Biosynthesis of Mannosylinositolphosphoceramide in *Saccharomyces cerevisiae* Is Dependent on Genes Controlling the Flow of Secretory Vesicles from the Endoplasmic Reticulum to the Golgi

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Abstract. *Saccharomyces cerevisiae* contains several abundant phosphoinositol-containing sphingolipids, namely inositolphosphoceramides (IPCs), mannosylinositolphosphoceramide (MIPC), which is substituted on the headgroup with an additional mannose, and $M(IP)_2C$, a ceramide substituted with one mannose and two phosphoinositol groups. Using well-defined temperature-sensitive secretion mutants we demonstrate that the biosynthesis of MIPC, $M(IP)_2C$, and a subclass if IPCs is dependent on genes that are required for the vesicular transport of proteins from the ER to the Golgi. Synthesis of these lipids in intact cells is dependent on metabolic energy. A likely but tentative interpretation of the data is that the biosynthesis of these sphingolipids is restricted to the Golgi apparatus, and that one or more substrates for the biosynthesis of these sphingolipids (phosphatidylinositol, IPCs, or MIPC) are delivered to the Golgi apparatus by an obligatory vesicular transport step. Alternative models to explain the data are also discussed.

ASSEMBLY of lipids into membranes of eukaryotic cells can be viewed as a three step process involving biosynthesis, transmembrane movement of lipids across the bilayer, and movement of lipids between cellular membranes (Bishop and Bell, 1988). Since membranes differ in lipid composition, this movement must occur with some selectivity (Bell et al., 1981). Three mechanisms for transport of lipids from one membrane to the other are postulated: (*a*) vesicular transport; (*b*) transfer of lipid monomers through the cytosol either by spontaneous diffusion or by protein-facilitated transport; and (*c*) lateral diffusion of lipids between membranes at regions of direct membrane contact (for reviews see Dawidowicz, 1987; Bishop and Bell, 1988). Different transport mechanisms have been implied for different lipid species. Cholesterol for instance is synthesized in the ER and seems to be brought to its final destination, the cell surface, by vesicular transport since transport is relatively slow ($t_{1/2}$ = 10 min), energy dependent and blocked at 15°C (Kaplan and Simoni, 1985*a*), although its vesicular route might be distinct from the one used by secretory proteins since cholesterol transport is not inhibited by brefeldin A (Urbani and Simoni, 1990). On the other hand, phosphatidylcholine transport from the ER to the cell surface occurs with a $t_{1/2}$ of 1 min, is independent of metabolic energy and continues at temperatures <15°C (Kaplan and Simoni, 1985*b*) and is therefore believed to be transported by a nonvesicular mechanism.

Lipid transport has not been studied extensively in the yeast *Saccharomyces cerevisiae* probably because the cell wall prohibits access to the plasma membrane. In one study, the transport of phosphatidylinositol (PI)[1] to the cell surface has been reported to be independent of vesicular transport (Daum et al., 1986), since it occurred in the secretion mutant *sec18* in which the traffic of secretory vesicles is blocked (Novick et al., 1980, 1981; Esmon et al., 1981). PI synthase activity is found in about equal amounts in the ER and the mitochondria of *S. cerevisiae* (Kuchler et al., 1986) and PI is an essential lipid (Culbertson and Henry, 1975; Henry, 1982; Nikawa et al., 1987). PI and its phosphorylated derivatives make up for 60 and 1%, respectively, of inositol-containing lipids of *S. cerevisiae*, whereas the remainder consists of three classes of PI-containing sphingolipids (Steiner et al., 1969; Smith and Lester, 1974): The inositolphosphoceramides (IPCs) contain a single inositolphosphate, mannosylinositolphosphoceramide (MIPC) contains an additional mannose unit attached to the inositol; the major sphingolipid, has the composition mannose-$(inositolphosphate)_2$-ceramide ($M(IP)_2C$). IPC's can be further divided into three major subclasses based on the type of long-chain base and the degree of hydroxylation and chain length of the fatty acids (Smith and Lester, 1974). Yeast microsomes contain an activity that transfers inositolphosphate from PI onto endogenous ceramides thereby generating IPC's (Becker and Lester, 1980).

1. *Abbreviations used in this paper*: IPC, inositolphosphoceramide; MIPC, mannosylinositolphosphoceramide; PI, phosphatidylinositol; PLC, phospholipase C; PLD, phospholipase D; TLC, thin-layer chromatography.

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Here we report on the observation that secretion mutants blocked at early stages of the secretory pathway cannot make several classes of inositol-containing sphingolipids at the restrictive temperature. Several interpretations of this observation will be discussed.

Materials and Methods

Strains, Growth Conditions, and Materials

Haploid *Saccharomyces cerevisiae* strains were used: The secretion mutants originally developed by Peter Novick and Randy Schekman were provided by Howard Riezman (Biozentrum, University of Basel, Switzerland): HSMF136, a *sec6-4*; SF294-2B, a *sec7-1*; SF226-1C, a *sec12-4*; HMSF163, a *sec13-1*; HMSF169, a *sec14-3*; HMSF174, a *sec16-2*; HMSF175, a *sec17-1*; HMSF176, a *sec18-1*; HMSF179, a *sec20-1*; HMSF180, a *sec21-1*; HMSF183, a *sec22-3*; HMSF190, a *sec23-1*; HMSF331, a *sec53-6*; SF-402-4D, a *sec59-1* and the corresponding wild-type strains X2180-1A, a *SUC2 mal gal2 CUP1* and X2180-1B, α *SUC2 mal gal2 CUP1*. Cells were kept on YPD plates containing 1% Bacto yeast extract, 2% casein hydrolysate (peptone 140), 2% Bacto-Agar, and 2% glucose. Wickerham's minimal medium (Wickerham, 1946) with 2% glucose as a carbon source but omitting myoinositol was used. The OD of dilute cell suspensions was measured in a 1-cm cuvette at 600 nm. 1 OD_{600} unit of cells corresponds to 1–2.5 × 10^7 cells depending on the strain. Reagents were obtained from the sources described recently (Conzelmann et al., 1990). Pure PI-PLC from *B. thuringiensis* prepared by Sapporo Beer was obtained through Pharmacare (Lucerne, Switzerland). PI-PLC from *B. cereus* was from Boehringer (Mannheim, FRG). PLD from *S. chromofuscus* and from cabbage as well as ceramide type III from bovine brain and phytosphingosine from yeast were from Sigma Chemical Co. (St. Louis, MO). Jack bean α-mannosidase was a gift from Dr. S. Kornfeld (St. Louis, MO).

Radiolabeling of Cells

All cultures and labelings were done in a shaking waterbath at 250 rpm. Cells were precultured overnight in minimal medium. For energy deprivation experiments (Fig. 6), the cells were precultured and labeled in minimal medium containing sodium pyruvate (20 g/liter) instead of glucose as a carbon source and containing 24 mg/liter of ergosterol, 0.05% Tween 80, and 0.05% ethanol as additional supplements. For labeling with [^{3}H]myoinositol, exponentially growing cells were centrifuged and resuspended at 10 OD_{600} units/ml in fresh medium and aliquots of 0.1–0.5 ml put into large 50-ml plastic tubes (Falcon Labware, Oxnard, CA). The cells from secretion mutants were preincubated for 10–15 min at 37°C (to induce the secretion block) or at 24°C (for control) before the addition of [^{3}H]myoinositol (0.5–5 μCi/OD_{600} unit, 0.36–3.6 μM). Unless indicated otherwise, the cells were labeled for 10 min and then chased by the addition of 4 vol of minimal medium containing myoinositol (200–500 μM) and further incubation for 50–60 min. Labeling with [^{3}H]palmitic acid was done in minimal medium for 2 h using 40–50 μCi/ml and 1 OD_{600} unit of cells per ml. At the end of the incubations, NaF and NaN_3 were added (10 and 3 mM final), cells were placed on ice and centrifuged and the cell pellet was resuspended in chloroform/methanol (1:1) to achieve a final concentration of chloroform/methanol/aqueous cell suspension of 10:10:3 (vol/vol/vol). Cells were broken by vortexing with glass beads (three times for 1 min each). Broken cells were transferred to fresh tubes and precipitated proteins and cell walls were pelleted by centrifugation (at 10,000 *g* for 5 min). The pellet was reextracted twice for 45 min with additional chloroform/methanol/H_2O (10:10:3). The pooled lipid extracts were dried under nitrogen and freed of salts by butanol extraction as described (Krakow et al., 1986). Lipids were stored at −20°C.

