Intracellular transport of inositol-containing sphingolipids in the yeast, *Saccharomyces cerevisiae*

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Abstract Organelles of the early protein secretion pathway (ER, Golgi) are involved in biosynthesis and intracellular migration of the yeast sphingolipids, inositolphosphorylceramide (IPC), mannosylinositolphosphorylceramide (MIPC), and mannosyl-diinositolphosphorylceramide ($M(IP)_2C$). Cycloheximide and nocodazole neither block biosynthesis of sphingolipids, nor ER to Golgi transport of IPC. In contrast, treatment of yeast cells with brefeldin A, which affects integrity of the Golgi, decreases formation of IPC and MIPC. Interruption of late steps of protein secretion (Golgi to plasma membrane transport) in temperature-sensitive secretory mutants prevents sphingolipids from being transported to the cell periphery.

Key words: Sphingolipid; Lipid transport; Protein secretion; Plasma membrane; *Saccharomyces cerevisiae*

1. Introduction

In yeast as in higher eukaryotic cells sphingolipids are essential membrane components. The yeast, *Saccharomyces cerevisiae*, contains three major classes of sphingolipids, namely inositolphosphorylceramide (IPC), mannosylinositolphosphorylceramide (MIPC), and mannosyl-diinositolphosphorylceramide ($M(IP)_2C$). The role of these lipids in cell growth and viability is still obscure. They are thought to be involved in the modulation of plasma membrane H^+-ATPase activity [1], membrane signalling [2], regulation of phospholipid biosynthesis [3], and anchoring of cell surface glycoproteins [4]. IPC is synthesized in the endoplasmic reticulum [5]. Mannosylation and further addition of inositolphosphate, leading to the formation of M(IP)C and $M(IP)_2C$, occur in the Golgi. Puoti et al. [6] and Hechtberger et al. [7] reported that temperature-sensitive yeast secretory mutants [8], blocked in early steps of the secretory pathway of proteins, are unable to synthesize a subclass of IPC, MIPC and $M(IP)_2C$ at the non-permissive temperature. This observation supported the notion that sphingolipids are transported from the ER to the Golgi via the secretory pathway of proteins.

In yeast cells the major location of mannosylated sphingolipids is the plasma membrane [9,10]. The fact that IPC, MIPC, and $M(IP)_2C$ are also present in secretory vesicles [10] gave raise to the idea that also late steps of sphingolipid transport (Golgi to plasma membrane) occur via the secretory pathway of proteins. In order to address this question we performed experiments using late secretory mutants (*sec1, sec6,* and *sec14)* and demonstrated, that appearance of IPC, MIPC, and $M(IP)_2C$ in the plasma membrane depends on an intact protein secretory machinery. Furthermore, we tested the influence of brefeldin A (BFA), cycloheximide (CHI), and nocodazole on sphingolipid synthesis and intracellular transport. The toxin BFA dramatically alters structure and function of organelles of the secretory pathway in mammalian cells [11,12]. In *Saccharomyces cerevisiae* BFA has be shown to be active only in strains with a defect in ergosterol biosynthesis, e.g. the *erg6* mutant [13]. CHI blocks synthesis of proteins on cytosolic ribosomes, but not the flux of secretory vesicles [14]. However, Ayscough and Warren [15] showed that treatment of *Schizosaccharomyces pombe* with CHI resulted in a lack of recognizable Golgi stacks. Nocodazole causes disruption of microtubules [16] and may thus influence the protein flux through the secretory pathway. In this paper we demonstrate, that BFA affects transport of IPC between the endoplasmic reticulum and Golgi. In contrast, neither nocodazole nor CHI have an influence on sphingolipid biosynthesis and translocation in yeast.

2. Materials and methods

2.1. Yeast strains and culture condition

The wild-type yeast strain *Saccharomyces cerevisiae* X2180–1A (a, SUC2, mal, gal2, CUP1), the mutant strain *Saccharomyces cerevisiae erg6* FKY 213 (α, erg-Δ, ura3–1, leu2–3,-112, his3–11,-15, trp1–1, ade2–1, CanR) and the corresponding wild-type strain FKY 131 (α, ura3–1, leu2–3,-112, his3–11,-15, trp1–1, ade2–1, CanR) (provided by F. Kepes), and the temperature-sensitive secretory mutants *Saccharomyces cerevisiae sec1*, *sec6* and *sec14* (provided by R. Schekman) were used throughout this study. Cells were grown aerobically in 0.5- or 2-liter flasks either on YPD medium containing 3% glucose, 1% yeast extract (Difco), and 2% peptone (Difco), or on inositol-free medium [17]. Wild-type strains and the *erg6* mutant were cultivated at 30°C, temperature-sensitive secretory mutants at 24°C, and shifted to 37°C to induce the secretion block.

