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Interorganelle transfer and glycosylation of yeast invertase *in vitro*

(endoplasmic reticulum/Golgi body/*SEC* gene/α-1→3-mannosyltransferase)

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ABSTRACT **Core glycosylated proteins formed in the yeast endoplasmic reticulum (ER) are transported to the Golgi body, where oligosaccharides are elongated by addition of outer-chain carbohydrate. The transport process is blocked in a temperature-sensitive secretion mutant (*sec18*) of *Saccharomyces cerevisiae*, which accumulates core glycosylated invertase (product of *SUC2*; EC 3.2.1.26) in the ER. To approach the molecular mechanism of this transport process, we have devised a reaction in which core glycosylated invertase, accumulated in *sec18* cells, is transferred to the Golgi body *in vitro*. For this purpose, membranes from *sec18*, *SUC2* cells that are also defective in an outer chain α-1→3-mannosyltransferase (*mnn1*) are mixed with membranes from a strain that contains the transferase but is deficient in invertase (*MNN1*, Δ*SUC2*). Transfer is detected by the acquisition of outer-chain α-1→3-linked mannose residues dependent on both donor and recipient membranes. The reaction is temperature and detergent sensitive and requires ATP, GDP-mannose, Mg^{2+}, and Mn^{2+}, and the product invertase remains associated with sedimentable membranes. Treatment of donor, but not acceptor, membranes with *N*-ethylmaleimide or trypsin inactivates transfer competence. These characteristics suggest that the ER, or a vesicle derived from the ER, contributes invertase to a chemically distinct compartment where mannosyl modification is executed.**

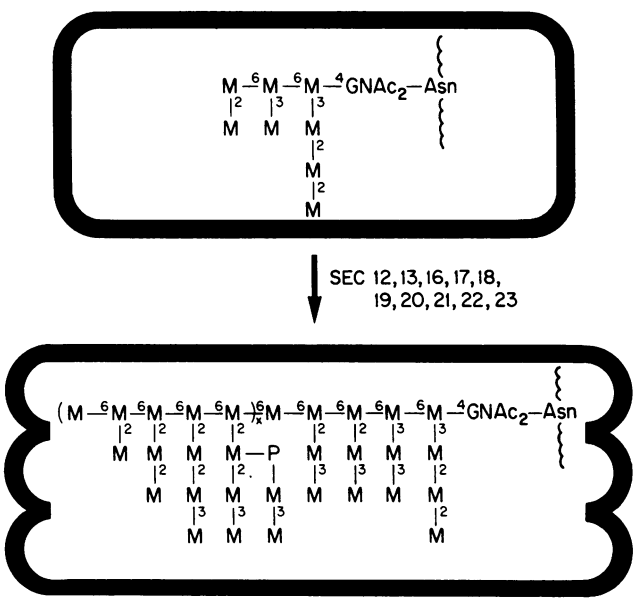

FIG. 1. Glycosylation coupled to transport from the ER to the Golgi body. M, mannose; GNAc, *N*-acetylglucosamine; *SEC*, wild-type gene; Asn, asparagine.

The process of intracellular protein transport and secretion involves several complex organelles and a large number of gene products (1, 2). In *Saccharomyces cerevisiae* four stages have been detected that can be blocked reversibly in secretory mutants (3, 4). At a restrictive growth temperature these mutants accumulate organelles and glycoprotein precursors, which are discharged to a succeeding stage in the secretory pathway upon return to a permissive temperature. The availability of *sec* mutants provides a unique opportunity to examine the mechanism of intercompartmental transport. A cell-free protein transport system that reproduces the requirement for *SEC* gene products will be essential in evaluating the function of these proteins.

In designing a cell-free protein transport reaction, we have been guided by the work of Rothman and colleagues (5, 6) who reconstituted *in vitro* transport of vesicular stomatitis virus (VSV) G protein from *cis* to *trans* compartments of the mammalian Golgi body. In their reaction, transfer is monitored by the acquisition of oligosaccharide modifications dependent upon a donor membrane that contributes an incompletely glycosylated form of VSV G protein and an acceptor membrane that contains a glycosyltransferase that is missing in the donor fraction.