Analytical Methods

Lipid mixtures or isolated lipids were deacylated by mild alkaline hydrolysis as described (Becker and Lester, 1980). After neutralization with acetic acid, lipids were desalted by partitioning between butanol and water as described (Krakow et al., 1986). Strong acid hydrolysis of [^{3}H]palmitic acid labeled lipids was done in 1 N HCl in CH_3OH/H_2O (82:18) for 16 h at 80°C in Teflon-lined glass tubes. Solvent was evaporated and the products subjected to Folch partitioning and the dried organic phase was analyzed by TLC. Strong alkaline hydrolysis was done in 1 N KOH in CH_3OH/H_2O (2:1) at 80°C for 18 h.

Ascending TLC was performed on 0.2-mm-thick silica gel 60 plates (Merck, Darmstadt, FRG) using the following solvent-systems: $CHCl_3$/CH_3OH/4.2 N NH_4OH (9:7:2); $CHCl_3$/CH_3OH/0.25% KCl in water (55:45:10 or 55:45:5); $CHCl_3$/CH_3OH/2 N NH_3 (40:10:1). Lipids were taken up in $CHCl_3$/CH_3OH/water (10:10:3) and 60,000–150,000 cpm were routinely spotted per lane. The developed TLC plates were sprayed with EN^{3}HANCE (New England Nuclear, Boston, MA) and fluorograms were obtained using X-OMAT film exposed for 3–6 d at −80°C. The relative mobilities of [^{3}H]myoinositol-labeled lipids were very similar in the 55:45:10 and the 9:7:2 systems since all lipids were close to the diagonal upon two-dimensional chromatography using solvent 9:7:2 for the first and solvent 55:45:10 for the second dimension. We preferred the solvent 55:45:10 since this solvent separated lipid E from lipid F and this solvent was used unless indicated otherwise. HPLC separation of lipids was performed as described (Wells and Lester, 1983), except that we used a Radial-Pak cartridge containing 10-μm-diam silica gel particles (Water Associates, Inc., Milford, MA) and that the column was run at room temperature. The fractions were neutralized by the addition of acetic acid. Fractions containing radioactivity were pooled and the solvent was evaporated under N_2 gas and the lipids were desalted by phase separation in *n*-butanol and water (Krakow et al., 1986).

Treatment of lipids with PLC was done in the following buffers: PI-PLC *B. thuringiensis* and *B. cereus*: 20 mM Tris-HCl, pH 7.4, 0.1% TX-100, 1 mM EDTA (50 μl/tube). PLD from *S. chromofuscus*: 20 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.05% TX-100, 0.02% NaN_3, 20 mM NaCl, (50 μl/tube). PLD from cabbage was used in 300 μl of 80 mM sodium acetate, pH 5.6, 40 mM $CaCl_2$, and 1% ethanol. Reactions were started by the addition of enzymes; in the case of cabbage PLD, 200 μl of ether was added in addition.

Treated lipids were extracted with butanol and analyzed by TLC. The remaining aqueous phase containing the cleaved headgroups was desalted by the addition of 500 μl water and 200 μl of AG 501-X8 mixed bed ion exchange resin (Bio-Rad Laboratories, Richmond, CA) prewashed with 10 mM myoinositol in water. After incubation, the supernatant was removed, dried and analyzed by descending paper chromatography on filter paper (no. 1; Whatman Instruments Co., Clifton, NJ) using one of the following solvent systems: Ethyl acetate/pyridine/water (12:5:4, or 8:2:1, vol/vol) in a nonequilibrated tank for 15 h. The sample lanes were cut into 1-cm sections and the distributions of radioactivity were determined by scintillation counting. Standards were located by the silver nitrate dip assay (Trevelyan et al., 1950). Headgroups were treated with jack bean α-mannosidase in 50 μl of 5 mM sodium acetate, pH 4.5, 1 mM $ZnCl_2$, 0.01% BSA, 3 mM NaN_3, and 0.5 U of α-mannosidase at 37°C for 16 h.

Results

(a) Arrest of Vesicular Traffic between ER and Golgi Interferes with the Biosynthesis of Certain IPCs

When cells were incubated with [^{3}H]myoinositol, the label got incorporated into PI and a range of more polar lipids which were named by *A–H*, as indicated in Fig. 1, lane *1*. Lipids *C*, *D*, *E*, and *H* were completely resistant to deacylation by mild alkaline hydrolysis whereas lipid *F* only occasionally contained some minor base resistant component and lipids *A*, *B*, and *G* were completely base-sensitive (Fig. 1, lane *4*; Fig. 9). Lipids *A–H* correspond to PI, IPC, MIPC, and $M(IP)_2C$ as indicated in Fig. 1, lane *1*, based *a*) on a comparison of their mobilities in thin layer chromatography (TLC) with those reported in the literature (Smith and Lester, 1974); (*b*) their relative abundance; (*c*) their sensitivity to mild alkaline hydrolysis (Fig. 1); and (*d*) the analysis of their headgroups (see sections *e* and *f*). We did not attempt to assign lipids contained in bands *C* and *D* to the three known subclasses of IPC (IPC-I, -II, -III; Smith and Lester, 1974).

Uptake of low micromolar concentrations of [^{3}H]myoinositol into cells and incorporation into PI is in most experiments complete (80%) within a few minutes (Conzelmann et al., 1990). This is explained by the presence of an

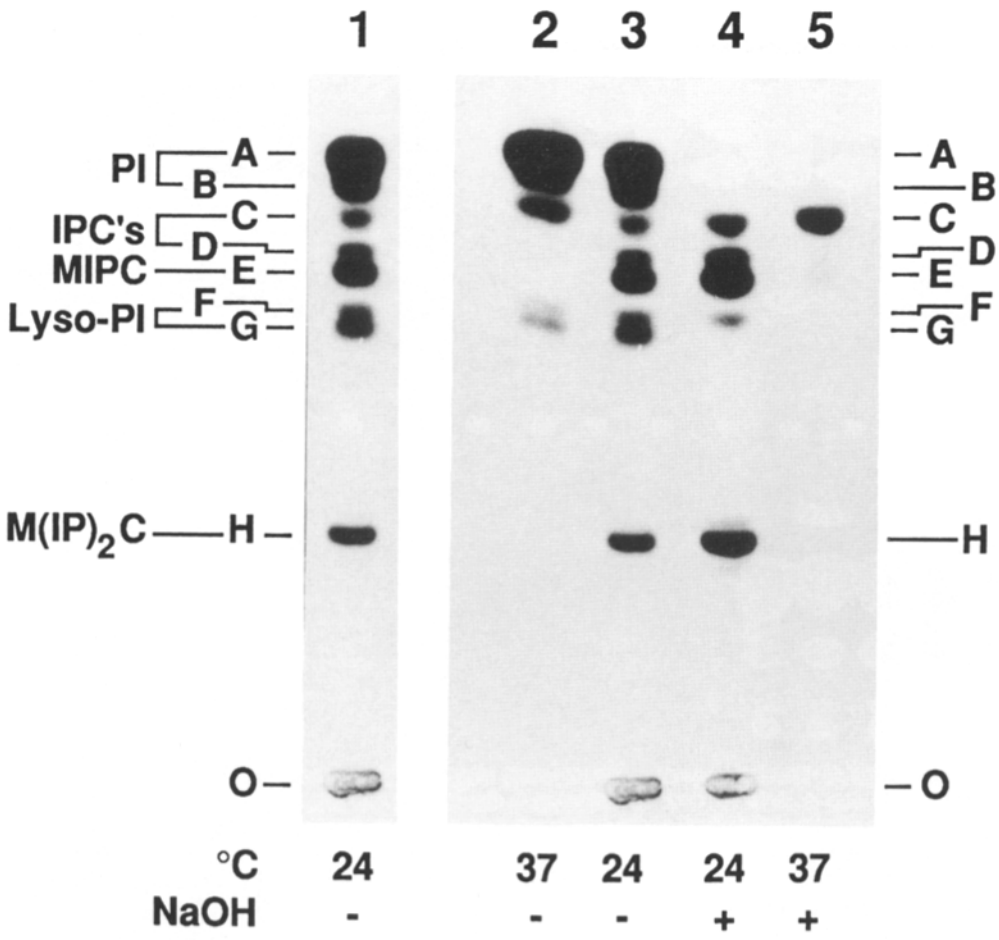

Figure 1. Mild alkaline hydrolysis of [^{3}H]myoinositol-labeled lipids. *Sec23* cells were labeled with [^{3}H]myoinositol at 24°C or 37°C as indicated at the bottom (°*C*) and the lipid extracts either deacylated by mild alkaline hydrolysis with NaOH (+) or control incubated (−). Lipids were extracted with butanol and analyzed by TLC and fluorography. The lipids are designated by letters and belong to the lipid classes indicated at the left. *O* = origin.

active transporter for myoinositol in the plasma membrane of yeast cells (Nikawa et al., 1982), by the fact that the K_M of PI-synthase for myoinositol (0.21 mM) is about nine times higher than the steady-state level of endogenously produced myoinositol in the cytoplasm (Kelley et al., 1988) and by the fact that the cosubstrate for PI biosynthesis, CDP-diacylglycerol appears not to be limiting. IPC/C, IPC/D, MIPC/E, and $M(IP)_2C/H$ also start to appear within a few minutes after addition of ^{3}H-myoinositol (Fig. 5, lane *1*; not shown) but continue to increase in intensity even when the radioactivity in PI has reached a plateau level.