2.2. Labelling of total cellular sphingolipids with [^{3}H]inositol

Cells were grown in inositol-free medium for 16 h, transferred to new medium, and adjusted to an A_{600} of 10 in a volume of 0.5 ml. Preincubation of the *erg6* mutant and the corresponding wild-type strain with BFA (Sigma Chemical Co., St. Louis, MO; final concentration 100 μg/ml; stock solution in ethanol) was carried out at 30°C for 90 min. Treatment of the wild-type strain *Saccharomyces cerevisiae* X2180–1A with CHI (Boehringer Mannheim GmbH, Germany; final concentration 23 μg/ml; stock solution in water) or nocodazole (Sigma Chemical Co., St. Louis, MO; final concentration 15 μg/ml; stock solution in DMSO) was performed at 30°C for 2 h. Control cells were treated with the corresponding volume of the respective solvent. Then, cells were labeled for 3 h with 10 μCi [^{3}H]inositol (spec.act. 20 Ci/mmol). After the labelling period NaN_3 (final concentration 10 mM) was added, cells were harvested by centrifugation, suspended in 3 ml chloroform/methanol/pyridine/water (60:30:1:6; per vol.), and frozen in liquid nitrogen. Glass beads (1 ml; 0.3–0.4 mm diameter) were added, and cells were disintegrated at room temperature by vigorous shaking on an EVAPO-MIX (Buchler Instruments, Fort Lee, NJ) for 10 min. After two hours extraction at room temperature, glass beads and cell debris were removed by centrifugation and reextracted. The combined supernatants containing the inositol-labeled lipids (phosphatidylinositol,

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sphingolipids) were taken to dryness. Lipids were dissolved in 0.5 ml chloroform/methanol/water (16:16:5; per vol.).

2.3. Labelling of secretory mutants with [^{3}H]inositol and isolation of the plasma membrane

Yeast secretory mutants were grown on YPD medium in 2 liter flasks at 24°C for 16 h. Cells were harvested and resuspended in 1 liter inositol-free medium. One half of the culture was further incubated at 24°C, the other half at 37°C (induction of the secretion block) for two hours. Then, cells were labeled with [^{3}H]inositol (200 μCi per flask; spec. act. 20 Ci/mmol) for 2 h. Metabolism was stopped with NaN_3 (final concentration 10 mM), and the plasma membrane was isolated by the method of Serrano [18]. Inositol-containing lipids were extracted with chloroform/methanol/pyridine/water (60:30:1:6; per vol.) at 57°C for 16 h. Membrane remnants were removed by filtration, and the solvent was removed under a stream of nitrogen. Lipids were dissolved in 1 ml chloroform/methanol/water (16:16:5; per vol.), and mild saponification was carried out with 1 ml 0.2 M NaOH in methanol at 30°C for 1 h. Then, 1 ml of 0.5 M EDTA was added, and samples were neutralized with 200 μl of 1 M acetic acid. Unsaponified lipids (among them sphingolipids) were extracted with 1 ml chloroform. Samples were taken to dryness and resuspended in 0.5 ml chloroform/methanol/water (16:16:5; per vol.).

2.4. Labelling of lipids with [^{3}H]inositol in vitro

A microsomal fraction (40,000 × *g* membrane pellet) was isolated from *Saccharomyces cerevisiae* X2180–1A as described elsewhere [19]. Microsomes (200 μg membrane protein) were incubated in 0.5 ml 50 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 1 mM PMSF, either in the absence or presence of BFA (final concentration 73 μg/ml) for 30 min at 30°C. Then, samples were labeled with 5 μCi of [^{3}H]inositol (spec.act. 20 Ci/mmol) for 3 h. Lipids were extracted with 6 ml chloroform/methanol/pyridine/water (60:30:1:6; per vol.) at 57°C for 16 hours. Membrane remnants were removed by filtration, extracts were taken to dryness, and lipids were dissolved in 0.5 ml chloroform/methanol/water (16:16:5; per vol.).

2.5. Analytical procedures

Thin-layer chromatography of sphingolipids was carried out on Polygram Sil G plates (Macherey-Nagel) using chloroform/methanol/4.2 M NH_3 (9:7:2; per vol.) as a developing solvent. Plates containing radioactive lipids were scanned on an Automatic TLC-Linear Analyzer (Berthold). For autoradiography plates were sprayed with Amplify (Amersham) and exposed to X-ray films for several days at −70°C. X-Ray films were scanned on a Dual Wavelenght Chromato Scanner CS-930 (Shimadzu).