Compartmental modification of glycoprotein oligosaccharide chains has also been documented in yeast. Core glycosylated forms of secretory proteins, such as invertase, are assembled in the endoplasmic reticulum (ER) and are modified further by addition of outer-chain carbohydrate at some point during transit through the Golgi body (7). On the basis of oligosaccharide structural analysis (8, 9) and evaluation of the influence of *sec* mutations on oligosaccharide maturation (7, 10), we proposed the pathway of glycoprotein assembly shown in Fig. 1. One of the several unique determinants formed in the Golgi body is an α-1→3-mannose linkage at the nonreducing end of oligosaccharide side chains (8). An α-1→3-mannosyltransferase, encoded by the *MNN1* gene, executes this modification (11); *mnn1* mutant cells secrete glycoproteins that are missing only this linkage.

In this report we describe a strategy for detecting protein transport that exploits the compartmental nature of yeast oligosaccharide maturation. Core glycosylated invertase (EC 3.2.1.26) accumulated in an ER-blocked (*sec18*), *mnn1* double mutant strain is used as a source of donor membrane, and invertase-deficient (Δ*SUC2*), *MNN1* (wild-type) cells provide the recipient membrane. Addition of α-1→3-mannose units to invertase, detected by reaction with an antibody that recognizes this determinant (12), is used to monitor transfer. The characteristics of invertase transfer between the donor and recipient compartments suggest that a membrane protein-mediated, and possibly *SEC* gene-dependent, process has been reconstituted.

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Abbreviations: VSV, vesicular stomatitis virus; ER, endoplasmic reticulum; mU, milliunit.

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MATERIALS AND METHODS

Materials. *Saccharomyces cerevisiae* strain THY1-1B (*sec18, mnn1*) was derived from MBY12-R1A (*sec18-1*, MATα) and LB1-10B (*mnn1-2*, MATa). Strains SEY2102, SEY5078 (*sec7*), and SEY5180 (*sec18*) all contained a deletion in *SUC2* (*suc2-Δ9*) (13). Strain LB6-5D (*mnn4-1*) was provided by Lun Ballou (this department). X2180-1A was from the Yeast Genetic Stock Center (Department of Biophysics, University of California, Berkeley, CA).

ATP (grade I Na form, stock solution neutralized with NaOH), GDP-mannose (from yeast), DL-dithiothreitol, *N*-ethylmaleimide, bovine serum albumin (RIA grade), D-sorbitol, D-mannitol, phenylmethylsulfonyl fluoride, phospho*enol*pyruvate, pyruvate kinase (rabbit muscle, lyophilized), trypsin (type III-S), and trypsin inhibitor (type I-S) were from Sigma; Percoll was from Pharmacia Fine Chemicals; glass fiber filters (GF/A) were from Whatman. Lyticase was used to prepare spheroplasts as described elsewhere (14).

Evaluation of Secreted and Accumulated Invertase. X2180-1A and THY1-1B were grown overnight in YPD medium (1% Bacto-yeast extract/2% Bacto-peptone/5% glucose) at 24°C to an OD_{600} of 5–10 (measured in a 1-cm cuvette with a Zeiss PMQII spectrophotometer). Cell aliquots (100 OD_{600} units each) were centrifuged and resuspended in an equal volume of YP medium (1% Bacto-yeast extract/2% Bacto-peptone) containing 0.1% glucose for 1.5 hr at 24°C; these conditions derepress synthesis of secreted invertase. Another aliquot of THY1-1B was treated similarly except that incubation for 1 hr at 24°C in YP medium/0.1% glucose was followed by transfer to 37°C for 30 min. Cells were sedimented and converted to spheroplasts by incubation for 30 min at 24°C or 37°C in 12.5 mM Tris·HCl, pH 7.5/0.7 M sorbitol/10 mM 2-mercaptoethanol and 40 units of lyticase per OD_{600} unit of cells. Spheroplasts were sedimented at 1000 × *g* for 5 min and lysed by resuspension in 0.5 ml of 50 mM Tris·HCl, pH 7.4/0.5% (vol/vol) Triton X-100. Spheroplast supernatant fractions, which contained secreted invertase, were adjusted to 0.5% Triton X-100. Both fractions were clarified by centrifugation for 5 min in a Fisher microcentrifuge. Aliquots (5 μl) of each sample were evaluated by immunoprecipitation with antibody against α-1→3-mannosyl determinants (see In Vitro *Transport Assay*).