IPC/D, MIPC/E, and $M(IP)_2C/H$ were not made when we labeled *sec23*, a temperature-sensitive secretion mutant in which newly synthesized secretory proteins remain blocked in the ER at 37°C (Novick et al., 1980) (Fig. 1, lane *2*). A systematic survey of available temperature-sensitive secretion mutants showed that the majority of mutants in which the vesicular transport of proteins is blocked at the stage of the ER to Golgi transition, namely *sec12*, *16*, *17*, *18*, *22* and *23*, make neither IPC/D, MIPC/E, nor $M(IP)_2C/H$ at the restrictive temperature (Fig. 2, *A*). *sec13*, *sec20*, and *sec21* made reduced but still significant amounts of these lipids even at 37°C. The biosynthesis of IPC/D, MIPC/E, and $M(IP)_2C/H$ in these three latter mutants was not due to the presence of revertants since labelings were routinely performed with precultures derived from a single colony. When the time of preincubation at 37°C used to instore the secretion block before the addition of ^{3}H-myoinositol was prolonged to 30 or 90 min, labeling of IPC/D, MIPC/E, and $M(IP)_2C/H$ was almost completely abolished in *sec13* and *sec21* but *sec20* still produced a reduced amount of these lipids even after these prolonged preincubations (Fig. 2 *B*). Lipids *C*, *D*, *E*, and *H* were base resistant in all these mutants as well as in wild type cells (not shown). Whereas the absence of IPC/D, MIPC/E, and $M(IP)_2C/H$ in these secretion mutants was a consistent finding in dozens of experiments, the relative intensity of several lipids, but in particular of IPC/D and lyso-PI/FG was somewhat variable. Exclusion of O_2 from the preculture and/or during labeling had no significant influence on the synthesis of IPC/C, MIPC/E, and $M(IP)_2C/H$ but slightly increased the relative amount of IPC/D. *sec19*, a mutant that arrests secretion at multiple stages, made normal amounts of all lipids at 37°C.

(b) Arrest of Secretion at the Golgi Stage or Later Does Not Interfere with Biosynthesis of Inositol-containing Sphingolipids

SEC7 and *SEC14* encode for cytoplasmic proteins that are required for the transit of secretory proteins through the Golgi apparatus (Achstetter et al., 1988; Bankaitis et al., 1989; Franzusoff and Schekman, 1989), whereas *SEC6* is required for the fusion of secretory vesicles with the plasma membrane (Esmon et al., 1981). As can be seen in Fig. 3, wild-type cells show a moderate increase in production of lipids IPC/C, IPC/D, MIPC/E at 37°C as compared with 24°C. In the mutants, biosynthesis of IPC/D and MIPC/E is slightly

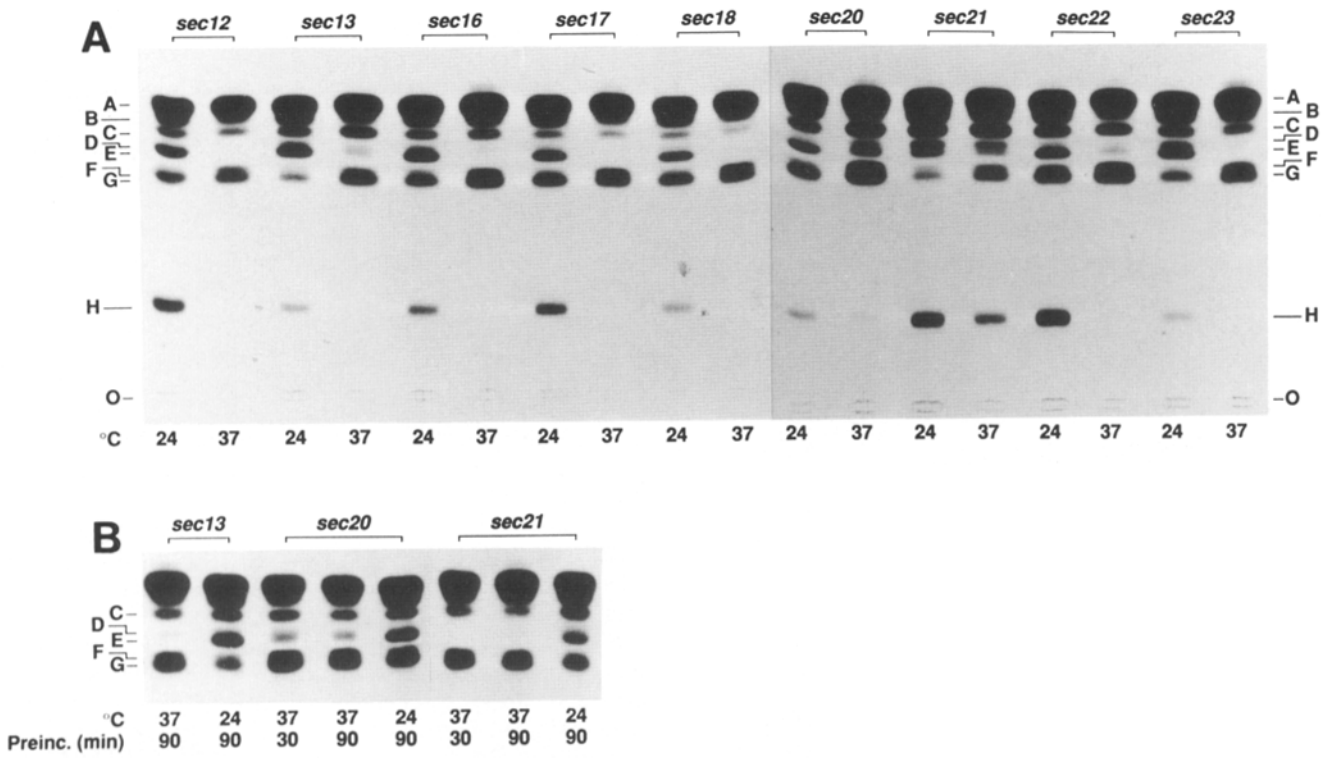

Figure 2. [^{3}H]Myoinositol incorporation into lipids in secretion mutants affecting early stages of the secretory pathway. (*A*) Secretion mutants were labeled with [^{3}H]myoinositol for 10 min at 24°C or 37°C as indicated at the bottom and after a preincubation of 10 min at the same temperature. After labeling, 4 vol of medium, prewarmed to the labeling temperature and supplemented with cold myoinositol (200 μM) were added and cells were further incubated at the labeling temperature for 50 min. After this chase, lipids were extracted and analyzed by TLC and fluorography. (*B*) Some of the mutants were labeled at 24°C or 37°C as above but preincubations before the addition of [^{3}H]myoinositol were 30 or 90 min, as indicated at the bottom.

