct from both the RER and the cis Golgi during incubation of infected cells at 15 \(^{\circ}\)C (Saraste and Hedman, 1983; Saraste and Kuusmanen, 1984); 2) coron-
avirous MHV59 buds into a morphologically unique compartment
during maturation in vivo (Toozie et al., 1994; Toozie et al.,
1988); 3) vesicles of unique density containing protein en
route to the Golgi have been detected in liver (Saraste et al.,
1988) and hepatocytes (Lodish et al., 1987); and 4), recent
results with the drug Brefeldin A suggests the existence of a
unique recycling compartment between the ER and the Golgi
(Lippincott-Schwartz, 1988, 1990). The combined results from
these studies have led to the speculation that the salvage
compartment, the 15 °C compartment, and the compartment
in which the MHV59 virus particles mature may be the same
(Warren, 1987; Pelham, 1989).

If the 15 °C intermediate is a novel compartment, we would
anticipate that transport between the RER and the cis Golgi
compartment may require at least two rounds of vesicular
transport (Fig. 14, panel B, upper pathway). In this model,
during incubation at 15 °C in vivo or in vitro VSV-G protein is
either target to, or form de novo, this novel compartment.
This step may be analogous to the delivery of coated vesicles
from the cell surface to the endosome, the recycling compart-
ment found in the endocytic pathway. Transport from this
novel "exosome" (Lippincott-Schwartz, 1989) to the cis Golgi
could occur by a second round of vesicle budding and fusion,
by or fusion of the entire structure to the cis Golgi. In this
interpretation, the lag period in transport reflects, in part,
migration of VSV-G protein through the exosome. All of the
properties attributed above to export of VSV-G protein
through a specialized transitional region of the ER could also
be rationalized for transport of VSV-G protein from the
exosome to the cis Golgi in the following way: 1) transport
requires reduced cytosol because it reflects export from a
different compartment in the pathway; 2) transport is homog-
enization resistant because VSV-G protein is now concen-
trated in a restricted collection of vesicular compartments,
which is essential for transport of VSV-G protein between
its site of synthesis in the RER and the 15 °C intermediate.
Experiments are currently in progress to address these issues.

**Table 1**

Biochemical properties distinguishing sequential transport of VSV-G proteins
from the ER and different intermediate steps to the cis Golgi compartment

<table>
<thead>
<tr>
<th>Condition</th>
<th>ER</th>
<th>15 °C Intermediate</th>
<th>Dilution-resistant Intermediate</th>
<th>NEM-resistant Intermediate</th>
<th>EGTA-sensitive Intermediate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag period</td>
<td>10-20 min</td>
<td>None</td>
<td>Required</td>
<td>ND*</td>
<td>None</td>
</tr>
<tr>
<td>ATP</td>
<td>Required</td>
<td>Required</td>
<td>Required</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td>Cytosol</td>
<td>Required</td>
<td>Reduced</td>
<td>Required*</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td>Homogenization</td>
<td>Sensitive</td>
<td>Insensitive</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GTPγS</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NEM</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>ND</td>
<td>Sensitive</td>
<td>ND</td>
</tr>
<tr>
<td>EGTA</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>ND</td>
<td>ND</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Peptide</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>ND</td>
<td>ND</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

*ND, not detected.