Preparation of Crude Membrane Fractions. Cells grown overnight at 24°C to an OD_{600} of 5–10 in YPD medium were sedimented, washed with distilled water, and resuspended at 5 OD_{600} units/ml in YP medium/0.1% glucose. After 1 hr, cells were converted to spheroplasts in a two-step procedure. Cells were sedimented and resuspended in 0.1 M Tris·HCl, pH 9.4/0.01 M dithiothreitol at a density of 50 OD_{600} units/ml and incubated at 24°C for 20 min. Next, cells were sedimented and resuspended in spheroplasting buffer (0.7 M sorbitol/12.5 mM Tris·HCl, pH 7.5 and 25 units of lyticase per OD_{600} unit of cells) at a cell density of 250 OD_{600} units/ml. Spheroplasts formed during a 30-min incubation at 24°C were sedimented through a cushion of 1.2 M sorbitol and then resuspended in YP medium containing 0.7 M sorbitol, 25 mM Tris·HCl at pH 7.5, and 0.1% glucose at a cell density of 10 OD_{600} units/ml. Donor spheroplasts (THY1-1B) were incubated for 30 min at 24°C followed by 10 min at 37°C to establish the *sec18* block; acceptor spheroplasts were incubated at 24°C only. Spheroplast samples (50 ml) were poured into chilled Sorvall GSA centrifuge bottles and kept on ice an additional 5 min before centrifugation for 5 min at 2500 × *g* in a Sorvall GSA rotor at 4°C. Sedimented spheroplasts were resuspended in 10 ml of chilled 25 mM Tris·HCl, pH 7.5/0.7 M sorbitol, transferred into a 10-ml culture tube, and centrifuged for 5 min in a clinical centrifuge at room temperature. Chilled lysis buffer (1 ml; 50 mM Tris·HCl, pH 7.5/0.1 M KCl/5 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride) was added to the spheroplast pellet and the suspension was mixed by trituration with a 1-ml Pipetman followed by agitation on a Vortex mixer at half speed for 5 sec. Lysis was achieved by dilution with more lysis buffer (final volume of 10 ml; 50 OD_{600} cell equivalent units/ml) and mixing by inversion followed by agitation on a Vortex mixer. After 5 min at 0°C, unlysed spheroplasts were removed by centrifugation at 100 × *g* for 5 min in a Sorvall SS34 rotor, and membranes remaining in suspension were sedimented onto a 1-ml shelf of Percoll by centrifugation at 17,000 × *g* (donor cells) or 60,000 × *g* (recipient cells) for 30 min in a Beckman type 40 rotor. The resulting membrane layer was removed and used directly or frozen in liquid nitrogen for storage at −80°C. Concentrated membranes contained 5–8 mg of protein per ml based on the dye-binding assay (15); donor membranes contained ≈15 milliunits of invertase per μl.

***In Vitro* Transport Assay.** A standard incubation mixture (40 μl) contained 5 μl each of donor and recipient membranes, 0.6 M mannitol, 10 mM Hepes/KOH at pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mM ATP, 5 mM phospho*enol*pyruvate, 4 units of pyruvate kinase, 0.5 mM GDP-mannose, 5 mM $MgCl_2$, and 5 mM $MnCl_2$. Incubations were conducted at 0°C, 24°C, 30°C, or 37°C with components that were incubated at the respective temperature for 5 min before mixing. Reactions were stopped by adding EDTA and Triton X-100 (to 20 mM and 0.1%, respectively) to samples transferred to 0°C. Debris was removed by centrifugation in a Fisher microcentrifuge for 15 min.