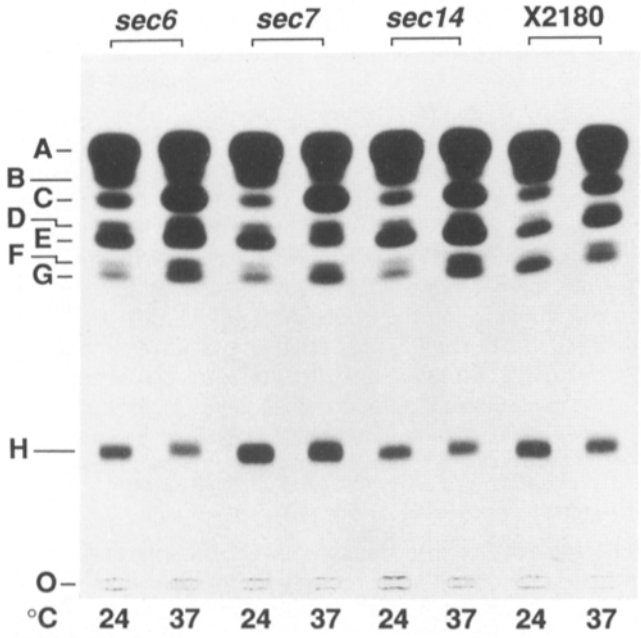

Figure 3. Mutations affecting late stages of the secretory pathway do not affect the biosynthesis of IPC/D, MIPC/E, and M(IP)$_2$C/H. *sec6*, *sec7*, *sec14*, or wild-type cells (X2180) were labeled with [^{3}H]myoinositol at 24°C or 37°C and the lipid extracts analyzed by TLC/fluorography.

higher than in wild type already at 24°C and labeling at 37°C enhances production of these lipids even more except for *sec7*, in which labeling of MIPC/E was found to be somewhat reduced at 37°C in comparison with the 24°C control in several experiments. On the whole, it appears that vesicular transport through the Golgi apparatus and to the plasma membrane is not required for the biosynthesis of IPC/D, MIPC/E, and M(IP)$_2$C/H.

(c) Kinetics of [^{3}H]myosinositol Incorporation into Base-Labile and Base-Resistant Lipids

The synthesis of phosphoinositol-containing lipids has been studied previously with yeast cells labeled overnight with [^{3}H]myoinositol which, after being placed into fresh myoinositol-free medium, incorporated radiolabel into M(IP)$_2$C for up to 6–10 h, whereas the label decreased in PI correspondingly (Angus and Lester, 1972; Becker and Lester, 1977). Increases in IPC's and MIPC were only small during chase. Subsequently, the transfer of phosphoinositol from PI onto ceramides was elegantly demonstrated also in an in vitro system containing crude yeast membranes plus radiolabeled PI and in which two subclasses of IPC's and M(IP)$_2$C (but no MIPC) were generated (Becker and Lester, 1980). This led to the proposal of the following biosynthetic scheme: PI + ceramide → IPC + diacylglycerol; IPC + GDP-mannose → MIPC + GDP; MIPC + PI → M(IP)$_2$C + diacylglycerol. The slow increase of radioactivity in M(IP)$_2$C upon

further culture of equilibrium-labeled cells (Angus and Lester, 1972) did not directly demonstrate that newly made PI is immediately able to serve as a substrate for the biosynthesis of inositol-containing sphingolipids. Since we observed significant [^{3}H]myoinositol incorporation into IPC's, MIPC, and M(IP)$_2$C already after 6 min of pulse (not shown), we asked whether this label is derived from PI or whether [^{3}H]-myoinositol could also get incorporated into inositol-containing sphingolipids directly.

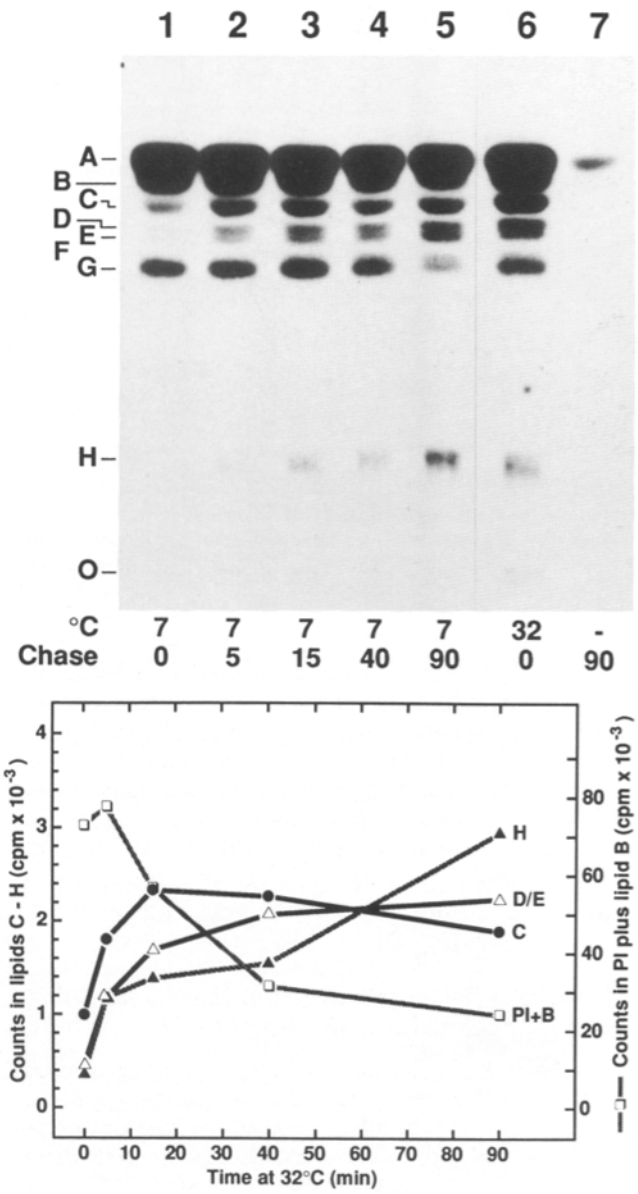

Figure 4. Incorporation of [^{3}H]myoinositol into IPC/D, MIPC/E, and M(IP)$_2$C/H in presence of high concentrations of cold myoinositol. (*Top*) X2180 cells were preincubated (25 min) and labeled with [^{3}H]myoinositol (25 min) at 7°C or 32°C as indicated below (°*C*) Cells were diluted with 6 vol of medium containing 500 μM myoinositol and further incubated at the labeling temperature for 13 min. In lane *7*, the [^{3}H]myoinositol was added only after cold myoinositol had been added. Tubes *2–5* and *7* were then further chased at 32°C for indicated times (chase indicated in minutes). (*Bottom*) Zones containing radioactivity were located using the fluorogram and scraped off the TLC plate for quantitation by scintillation counting.

We found that the incorporation of [^{3}H]myoinositol into PI was more rapid than incorporation into IPC's, MIPC and M(IP)$_2$C (not shown), this rate difference being even more pronounced when the labeling was performed at low temperature. Thus, labeling at low temperature for short periods resulted in the labeling of only PI/AB, lyso-PI/FG, and some IPC/C (Fig. 4, lane *1*). During a subsequent chase at 32°C in the presence of high concentrations of cold myoinositol, considerable amounts of IPC/C, IPC/D, MIPC/E, and M(IP)$_2$C/H were made (Fig. 4, lanes *2–5*). The kinetics and pulse–chase experiments are thus in agreement with the idea that [^{3}H]myoinositol gets first incorporated into PI and is then transferred from PI to IPCs, MIPC, and M(IP)$_2$C, as postulated by the in vivo and in vitro experiments of Lester and collaborators.

In *sec18* cells, the transfer of [^{3}H]myoinositol from PI onto ceramides in the presence of cold myoinositol proceeded only at 24°C but not at 37°C. As shown in Fig. 5, only small amounts of IPC/D and MIPC/E were present after 5 min of pulse and 5 min of chase at 24°C (lane *1*); if cells were subsequently shifted to 37°C, there was small increase of IPC/D, MIPC/E, and M(IP)$_2$C/H over starting levels. This increase was possibly due to synthesis during the few minutes elapsing until the secretion block was instored. Incubation of labeled cells at 24°C, on the other hand, led to the expected synthesis of large amounts IPC/D, MIPC/E, and M(IP)$_2$C/H (lanes *2* and *4*). More importantly, when the initial labeling was carried out at 37°C (lane *5*), IPC/D, MIPC/E, and M(IP)$_2$C/H were not present after labeling but these lipids could be observed after a subsequent chase at 24°C for 0.5 or 2 h (lanes *6* and *7*). The absolute amounts of IPC/D, MIPC/E, and M(IP)$_2$C/H made during a 2-h chase at 24°C, after labeling at 37°C, were somewhat lower than what was observed after a similar chase in cells labeled at 24°C (compare lanes *2* and *7*). This might be explained by the fact that in the former case cells require part of the chase period to reinstore vesicular traffic (Novick et al., 1981; Tschopp et al., 1984) or else that cells need to resynthesize enzymes required for the biosynthesis of lipids *D*, *E*, and *H*. This result clearly speaks against the possibility that the lipid moiety of PI made in *sec18* cells at 37°C is structurally different from the one made at 24°C to the extent that it cannot serve as donor of phosphoinositol, since PI molecules made at both temperatures can donate [^{3}H]myoinositol for the biosynthesis of IPC/D, MIPC/E, and M(IP)$_2$C/H, provided the vesicular transport from ER to Golgi is not blocked.