Proteins were quantified by the method of Lowry et al. [20]. SDS-PAGE was carried out as described by Laemmli [21]. Western blot analysis was performed by the method of Haid and Suissa [22]. Antiserum against yeast plasma membrane ATPase was a gift of R. Serrano, Valencia, Spain. Antibodies against other organelle specific proteins were raised in rabbits as described elsewhere [23]. Immunoreactive proteins were detected by ELISA using peroxidase-conjugated goat anti-rabbit IgG as secondary antibodies.

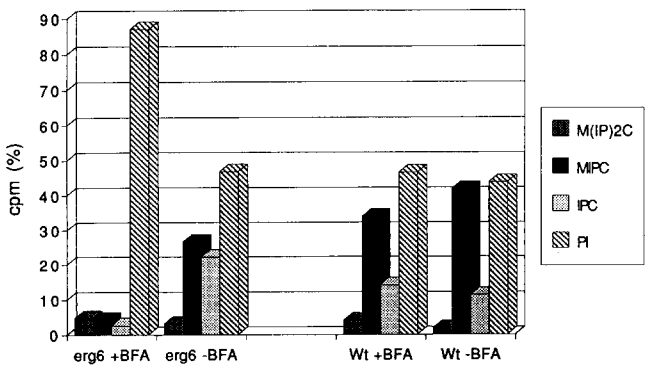

Fig. 1. Influence of BFA on synthesis and transport of yeast sphingolipids. The BFA-sensitive yeast strain *Saccharomyces cerevisiae erg6* (erg6) and the corresponding BFA-insensitive wild-type (Wt) were labeled with [^{3}H]inositol for 3 h as described in section 2. The distribution of the label among inositol-containing lipids in the presence (+BFA) or absence of BFA (−BFA) was calculated in % cpm.

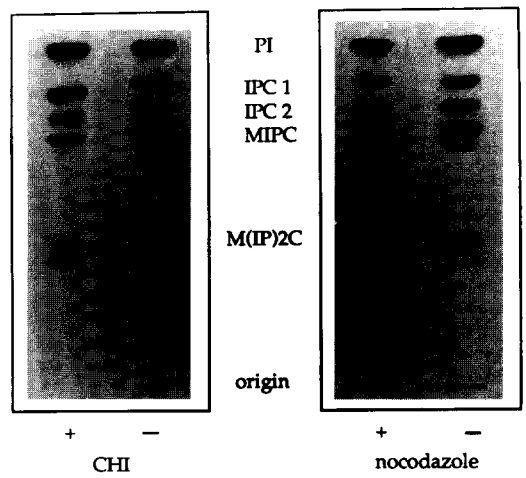

Fig. 2. Cycloheximide and nocodazole do not influence synthesis and transport of yeast sphingolipids. Treatment of the wild-type yeast strain *Saccharomyces cerevisiae* X-2180 with cycloheximide (CHI) and nocodazole, and labelling with [^{3}H]inositol were carried out as described in section 2. The autoradiogram shows the pattern of inositol-labeled lipids in treated (+) and untreated (−) cells after thin-layer chromatographic separation.

3. Results

Brefeldin A (BFA), which is known to cause disassembly of the Golgi in mammalian cells [11,12] is not able to penetrate the plasma membrane of wild-type yeast cells. Mutants of *Saccharomyces cerevisiae* defective in ergosterol biosynthesis with an altered permeability of the plasma membrane can incorporate BFA thus allowing studies comparable to those described for higher eukaryotes. Treatment with BFA caused growth inhibition of the *erg6* mutant strain, whereas the corresponding wild-type yeast strain was not affected (data not shown). Incorporation of [^{3}H]inositol into lipids (see section 2) was not affected by BFA, but the amount of label in IPC and MIPC was dramatically reduced in the presence of the drug (Fig. 1). Concomitantly, [^{3}H]inositol accumulated in phosphatidylinositol, which is known to act as a precursor of IPC [24]. This effect could only be observed in the *erg6* mutant strain, but not in wild-type cells. Similar amounts of radioactivity were detected in $M(IP)_2C$ of cells grown in the presence or absence of BFA.

The decreased amount of [^{3}H]inositol detected in IPC of *erg6* cells treated with BFA could be attributed either to an inhibition of IPC systhesis caused by the drug, or to a disturbance of transport-linked metabolic conversion. The former possibility was ruled out by an in vitro assay (see section 2) measuring incorporation of [^{3}H]inositol into phosphatidylinositol and IPC using 40,000 × *g* microsomes as an enzyme source. In the presence as well as in the absence of BFA 85 ± 4% of the label were incorporated into phosphatidylinositol, and 15 ± 4% into IPC.