Invertase in the supernatant fractions was treated with antibody directed against α-1→3-mannose linkages of the outer chain carbohydrate. A polyclonal serum directed against antigenic determinants on the surface of intact *mnn4* mutant yeast cells (largely, though not exclusively, specific for α-1→3-mannosyl units) was prepared as described (11). This serum was purified by absorption with heat-treated (70°C for 1 hr) *mnn1* mutant cells (20 OD_{600} units of cells per ml of serum) three times for 1 hr each at room temperature. A titer of 2^{-6} to 2^{-8} was determined in agglutination assays with 50 μl each of *MNN1* cells at 10 OD_{600} units/ml in 50 mM Tris·HCl, pH 7.4 (16).

Samples to be treated with antibody were diluted to 1 ml with buffer A (10 mM Tris·HCl, pH 7.5/0.2 M NaCl/4 mM

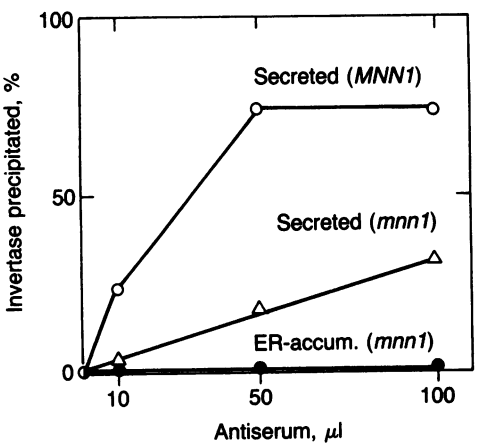

FIG. 2. *MNN1* and *SEC18*-dependent glycosylation of invertase. Invertase secreted at 24°C by X2180-1A (*MNN1*) and THY1-1B (*mnn1*) and accumulated in THY1-1B at 37°C was prepared by converting cells to spheroplasts. Secreted proteins are released in a soluble form and accumulated proteins are retained in sedimentable spheroplasts. Aliquots containing 38–44 mU of invertase were exposed to increasing amounts of α-1→3-mannose-specific antiserum. Immune complexes were formed with IgGsorb (5 μl for 0 or 10 μl of serum; 25 μl for 50 μl of serum; 50 μl for 100 μl of antiserum).

NaN_3/1 mg of bovine serum albumin per ml/1% Triton X-100) and incubated with 100 μl of IgGsorb, reconstituted as described by the supplier, for 30 min at 0°C. After centrifugation the supernatant solution was mixed with 50 μl of antiserum and stored for 16 hr at 0°C. A fresh aliquot (25 μl) of IgGsorb was added and after 30 min at 0°C the precipitate was collected on a Whatman GF/A filter. Filters were washed with buffer A (twice, 5 ml each), buffer B (0.1 M Tris·HCl, pH 7.5/0.2 M NaCl/2 M urea/1% Triton X-100; twice, 5 ml each) and buffer A again (twice, 5 ml each).

Invertase was assayed as before (17), except with GF/A filters suspended in 0.4 ml of reaction buffer (0.1 M sodium acetate, pH 5.1/0.1 M sucrose). Activity is expressed in milliunits (mU; 1 mU corresponds to 1 nmol of glucose released per min at 30°C). A correction for background was established with transport reactions in mixtures incubated at 0°C. Typically, the background value was around 0.08 mU of invertase.

RESULTS

Design of the Transport Assay. In previous work we showed that the α-1→6-mannose-linked backbone of the outer chain carbohydrate is added to glycoproteins during transit through the yeast Golgi body (7). *sec18*, a mutant blocked in transport of proteins from the endoplasmic reticulum, accumulates a core glycosylated form of invertase that is not precipitated by an antibody that reacts with the α-1→6-linked polymannose backbone. Immunodetection of the backbone requires transport to a Golgi body that is deficient in addition of α-1→2-linked oligosaccharide side chains (7).