(d) Energy Dependence of Biosynthesis of IPC/D, MIPC/E, and M(IP)$_2$C/H

Incorporation of [^{3}H]myoinositol into PI was found to be completely blocked in the presence of metabolic inhibitors, probably because myoinositol is taken up by cells by an energy-dependent, active transport mechanism (Nikawa et al., 1982). As mentioned, low temperature reduces the incorporation of [^{3}H]myoinositol into inositol-containing sphingolipids more than the incorporation into PI. This differential effect allowed to measure an energy dependence of IPC biosynthesis in intact cells. While a pulse at 7°C still resulted in the biosynthesis of low amounts of IPC/D and MIPC/E

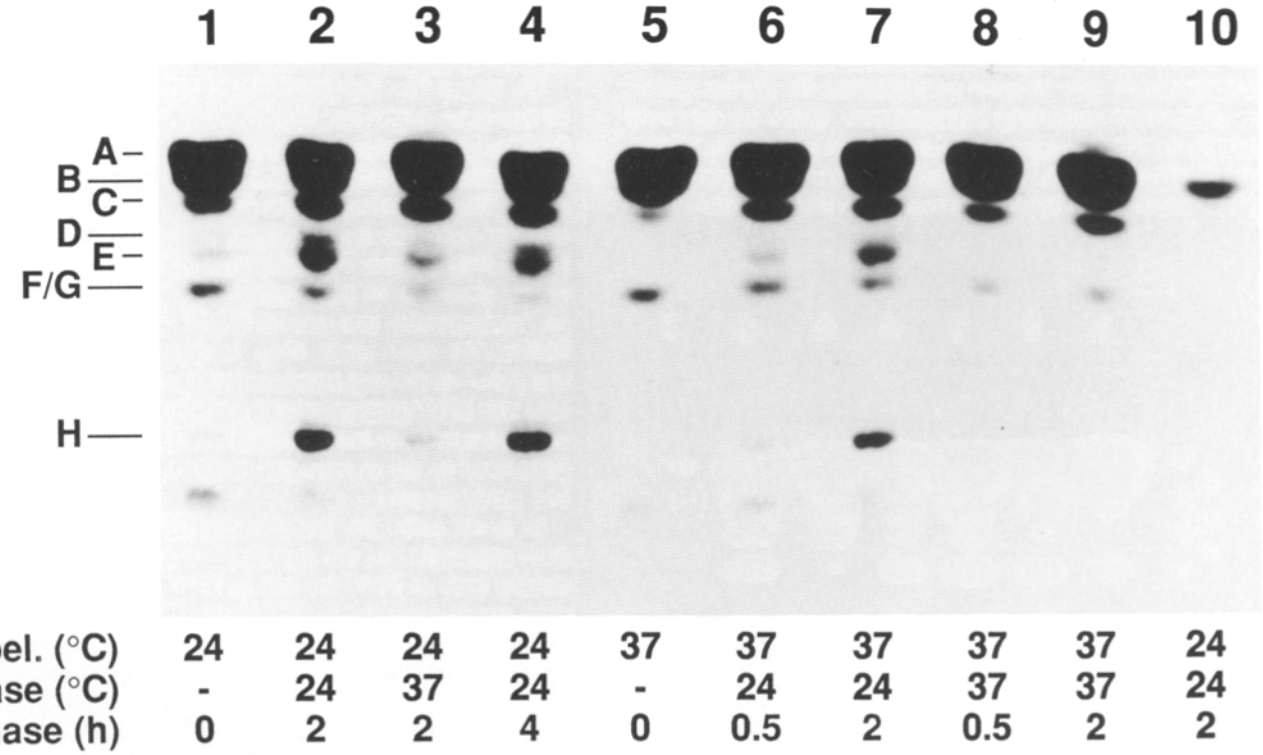

Figure 5. Synthesis of IPC/D, MIPC/E, and $M(IP)_2C/H$ from PI during recovery from the secretory block. *sec18* cells were preincubated for 15 min and labeled with [^{3}H]myoinositol for 5 min at the indicated temperatures. Cells were diluted with 20 vol of minimal medium containing 200 μM myoinositol and chased at the labeling temperature for 5 min. At this point the incubations were either stopped (lanes *1* and *5*) or tubes were further incubated for chase at the temperature and for the time periods (in hours) indicated at the bottom. In lane *10*, radioactivity was added at the start of the chase only.

(Fig. 4), cells only made PI/AB, lyso-PI/FG, and some IPC/C during a pulse at 3°C (Fig. 6, lane *1*). As expected, cells produced IPC/C, IPC/D, MIPC/E, and $M(IP)_2C/H$ during a subsequent chase at 24°C (Fig. 6, lane *3*). When NaN_3 was present during this chase period, the biosynthesis of IPC/D, MIPC/E, and $M(IP)_2C/H$ was drastically reduced, whereas biosynthesis of IPC/C was less severely affected (lane *2*). Thus, the biosynthesis of IPC/D, MIPC/E, and $M(IP)_2C/H$ clearly requires metabolic energy, whereas this requirement is slightly less pronounced for IPC/C.

(e) Radiolabeling of IPC/D, MIPC/E, and $M(IP)_2C/H$ with [^{3}H]Palmitic Acid and [^{3}H]Mannose

The flux of [^{3}H]myoinositol via newly made PI into certain inositol-containing sphingolipids being blocked in the early secretion mutants, we wondered whether the biosynthesis of these lipids halted completely in these mutants or whether it continued drawing from the pool of cold PI which had been made before the instoration of the secretion block. In an attempt to resolve this, we tried to radiolabel IPCs in their lipid moiety by offering [^{3}H]palmitic acid to cells. Labeled lipids were extracted and analyzed by TLC (Fig. 7). *sec18* labeled at 24°C or 37°C incorporated [^{3}H]palmitic acid into a large variety of lipids, the profile being very similar at the two labeling temperatures (lanes *2* and *4*). Upon mild alkaline hydrolysis of these lipid mixtures, most of the radioactivity of both samples comigrated with [^{3}H]palmitic acid (Fig. 7, lanes *1* and *3*). The major base-resistant lipids from cells labeled at 24°C comigrated with bands IPC/C, MIPC/E and $M(IP)_2C/H$ (Fig. 7, lanes *1*, *5*, and *6*), whereas the resistant lipids from cells labeled at 37°C mainly consisted of a species comigrating with IPC/C (Fig. 7, lanes *3*, *5*, and *7*). In addition, minor base-resistant bands (labeled *X* and *Y*) were found in both extracts, whereas a minor band at position D was only made by cells at 24°C (lanes *1* and *3*). This suggested that [^{3}H]palmitic acid was incorporated into the same lipids IPC/C, IPC/D, MIPC/E, and $M(IP)_2C/H$ as

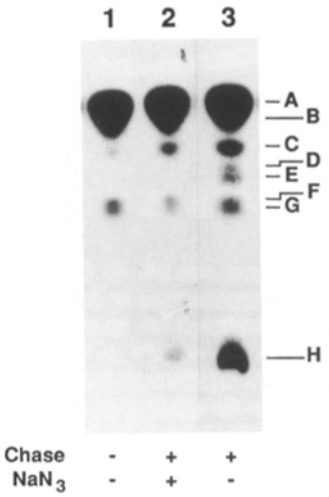

Figure 6. Biosynthesis of IPC/D, MIPC/E, and $M(IP)_2C/H$ requires metabolic energy. X2180 cells were precultured overnight in glucose-free minimal medium containing pyruvate as a carbon source at 30°C. Cells were preincubated at 3°C for 20 min and labeled with [^{3}H]myoinositol at 3°C without agitation in the same medium. After 30 min, all tubes received cold myoinositol and were further chased at 3°C for 20 min to allow for depletion of ATP. Subsequently sodium azide (3 mM final) was added to one tube (lane 2). The incubations were either continued for 20 min at 24°C (*chase*, +) or stopped (*chase*, −) after dilution with 4 vol of fresh medium.