1. A dilution-sensitive, semi-intact cell associated factor is required to form the intermediate but is not required for its consumption.
2. At least one NEM-sensitive factor is required to form the intermediate but is not required for its consumption.
3. Rab3A effector domain peptide (see Plutner et al., 1990).
preceding fusion with the cis Golgi which requires Ca\textsuperscript{2+} and is sensitive to a synthetic peptide to the putative effector domain of Rab proteins (Plutner et al., 1990). Since transport from the 15 °C intermediate is also sensitive to NEM and GTP\textgamma{}S, it is likely that incubation at 30 °C for 20 min results in the trafficking of VSV-G through this compartment. Sequential intermediates have also been identified in the transport of protein between Golgi compartments. Incubation of Golgi in the presence of GTP\textgamma{}S results in the accumulation of coated vesicles (Meliacon et al., 1987; Malhotra et al., 1988; Malhotra et al., 1989) which subsequently lose their coats to form uncoated vesicles which require NSF for fusion (Orci et al., 1989). Formation of both coated and uncoated vesicles during inter-Golgi transport is rapid relative to fusion (Balch et al., 1984; Malhotra et al., 1988; Orsi et al., 1989). By analogy to these results, the first category of transport steps involved in RER to Golgi transport is likely to correspond to the export of VSV-G protein from the RER (through the 15 °C intermediate); the second to events involved in the fusion of transport vesicles to the cis Golgi compartment. This interpretation is supported by the observation that the SEC18 (yeast NSF) temperature-sensitive mutants defective in ER to Golgi transport accumulate large populations of uncoated vesicles when incubated at the restrictive temperature (Kaiser and Schekman, 1990).

A more refined order to transport (Fig. 14, panel B) was established by accumulation of VSV-G protein in each of the transport intermediates. The biochemical properties of transport from these intermediates are summarized in Table I. We have detected at last four sequential and reversible intermediate steps in the transport of VSV-G protein from the RER to the Golgi including the 15 °C intermediate discussed above, the dilution-resistant intermediate, the NEM-sensitive intermediate, and the EGTA-sensitive intermediate. Two other sequential steps in transport which can be detected by the addition of inhibitors are the GTP\textgamma{}S-sensitive step (Balch and Beckers, 1989) and a step sensitive to inhibition by a synthetic peptide homologous to the putative effector domain of members of the rab gene family of small GTP-binding proteins (Plutner et al., 1990). The latter two steps are presently irreversible. Since transport from the Ca\textsuperscript{2+}-dependent step was insensitive to GTP\textgamma{}S, we have placed the GTP\textgamma{}S-sensitive step preceding the Ca\textsuperscript{2+}-sensitive step(s). Although the kinetic data argue that NSF (or additional NEM-sensitive component(s)) and ATP are required early in transport, we have previously shown that NSF and ATP are also required for consumption of the EGTA intermediate (Beckers et al., 1989). The seemingly contradictory observation that NEM and ATP-sensitive components are only necessary during an early transport step, in contrast to the requirement for NSF and ATP for the consumption of a late transport intermediate which accumulates in the presence of EGTA can be readily interpreted in one of several ways. One possibility is that the absence of Ca\textsuperscript{2+} disrupts a key step in the maturation of a fusion complex essential for VSV-G protein delivery to the cis Golgi compartment. Only upon readdition of Ca\textsuperscript{2+} are components including NSF, an ATP-dependent factor and a putative Rab effector protein (Plutner, 1990) able to complete their function. In this interpretation, Ca\textsuperscript{2+} serves as a key cofactor in both the integration of components required during early steps in transport, as well as the function of these components in a late step triggering vesicle fusion to the target membrane as discussed above (Fig. 14, panel B). An alternative interpretation is that Ca\textsuperscript{2+} is only essential at a step involved in vesicle fusion to the cis Golgi, but that components recycle following this event for reuse in subsequent rounds of transport. Such an hypothesis is consistent with the existence of the dilution-sensitive intermediate, and the proposed catalytic function for NSF (Block et al., 1988; Malhotra et al., 1988; Ori et al., 1989). If this were the case, incubation in the absence of Ca\textsuperscript{2+} might immobilize a limiting pool of these components and prevent their ability to function in earlier steps of the pathway. In any event, transit of protein through either the 15 °C intermediate or the EGTA intermediate is rapid relative to targeting and fusion.

An understanding of the enzymology of vesicular transport of VSV-G protein between the RER and the cis Golgi necessitates the functional dissection of transport into intermediate steps. The present analyses present preliminary insight into this pathway. Assays now focused on each of the reversible partial reactions found to be required for transport should provide new insight into the enzymology of vesicle formation, targeting and fusion.

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