An *in vitro* transport assay based on the addition of the α-1→6-mannose-linked backbone would require transfer into Golgi membranes defective in a specific mannosyltransferase. It was preferable, however, to combine membranes from a donor cell that could not perform a Golgi-limited modification with recipient membranes that could. For this reason, the addition of α-1→3-mannose units to oligosaccharides of the outer chain was examined. The outer chain α-1→3-mannose linkage can be diagnosed with rabbit antibody elicited in response to whole yeast cells (12). Invertase made in a wild-type strain was efficiently precipitated (≈75%) by antiserum directed against the α-1→3-linked mannoses (Fig. 2). Antibody-precipitable invertase was reduced but not eliminated in *mnn1* mutant cells, which are deficient in the outer chain α-1→3-mannosyltransferase activity. Partial reaction with the antiserum may be due to the presence of small amounts of antibodies that recognize other outer chain determinants. In contrast, invertase accumulated in *sec18*, *mnn1* cells at 37°C (or in *sec18, MNN1*, not shown) did not react with the antibody.

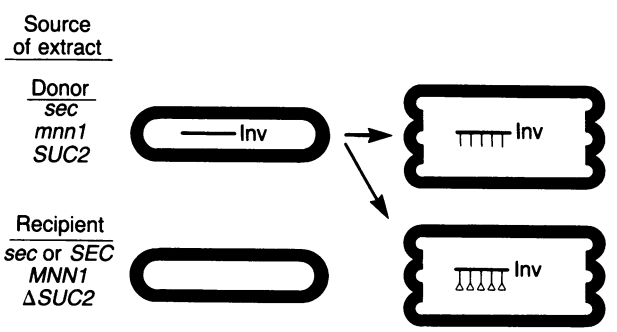

FIG. 3. Design of the *in vitro* transport assay. *SUC2*, wild-type invertase gene; *sec*, secretory mutant allele; *SEC*, wild-type allele; *MNN1*, wild-type α-1→3-mannosyltransferase gene; *SUC2*, deletion mutant; Inv, invertase; straight line to Inv, core oligosaccharide; branched line, oligosaccharide without terminal α-1→3-mannose units; branched line with triangles, complete oligosaccharide.

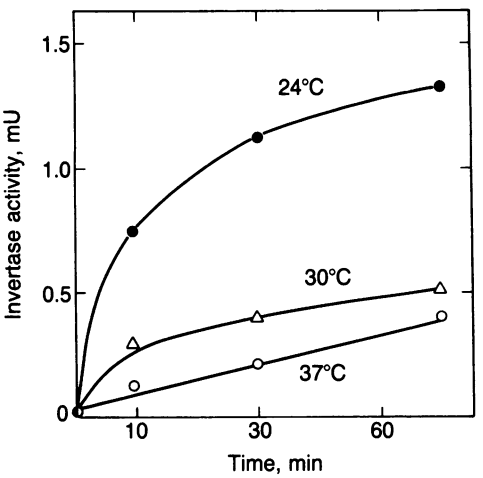

FIG. 4. Kinetics and temperature sensitivity of conversion.

Intercompartmental transfer of invertase was ensured by a strategy depicted in Fig. 3. Donor membranes were prepared from *sec18, mnn1* spheroplasts that had been derepressed for invertase synthesis at 37°C. Recipient membranes were from *sec* mutant or wild-type cells that lacked a functional *SUC* (invertase) gene but carried the wild type *MNN1* gene.

Characteristics of the Transfer Reaction. When equal portions of donor and recipient membranes were mixed along with ATP, an ATP-regenerating system, GDP-mannose, Mg^{2+}, and Mn^{2+}, a small fraction (2–5%) of the invertase was converted to a form precipitated by the α-1→3-mannose-specific antiserum. Fig. 4 shows a time course of conversion at three temperatures. The marked temperature sensitivity of conversion mimics the restrictive character of *sec18-1* mutant cells, which are growth and secretion defective at 30°C and above. Equally severe temperature sensitivity was seen with recipient membranes from wild-type or *sec* mutant cells (not shown). To account for this behavior either the defective *sec18* gene product is in the donor membrane and hence cannot be replaced by wild-type recipient membranes or the transfer reaction is inherently thermosensitive.