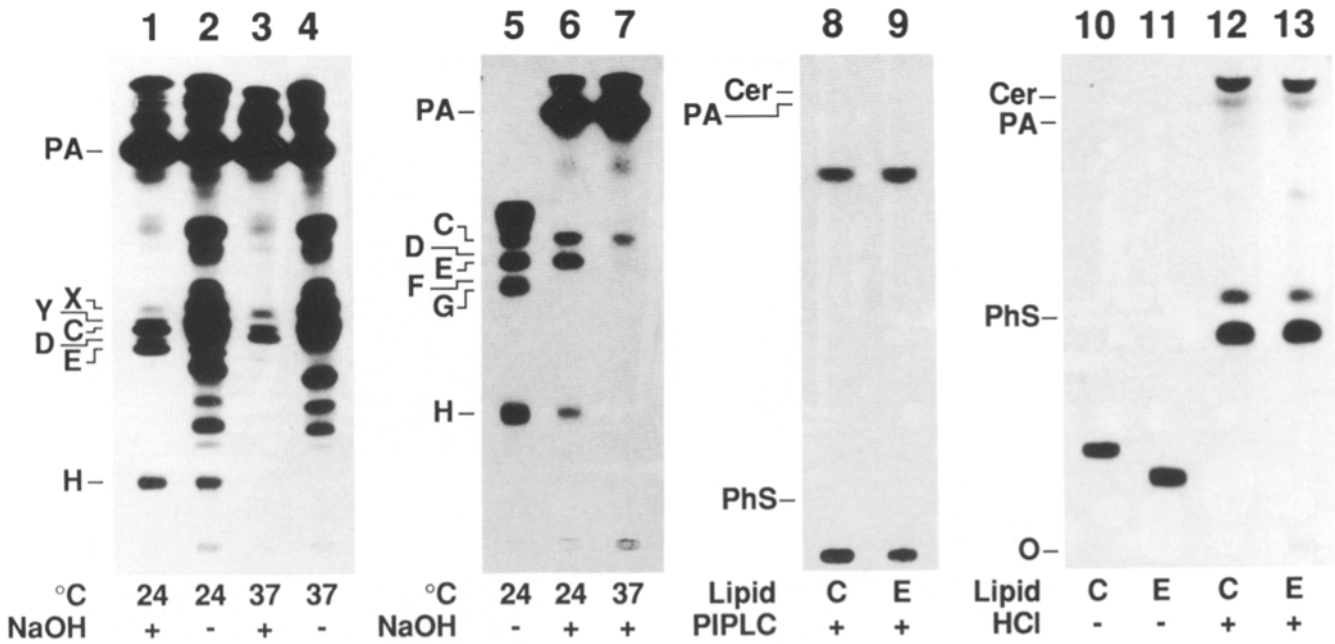

Figure 7. Analysis of [^{3}H]palmitic acid-labeled lipids. *sec18* cells were labeled with [^{3}H]palmitic acid at 24°C or 37°C. Lanes *1–7*: TLC analysis of the lipid extracts after mild alkaline hydrolysis with NaOH (*3+*) or after control incubation (−) in solvent 55:45:10. Lane *5* shows the mixture of [^{3}H]myoinositol-labeled lipids on the same chromatogram for comparison. Pure lipids *C* and *E* were obtained by preparative HPLC of the deacylated lipids from cells labeled at 24°C. Lanes *8* and *9*: Lipids *C* and *E* were treated with PI-PLC (*B. cereus*) and products analyzed by TLC (solvent 40:10:1). Lanes *10–13*: Purified lipids *C* and *E* were subjected to strong acid hydrolysis with HCl (+) or control incubated (−) and products run on TLC (solvent 55:45:5). Mobilities of lipid standards are indicated: *PA* = palmitic acid, *Cer* = ceramide type III from bovine brain, *PhS* = phytosphingosine, *X* and *Y* point to nonidentified base-resistant lipids.

[^{3}H]myoinositol since the labeled species comigrated on TLC, were base-resistant and their synthesis showed the same dependence on *SEC18*. To confirm this notion, the [^{3}H]palmitic acid labeled, base-resistant lipids comigrating with IPC/C and IPC/E were degraded by several hydrolytic procedures. TLC analysis of the resulting products did not reveal any difference in the lipid moieties of IPC/C and IPC/E. Thus, after treatment with PI-PLC, the products had identical mobilities in two solvent systems (Fig. 7, lanes *8* and *9*). Strong alkaline hydrolysis resulted in the generation of several products, the major one of which comigrated with phytosphingosine (not shown). Strong acid hydrolysis of IPC/C and IPC/E also yielded a band comigrating with phytosphingosine in two solvent systems and some minor bands which might represent partial breakdown products or hydroxylated fatty acids (Fig. 7, lanes *10–13*). Nitrous acid treatment of these lipids (Krakow et al., 1986) did not influence their mobility (not shown). Thus, the results suggested that early *SEC* genes not only control the flux of [^{3}H]myoinositol (via PI) but also the flux of [^{3}H]palmitic acid (via phytosphingosine) into IPC/D, MIPC/E and M(IP)$_2$C/H and hence that defects in these genes halt the biosynthesis of these lipids completely. Results also suggested that [^{3}H]palmitic acid was incorporated by yeast cells into phytosphingosine as well as into other components, possibly long chain fatty acids, and that the difference between IPC/C and MIPC/E resided outside the lipid portion. Also, the presence of phytosphingosine in lipids C and E was consistent with the assignments *C* = IPC and *E* = MIPC.

[^{3}H]Mannose labeled only lipids MIPC/E and M(IP)$_2$/H and these lipids were not labeled in *sec18* cells at 37°C (not shown). Thus, *SEC18* also regulates the flow of [^{3}H]mannose into these lipids.

(f) Structural Analysis of Lipids C, D, E, and H

Lipids *C*, *D*, *E*, and *H* fit the description of IPCs, MIPC and M(IP)$_2$C in that they are abundant, base-resistant myoinositol-containing lipids (Steiner et al., 1969; Smith and Lester, 1974). To confirm the assignments shown in Fig. 1, we analyzed the headgroups of these various lipids. For this, base-resistant lipids were separated by HPLC (Fig. 8). HPLC revealed the presence of an additional base-resistant, inositol-containing lipid (labeled *D'*) which migrates on TLC with an R_f intermediate between *C* and *D* (Fig. 8 *a*), but which is usually undetectable in TLC separation of total lipids, probably because of its lower abundance and its partial overlap with MIPC/D on TLC.

The total extracts of [^{3}H]myoinositol-labeled lipids from *sec6*, *sec7*, *sec17* and *sec18* cells (labeled at 37°C) and wild-type cells were digested with different phospholipases. Although PI-PLC from *B. thuringiensis* and from *B. cereus* were less efficient in cleaving the base-resistant IPC/C, IPC/D, MIPC/E, and M(IP)$_2$C/H than PI/AB and lyso-PI/FG, these enzymes achieved at least partial hydrolysis of IPC/C and to a lesser degree of MIPC/E, whereas M(IP)$_2$C was not cleaved (0.5 U/ml, 1 h, 30°C). Moreover, almost quantitative cleavage of base-resistant lipids could be achieved by high concentrations of PLD from *S. chromofuscus* (50 U, 16 h, 30°C), or PLD from cabbage (30 U, 4 h, 37°C) (not shown). The susceptibility of these lipids to phospholipases suggests that they are phospholipids, but it would appear that