In order to test if an intact cytoskeleton and ongoing protein synthesis are required for passage of yeast sphingolipids through the early stages of the secretory pathway, experiments

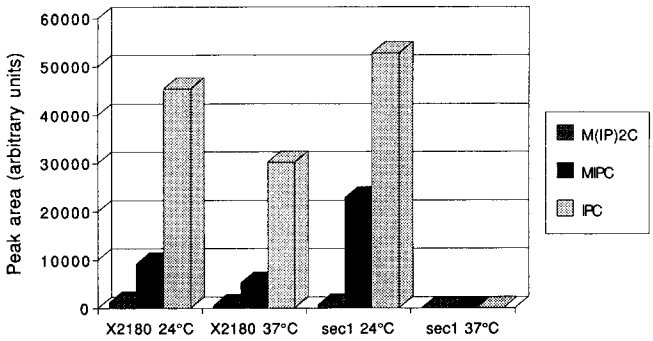

Fig. 3. Migration of sphingolipids to the plasma membrane is blocked in the late secretory mutant *Saccharomyces cerevisiae sec1* under non-permissive conditions. The temperature-sensitive mutant strain *Saccharomyces cerevisiae sec1* and the corresponding wild-type strain *Saccharomyces cerevisiae* X2180 were grown at the permissive (24°C) and non-permissive (37°C) temperature, respectively. Cells were labeled with [^{3}H]inositol, and plasma membrane was isolated as described in the section 2. Labeled sphingolipids of the plasma membrane were separated by thin-layer chromatography, visualized by autoradiography, and quantified by optical scanning. Values are expressed as arbitrary units (peak area).

with the microtubules-disrupting drug nocodazole and with cycloheximide (CHI) were carried out. Neither nocodazole nor CHI had a marked influence on sphingolipid synthesis and transport-linked conversion of IPC to M(IP)C and $M(IP)_2C$ (Fig. 2). Minor changes were observed in the M(IP)C to $M(IP)_2C$ ratio in CHI-treated cells.

The involvement of secretory vesicles in transport of sphingolipids to the cell periphery was examined using the late secretory mutants *Saccharomyces cerevisiae sec1, sec6,* and *sec14*. In these temperature-sensitive mutants the secretory pathway of proteins is interrupted between the Golgi and the plasma membrane at 37°C. As can be seen from Fig. 3, inositol-containing sphingolipids do not reach the plasma membrane of the *sec1* strain under non-permissive conditions. Similar results (data not shown) were obtained with *Saccharomyces cerevisiae sec6* and *sec14*.

4. Discussion

Using temperature-sensitive secretory yeast mutants, Puoti et al. [6] and Hechtberger et al. [7] demonstrated that early steps of the secretory pathway are identical for protein and IPC transport from ER to Golgi. In the yeast as in higher eukaryotes brefeldin A (BFA) inhibits protein transport from the ER to Golgi [25]; later steps of the secretory pathway are not affected. In both types of cells incubation with BFA results in an inhibition of growth, a block in secretion of proteins at the level of the ER, and structural alterations [26]; the BFA block is rapidly reversible. The fact that labelling of IPC and MIPC was reduced in BFA treated yeast cells (*erg6* mutant strain) (see Fig. 1) is in line with the observations mentioned above. During retrograde migration of Golgi components to the ER degradation of IPC may occur. As a consequence MIPC is formed only at a minimum level. Interestingly, conversion of MIPC to $M(IP)_2C$ seems to be unaffected by the BFA treatment. The limited amount of MIPC, which is synthesized even in the presence of BFA, obviously represents a sufficiently high substrate concentration for the enzyme catalyzing the last step in this sequence leading to the formation of $M(IP)_2C$.

Cycloheximide (CHI) blocks the synthesis of proteins, but the flux of secretory vesicles is not affected [14]. Neither synthesis nor transport of sphingolipids are inhibited by treatment of yeast with CHI (see Fig. 2). These results indicate that sphingolipid synthesis and transport are not directly linked to protein synthesis, but leaves us with the possibility of the migration of sphingolipids via secretory pathway.

Microtubules are thought to be involved in polarized movement of vesicles in animal cells [27], but they do not directly govern the growth dependent polarization in the yeast *Saccharomyces cerevisiae* [16]. Our results demonstrating that nocodazole has no influence on sphingolipid synthesis and transport (see Fig. 2) is in line with the observations mentioned above.

High concentrations of sphingolipids in secretory vesicles, which serve as transport vehicles for proteins from the Golgi to the plasma membrane, served as an indication that also late steps of the migration of sphingolipids to the cell periphery may depend on a functional secretory machinery [10]. This idea was confirmed by the finding, that a temperature-dependent block in the late secretory pathway of the yeast (*sec1* mutation) prevents sphingolipids from reaching the plasma membrane (see Fig. 3). These results complete the view that sphingolipid transport and protein secretion are linked processes in yeast.

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