A strong and proportional stimulation of conversion was seen when donor membranes were incubated with increased levels of *MNN1* recipient membranes (Fig. 5). The low level

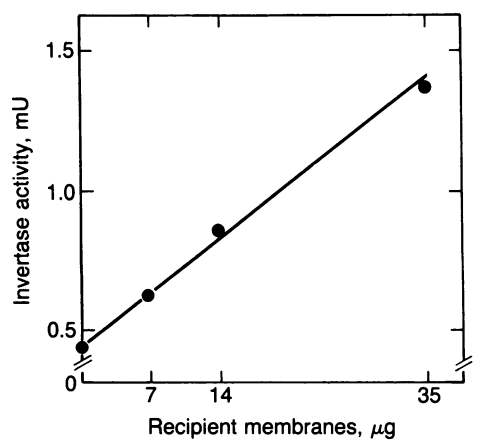

FIG. 5. Stimulation of conversion by incubation with recipient membranes. Donor membranes (5 μl, 35 μg of protein) were incubated with increasing amounts of recipient membranes for 30 min at 24°C. After the reaction had been stopped with EDTA and Triton X-100, additional recipient membranes were added to adjust each sample to the same amount of protein.

Table 1. Dependence of conversion on GDP-mannose and divalent cation

Assay*	Invertase converted, mU 24°C	37°C
Complete	0.8	0.1
− GDP-mannose	<0.1	<0.1
− Mg^{2+}/Mn^{2+}	<0.1	<0.1
− Mn^{2+}	<0.1	<0.1

*Standard reactions were carried out for 30 min.

of conversion in the absence of recipient membranes was temperature sensitive and 5-fold above the 0°C control incubation (0.08 mU of invertase). This conversion may represent glycosylation in Golgi membranes present in the donor fraction and be detected by the small amount of antibodies that react with other outer-chain determinants. Furthermore, some fraction of the molecules converted in a complete transfer reaction may be detected by these less-specific outer-chain antibodies.

Analysis of the distribution of membrane-associated and soluble invertase after the *in vitro* incubation indicated that converted invertase was retained in a sedimentable membrane. Freshly isolated and frozen donor membranes were incubated with recipient membranes and then the mixtures were centrifuged at 100,000 × *g* for 60 min. Less than 5% of the total invertase was released from the freshly isolated donor membrane; ≈15% remained in the supernatant from frozen donor membranes. In both cases all of the converted invertase was in the sedimented fraction. Moreover, the presence of detergent (1% Triton X-100) during the transfer reaction abolished conversion and released all of the invertase. The α-1→3-mannosyltransferase is active on artificial acceptors in the presence of 1% Triton X-100 (P. Esmon and R.S., unpublished data), so transfer inhibition was not due to inactivation of glycosylation. From this we concluded that invertase released by rupture of donor membranes was not a substrate for outer-chain glycosylation.

Conversion showed an absolute requirement for GDP-mannose and Mn^{2+} (Table 1), the substrate and cofactor required for outer-chain biosynthesis (18). In addition, the reaction was strongly stimulated by ATP and Mg^{2+} (Fig. 6 *Left*); GTP·Mg^{2+} did not substitute, and the Mg^{2+} requirement was not diminished with increased Mn^{2+}. The degree of ATP stimulation varied with each membrane preparation, as might be expected for variable contamination with nucleotide or ATPase activity in the sedimented membrane fractions. A small but reproducible stimulation by unphysiologically high levels of Ca^{2+} was observed, and this effect required ATP (Fig. 6 *Right*).