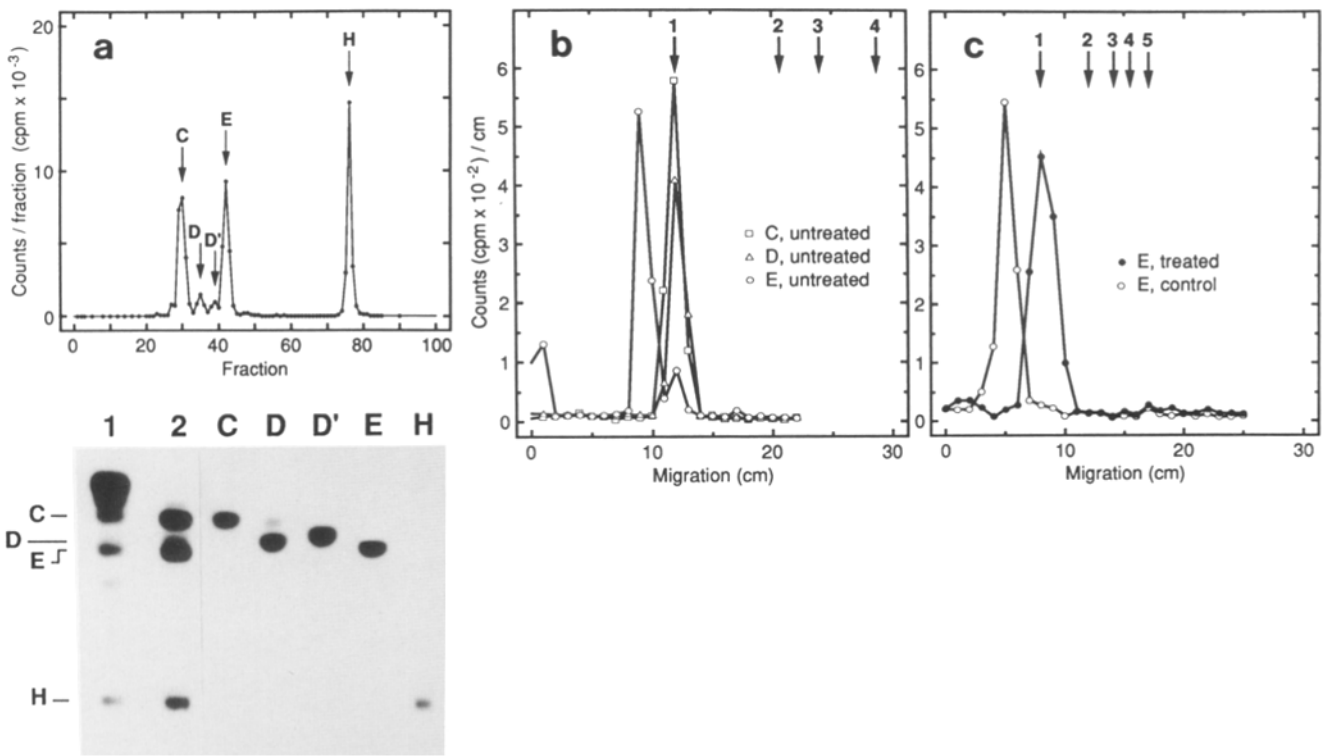

Figure 8. Analysis of [^{3}H]myoinositol-labeled headgroups. (*a*): Lipids from [^{3}H]myoinositol-labeled X2180 cells were deacylated by mild alkaline hydrolysis and separated by HPLC. 1-ml fractions were collected and aliquots of each fraction used for determination of tritium. *C, D, D', E,* and *H* were given to peaks containing lipids *C, D, D', E,* and *H* based on the analysis by TLC of an aliquot of each peak (fluorogram of TLC below *a*). The TLC also contains an aliquot of untreated (lane *1*) and NaOH-treated (lane *2*) lipids (starting material) for comparison. Each lipid was treated with PLD from *S. chromofuscus* and the released headgroups from lipids *C, D,* and *E* were analyzed by paperchromatography (*b*, solvent 12:5:4). The headgroup of lipid *E* was treated with jack bean α-mannosidase or control incubated (*c*, solvent 8:2:1). Paperchromatograms were cut into 1-cm sections and counted by scintillation counting. Standards were: myoinositol (*1*); glucosamine (*2*); galactose (*3*); glucose (*4*); mannose (*5*).

their ceramide moiety is much less well recognized by PLCs than diacylglycerol. Treatment of PI and lipid *B* with phospholipase A_2-generated lipids comigrating with *F* and *G*, thus demonstrating that lipids *F* and *G* are lysoforms of PI and lipid *B*.

The cleavage by PLD was exploited to prepare headgroups from [^{3}H]myoinositol-labeled lipids *A/B, C, D, D', E, F/G,* and *H* for paper chromatography analysis. The headgroups of lipids *A/B, C, D, D',* and *F/G* comigrated with myoinositol, whereas *E* migrated less fast and *H* stayed at the origin (Fig. 8 *b*, data not shown). After treatment of headgroup *E* with α-mannosidase the product comigrated with myoinositol (Fig. 8 *c*) whereas headgroup *H* still stayed at the origin after α-mannosidase treatment (not shown). The generation of myoinositol from the headgroup of lipid *E* was blocked by the presence of 100 mM α-methylmannoside, indicating that the effect was due to α-mannosidase rather than to a contaminating activity. These results clearly confirm the assignments *C, D, D'* = IPCs, *E* = MIPC. The assignment *H* = $M(IP)_2C$ relies on the migration of lipid *H* on TLC, its relative abundance, the size of its headgroup, which is bigger or more polar than the one of MIPC (lipid *E*) and the resistance of this headgroup to α-mannosidase, and the fact that it can be labeled with [^{3}H]mannose. The assignment of lipid *E* to MIPC suggested that *sec53*, a temperature-sensitive mutant which does not make GDP-mannose at 37°C (Kepes and Schekman, 1988), would be unable to make MIPC/E at 37°C. As shown in Fig. 9, synthesis of MIPC/E as well as $M(IP)_2C/H$ was significantly but not completely depressed in *sec53*, whereas a related mutant, *sec59* (Bernstein et al., 1989), made normal amounts of all lipids.

(g) Biosynthesis of Phosphatidylcholine and Sulfated Lipids

We tried to investigate whether the biosynthesis of lipids other than the inositol-containing sphingolipids is dependent on *SEC* gene products. In the absence of exogenously added ethanolamine, *S. cerevisiae* make phosphatidyl-ethanolamine (PE) by decarboxylation of phosphatidyl-serine (PS), a reaction that is confined to the mitochondrial inner membrane (Kuchler et al., 1986). In the absence of exogenous choline, phosphatidylcholine is made by methylation of PE, a reaction which is carried out by two methyl-transferases (*CHO2* and *OPI3*), which are localized in the microsomal fraction (Kuchler et al., 1986) and which have recently been cloned (Summers et al., 1988; McGraw and Henry, 1989). Therefore, if *S. cerevisiae* are labeled with [^{14}C]serine in minimal medium, the label is expected to get incorporated into PS, PE, and PC, the labeling of PC being dependent on the transfer of PE from the mitochondria to some microsomal com-

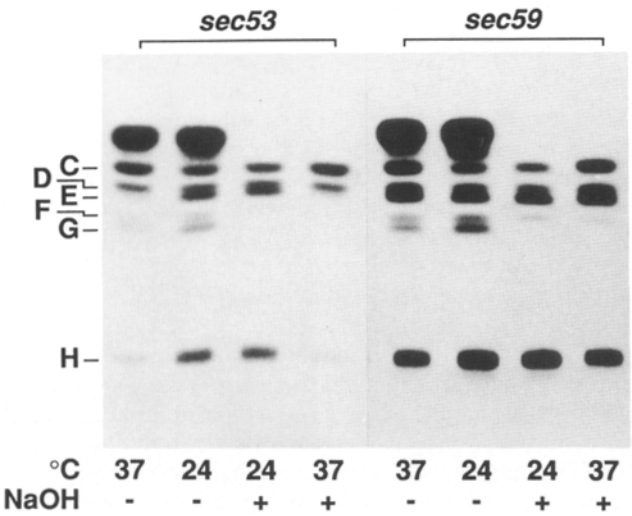

Figure 9. *sec53* does not make MIPC/E but makes some IPC/D at 37°C. *sec53* and *sec59* cells were labeled with [^{3}H]myoinositol at 24°C or 37°C and the lipid extracts analyzed as described in legend of Fig. 1.

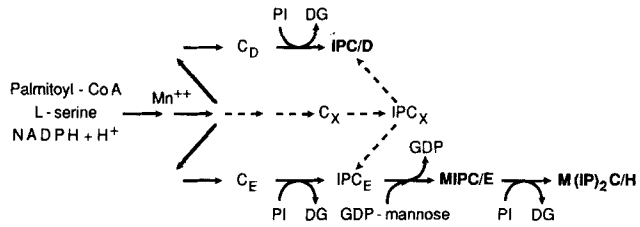

Figure 10. Scheme of probable reactions involved in biosynthesis of IPC/D, MIPC/E, and M(P)$_2$C/H. Abbreviations used: C_D, C_E: ceramides of IPC/D and MIPC/E, respectively; IPC_E: IPC containing the ceramide moiety typical of MIPC/E; C_X, IPC_X: hypothetical intermediates; *DG*: diacylglycerol. Black arrows indicate the probable biosynthetic pathways based on Steiner et al., 1969; Smith and Lester, 1974; Becker and Lester, 1980. Some alternative steps are shown with stippled arrows since it cannot be ruled out at present that modifications of ceramide moieties occur after addition of inositolphosphate.

partment. *sec18*, *sec7*, and *sec6*, when labeled with [^{14}C]serine, made normal amounts of PC at 37°C, suggesting that PE is transported from mitochondria to the microsomes in a way which does not require *SEC18* and also that a block in the later stages of secretion is without effect on the methylation of PE (not shown). Moreover, chloroform/methanol/water (10:10:3) extracts from *sec18* cells labeled at 24°C or 37°C with $^{35}SO_4$ yielded identical profiles of labeled components when analyzed by TLC (data not shown).

Discussion

Recent EM analysis of early secretion mutants shows that some mutants fail to form secretory vesicles off the ER membranes (class I mutants, *sec12*, *13*, *16*, *23*), whereas other mutants fail to fuse these vesicles with the Golgi apparatus (class II mutants, *sec17*, *18*, *22*) (Kaiser and Schekman, 1990). Our results clearly indicate that the biosynthesis of MIPC, of M(IP)$_2$C and a subclass of IPCs (IPC/D) is severely affected in both of these classes of mutants, whereas mutations affecting transport at later stages of the secretory pathway do not interfere with the biosynthesis of these lipids. Data presented in Fig. 5 also show that the defect is reversible in the sense that PI made at 37°C can be used for the biosynthesis of IPC/D, MIPC/E, and M(IP)$_2$C/H after a shift of labeled cells to the permissive temperature. This implies that the PI made at 37°C gets and maintains the potential to serve as a donor substrate for the biosynthesis of IPC/D, MIPC/E, and M(IP)$_2$C/H.

Why Do SEC Genes Influence the Biosynthesis of IPC/D, MIPC/E, and M(IP)$_2$C/H?

Previous structural analysis has shown that the three subclasses of IPC's and MIPC each contain a different type of ceramide; in particular IPCs mainly contain saturated and unsaturated forms of phytosphingosine (hydroxysphinganine), whereas the MIPC contains mainly sphinganine (91%) and eicosasphinganine (5%) (Smith and Lester, 1974). This finding suggests that separate biosynthetic pathways operate for IPC/D and MIPC/E as outlined in Fig. 10.

In analogy to the assignment of various glycosylation reactions to ER or Golgi compartments made possible through the analysis of structures of *N*-glycans of glycoproteins accumulating in different *sec* mutants (Esmon et al., 1981; Franzusoff and Schekman, 1989) we are tempted to propose that *SEC* genes control the biosynthesis of IPC/D, MIPC/E, and M(IP)$_2$C/H by controlling the flow of substrates and that biosynthetic reactions are compartmentalized. The most likely version of such a "vesicular traffic" model would say that IPC/D, MIPC/E, and M(IP)$_2$C/H are made in the Golgi and that substrates necessary for their biosynthesis reach the Golgi by vesicular traffic only. (The organelle(s) where IPC/D, MIPC/E, and M(IP)$_2$C/H are made could be different from Golgi, but would have to receive substrates by a pathway controlled by the same nine *SEC* genes that control traffic from the ER to the Golgi. In the following, this hypothetical biosynthetic compartment will be referred to as "Golgi" for simplicity.) At present, however, we cannot rule out other possibilities, namely, (*a*) that some of the enzymatic reactions required for the biosynthesis of IPC/D, MIPC/E, and M(IP)$_2$C/H are critically dependent on normal physiological conditions which are perturbed by the secretion block, i.e., the fragmentation, distension, and ionic changes in the biosynthetic compartment, which are induced by the secretion block, could suffice to stop biosynthesis of these lipids. (*b*) Also, IPC/D, MIPC/E, and M(IP)$_2$C/H might continue to be made in the early secretion mutants at 37°C but might be quantitatively degraded or further metabolized when the secretion is blocked, At present, the only argument in favor of the vesicular traffic model comes from the fact that the block of vesicular traffic affects the biosynthesis of IPC/D, MIPC/E, and M(IP)$_2$C/H very rapidly. In fact, in wild type cells or in *sec18* cells labeled at 24°C, lipids *D*, *E*, and *H* are detectable already 6 min after addition of [^{3}H]myoinositol whereas these lipids are not made when *sec18* cells are shifted to 37°C at the moment of the addition of ^{3}H-myoinositol (not shown). Thus, the secretion block affects biosynthesis of IPC/D, MIPC/E, and M(IP)$_2$C/H rapidly, i.e., within 6 min at most and at a time at which the morphological appearance of the ER might still be normal.

Two subclasses of IPCs and low amounts of $M(IP)_2C$ were observed to accumulate when extensively washed yeast membranes were incubated with [^{32}P]phosphatidyl-[^{3}H]myo-inositol in vitro (Becker and Lester, 1980). This reaction occurs in the absence of cytosol, ATP, and GDP-mannose and in the presence of a simple Tris-HCl buffer and of 0.2% Triton-X-100, i.e., under quite nonphysiological conditions. In our hands, microsomes only make IPC/C and traces of $M(IP)_2C/H$ under these conditions. Thus, the lipids which are made under the control of SEC genes in vivo are not efficiently made by the in vitro system. It is, however, impossible to interpret this finding in favor of the one or the other of the various models. The energy requirement for synthesis of IPC/D, MIPC/E, and $M(IP)_2C/H$ in living cells is compatible with the "vesicular traffic" model since vesicular flow from ER to Golgi has been shown to require ATP (Novick et al., 1981; Balch et al., 1986) but the other possibilities mentioned above might also imply ATP-dependent processes. Since ceramide biosynthesis requires ATP (Kishimoto, 1983), energy deprivation might affect biosynthesis of IPC's if ceramide is limiting but since considerable amounts of IPC/C are still made in the presence of NaN_3 this effect by itself may not explain the almost complete depression of IPC/D, MIPC/E, and $M(IP)_2C/H$ by NaN_3. We are currently trying to get more definite evidence in favor or against the vesicular traffic model by measuring biosynthetic activities for MIPC/E and $M(IP)_2C/H$ in various subcellular fractions.

One secretion mutant (*sec20*) which is blocked between the ER and the Golgi still makes a reduced amount of IPC/D, MIPC/E, and $M(IP)_2C/H$ at 37°C. Interestingly, the careful morphological analysis of the early secretion mutants by EM reveals that the vesicles accumulating in *sec20* display a unique spatial distribution (Kaiser and Schekman, 1990).

What Substrate Could Be Transported by Vesicular Flow Only?

Since the vesicular traffic model seems to be the most likely, one might ask what substrate(s) needed for the biosynthesis of IPC/D, MIPC, or $M(IP)_2C$ could possibly reach the compartment where this biosynthesis takes place by an obligatory vesicular transport step (in the following called the "critical substrate"). Possible "critical substrates" are contained in Fig. 10 and comprise PI, ceramides, GDP-mannose, and some forms of IPC (IPC_x, IPC_E). Based on the current knowledge about biosynthesis, intracellular transport and bilayer distribution of PI (Kuchler et al., 1986; Imai and Gershengorn, 1987; Higgins et al., 1989, Daum et al., 1986; Aitken et al., 1990), of ceramides (Bell et al., 1981; Pagano and Sleight, 1985; Lipsky and Pagano, 1985; Futerman et al., 1989) and of GDP-mannose (Abeijon et al., 1989) we presently favor IPC's as the most likely "critical substrate."

If the vesicular traffic model will prove to be correct, the biosynthesis of IPC/D, MIPC/E, and $M(IP)_2C/H$ may eventually be taken as an indicator for vesicular flow between ER and the Golgi apparatus in the absence of protein synthesis or in glycosylation mutants in which glycans of proteins cannot serve as a reflection of the passage of molecules through secretory compartments. Thus, it is difficult to determine at what stage the vesicular flow is interrupted in *sec53-6* cells since secretory proteins remain unglycosylated in this mutant due to the lack of GDP-mannose at 37°C. As expected, synthesis of MIPC is severely reduced at 37°C in *sec53*, a fact that can be explained by the GDP-mannose requirement of the reaction IPC + GDP-mannose → MIPC (Becker and Lester, 1980). It is interesting, however, to note that some IPC/D and some $M(IP)_2C$ are still made in this mutant at 37°C (Fig. 9), which, if the vesicular traffic model is correct, would indicate that some vesicular traffic to the Golgi apparatus still occurs at 37°C. (Biosynthesis of $M(IP)_2C/H$ at 37°C in *sec53* probably indicates transfer of inositol-phosphate onto preexisting cold MIPC, a reaction that also explains the biosynthesis of low amounts of $M(IP)_2C/H$ in the absence of detectable synthesis of MIPC/E) in microsomes in vitro.

We hope that our observations will open the possibility to exploit existing yeast mutants to understand the mechanisms of lipid transport and intracellular flow of lipids in eukaryotic cells and vice versa, that this analysis of lipid flow in yeast secretion mutants will yield additional criteria allowing us to monitor vesicular flow between secretory organelles.

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