Balch *et al.* (19) showed that donor and recipient compartments in transport of VSV G protein could be distinguished by sensitivity of the former to the alkylating agent *N*-ethylmaleimide. Donor and recipient membranes in invertase transport were similarly distinct (Table 2). Treatment of donor membranes at 0°C with *N*-ethylmaleimide eliminated transfer activity, while pretreatment of recipient membranes alone was without effect. Inhibition was prevented by incubation with excess cysteine.

A similar experiment with trypsin treatment showed both donor and recipient membranes in VSV G protein transport to be protease sensitive (20). In contrast, only the donor membranes in invertase transfer were inactivated by trypsin treatment (Table 2). This experiment suggests that trypsin-sensitive protein(s) that participate in invertase transfer are exposed on the cytoplasmic surface of the donor membrane. Aside from the participation of *N*-ethylmaleimide- and protease-sensitive component(s) in the donor membrane, no requirement for or stimulation by a yeast cytosolic fraction was detected.

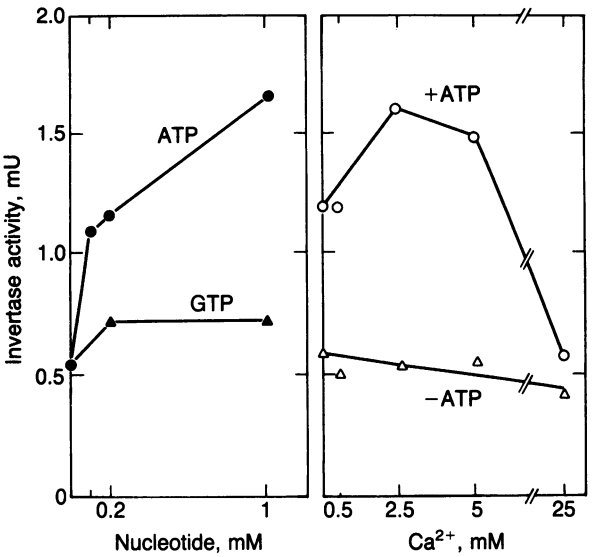

FIG. 6. Dependence of conversion on ATP and stimulation by Ca^{2+}. Standard incubation mixtures contained various levels of ATP, GTP (*Left*), or Ca^{2+} (*Right*). Mg^{2+} was 10 mM for *Left* and 5 mM for *Right*.

DISCUSSION

Transport of invertase and of other soluble and membrane-bound proteins from the endoplasmic reticulum to the Golgi body in yeast requires at least 10 genes (3). In no instance has an enzyme activity or molecular function been ascribed to the proteins encoded by these genes. An *in vitro* reaction that reproduces all or a portion of this transport process is an essential element in our effort to understand the role played by the *SEC* gene products.

We have adapted a strategy that was designed by Rothman and colleagues to detect transport of VSV G protein from *cis* to *trans* cisternae of mammalian Golgi stacks (6). Both schemes involve transfer of an incompletely glycosylated

Table 2. Donor, but not recipient, membranes are inactivated by *N*-ethylmaleimide or trypsin

Conditions	Invertase converted, mU Exp. 1, *N*-ethylmaleimide*	Exp. 2, trypsin†
i. Complete	0.7	1.5
ii. Complete + inhibitors and blocking agents	0.7	1.2
iii. Pretreat both	<0.1	0.2
iv. Pretreat donor only	<0.1	0.2
v. Pretreat recipient only	1.1	1.2

*Membranes (15 mg of protein per ml) were incubated with 10 mM *N*-ethylmaleimide for 30 min at 0°C. *N*-Ethylmaleimide was reduced by addition of excess cysteine (to 20 mM) either before (*ii*) or after (*iii, iv, v*) incubation with membranes. Control experiments showed that *N*-ethylmaleimide had no effect on invertase activity or on the immunoprecipitation. Treated or untreated membranes were diluted into standard reaction mixtures, which were incubated for 30 min at 24°C.

†Separate preparations of membranes (also at 15 mg of protein per ml) were incubated with trypsin at 250 μg/ml for 30 min at 0°C. Trypsin was inhibited by addition of excess trypsin inhibitor (500 μg/ml) either before (*ii*) or after (*iii, iv, v*) incubation with membranes. Control experiments showed that this level of trypsin had no effect on invertase activity or on the immunoprecipitation.

protein from a donor membrane that cannot perform a specific oligosaccharide modification to a recipient membrane that can. The reactions differ in the means used to define a donor compartment. In the VSV G system, the donor G protein is a kinetic intermediate that only transiently resides in a Golgi compartment preceding the one in which glycosyl modification is monitored *in vitro*. In our reaction, a *sec* mutant transport block is imposed by incubating donor cells at a restrictive temperature at which invertase accumulates in the ER. This by itself does not define the donor compartment as ER. The small amount of transfer-competent invertase could have escaped the *sec* block and reside in a distal compartment, possibly even in a Golgi cisterna.

Both reactions require ATP in addition to the sugar-nucleotide substrate for the glycosyl modification (UDP-GlcNAc for VSV G; GDP-Man for invertase). The ATP requirement is consistent with the effect of energy poisons on invertase transport in yeast cells and VSV G transport in mammalian cells (3, 5). Beyond a shared Mg^{2+} requirement, mannosyl modification of invertase requires Mn^{2+}, a known cofactor requirement for yeast outer-chain mannosyltransferases (18). Perhaps coincidentally, the compartments in both reactions can be distinguished by sensitivity of the donor, but not the acceptor, to *N*-ethylmaleimide.

Two important differences are that VSV G transport requires cytosolic proteins while invertase transfer does not, and that G protein transfer is quite efficient, sometimes exceeding 50% conversion (5), while only 2–5% of donor invertase is converted in the reaction with yeast membranes. Aside from the obvious possibility that our conditions of lysis and *in vitro* transport are not optimal, three other considerations may bear on these differences. First, transport from the ER may occur at a specialized region or transitional zone, which would contain only a fraction of the invertase accumulated in donor cells. Transfer into this part of the ER may not occur *in vitro* if it has been broken away during cell rupture. Second, outer-chain oligosaccharide addition could occur in a medial or *trans* cisterna of the yeast Golgi body. If so, invertase conversion would require multiple sequential transfers from a donor membrane, thus reducing the likelihood of high-efficiency modification. Finally, α-1→3-mannosyltransferase action may be rate-limiting so that only a fraction of transferred molecules are converted to a form that reacts with the α-1→3-mannose-specific antibody.

An additional problem with mixing crude membranes is the presence of Golgi elements in the donor fraction that should be competent as a recipient while contributing nothing to the apparent efficiency of transfer (Fig. 3 upper diagrams). If the invertase-containing donor membrane is distinct from the Golgi body, it should be possible to enrich separate donor and recipient vesicles from the respective lysates and improve the efficiency of invertase modification.

The invertase transfer reaction is as thermosensitive as secretion in the *sec18* donor cell and thus could reflect a direct participation of the mutant protein. Proof of this point requires a test of temperature sensitivity with donor membranes from a *SEC* wild-type cell. Such tests have given equivocal results. Normal cells contain too little invertase in transit from the ER to allow a reproducible signal of transfer above background. Wild-type donor membrane fractions that contained slow transport mutant forms of invertase [*SUC2-s1*, *-s2* (21)] were inactive in the transfer reaction. Until an independent test of the role of *SEC* gene products in the cell-free reaction can be developed, the possibility remains that *in vitro* conversion is inherently thermosensitive.

Compelling evidence for a protein-mediated intercompartmental transfer reaction is provided by the requirement for nucleotide beyond that necessary for the mannosyl modification alone, by the sensitivity of transfer to detergent, and by the involvement of *N*-ethylmaleimide- and trypsin-sensitive proteins in the donor membrane fraction. The issue of membrane specificity and the nature of the participating molecules can be pursued by further fractionation of donor and recipient membranes and by the application of molecular cloning techniques designed to identify *SEC* gene products.

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