Phospholipid-synthesizing enzymes in Golgi membranes of the yeast, *Saccharomyces cerevisiae*

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Abstract Golgi membranes of the yeast, *Saccharomyces cerevisiae*, were isolated by a method similar to the procedure described by Cleves et al. [Cell 64 (1991) 789–800]. Marker proteins of the Golgi, such as Kex2 protease and GDPase, are highly enriched in these preparations. The phospholipid and ergosterol content of Golgi membranes is low. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol are the major phospholipids of this compartment. The amount of phosphatidylserine in the Golgi is significantly higher than in yeast bulk membranes. Inositol-containing sphingolipids, especially inositolphosphorylceramide, are highly enriched in Golgi membranes. Two phospholipid-synthesizing enzymes, namely phosphatidylinositol synthase and *sn*-1,2-diacylglycerol cholinephosphotransferase, are detected in the Golgi at a specific activity which exceeds that of the endoplasmic reticulum.

Key words: Golgi; Endoplasmic reticulum; Phosphatidylinositol synthase; Cholinephosphotransferase; Sphingolipids; Yeast; *Saccharomyces cerevisiae*

1. Introduction

In yeast as well as in mammalian cells the majority of phospholipid-synthesizing enzymes is located in the endoplasmic reticulum (for reviews see [1,2]). Mitochondria contribute to the cellular phospholipid production insofar as they harbor phosphatidylserine decarboxylase, and enzymes of phosphatidylglycerol and cardiolipin biosynthesis [3–5]. In mammalian cells, enzymes of the CDP-choline and CDP-ethanolamine pathway of phosphatidylcholine and phosphatidylethanolamine biosynthesis were shown to be present in Golgi membranes with specific activities comparable to those in the endoplasmic reticulum [6]. Furthermore, mammalian Golgi was shown to be the major site of sphingolipid synthesis and the branching point of sphingolipid traffic to the cell periphery (for reviews see [1, 7]). Golgi of the yeast, *Saccharomyces cerevisiae*, fulfills a similar function. Using temperature-sensitive yeast secretory mutants [8] a connection between synthesis and intracellular transport of inositol-containing sphingolipids, and secretion of proteins was demonstrated [9,10]. Phospholipid-synthesizing enzymes have not been detected in the yeast Golgi so far.

The fact that the yeast phosphatidylinositol transfer protein (PITP), which is identical to the Sec14 gene product, is in part associated with the Golgi [11], suggested that this protein may serve as a sensor or/and as a regulator of Golgi membrane phospholipid composition. In a temperature-sensitive *sec14* mutant strain McGee et al. [12] observed an accumulation of phosphatidylcholine in Golgi membranes under non-permissive conditions. The imbalance between the phosphatidylcholine and phosphatidylinositol content in Golgi membranes caused by the mutation was regarded as the reason for the dysfunction of the protein secretory pathway. Skinner et al. [13] argued that PITP may serve as a direct modulator of the Golgi phospholipid composition by influencing the activity of choline-phosphate cytidylyltransferase.

Isolation of the yeast Golgi [11,14] is complicated by the fact that enzyme activities suffer from long centrifugation times. The aim of the present work was to design a procedure for the preparation of enzymatically active Golgi membranes thus allowing the measurement of enzymes involved in phospholipid biosynthesis in this subcellular compartment.

2. Materials and methods

2.1. Isolation of yeast subcellular membranes

The wild-type yeast strain *Saccharomyces cerevisiae* X-2180-1A (MATa, *SUC2, mal, gal2, CUP1*) was cultivated in 2-liter flasks at 30°C on a rotary shaker with vigorous aeration on YPD medium containing 1% yeast extract, 2% peptone and 3% glucose.

The method of Golgi preparation was similar to that described by Cleves et al. [11] with some modifications. Yeast cells grown for 14 h at 30°C in YPD medium were harvested by centrifugation and converted to spheroplasts as described by Daum et al. [15]. Spheroplasts were washed with 0.8 M sorbitol, 50 mM potassium phosphate, pH 7.5, 10 mM NaN_3, and suspended in ice-cold lysis buffer (0.6 M sorbitol, 10 mM triethanolamine, pH 7.2, 1 mM EDTA) at a final concentration of 3 g cell wet weight per ml. Then, cells were gently mixed using a Dounce homogenizer (1 stroke, loose-fitting pestle) and incubated for 20 min on ice with occasional agitation. The incubation mixture was adjusted to 1 M sorbitol, and centrifuged at $1,000 \times g$ for 6 min to remove unlysed spheroplasts and cell debris. The supernatant was centrifuged at $12,000 \times g$ for 15 min, and $30,000 \times g$ for 30 min, and the resulting pellets were discarded. Centrifugation at $100,000 \times g$ for 90 min yielded a pellet which was suspended in 10 mM Tris-HCl, pH 7.2, layered onto a sucrose density gradient (30–50%, step increments of 5%) in 10 mM MES, pH 7.2, and centrifuged at $200,000 \times g$ for 15 h in a Sorvall TH641 rotor. The gradient was fractionated from the bottom to the top (1 ml fractions). Fractions containing Kex2 protease detected by Western blot analysis (see below) were collected, diluted with 3 vols. of 10 mM Tris-HCl, pH 7.2, centrifuged at $100,000 \times g$ for 90 min and subjected to a second sucrose density gradient centrifugation under the same conditions as described above. The gradient was again fractionated from the bottom to the top (1 ml fractions), and Kex2 protein reactive fractions were collected.

Microsomal fractions were obtained from the post-mitochondrial supernatant [15] by successive steps of differential centrifugation at $30,000 \times g$, $40,000 \times g$, and $100,000 \times g$. The $30,000 \times g$ pellet exhibits the highest enrichment of proteins specific for the endoplasmic reticulum.

2.2. Protein analysis

Protein was quantified by the method of Lowry et al. [16] using bovine serum albumin as a standard. Prior to quantification, proteins

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were precipitated with trichloroacetic acid (10% final concentration) and solubilized in 0.1% sodium dodecyl sulfate, 0.1 M NaOH.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [17].

Immunological characterization of subcellular fractions was carried out after separating proteins by SDS-PAGE and transferring them to nitrocellulose filters (Hybond-C, Amersham) [18]. Proteins were detected by the ELISA technique using rabbit antibodies against the respective antigens, and peroxidase- or phosphatase-conjugated goat anti-rabbit secondary antibodies following the manufacturer's instructions. Stained immunoreactive bands were quantified by densitometric scanning at 500 nm using a Shimadzu dual-wavelength chromato scanner CS-930. Antisera against yeast plasma membrane ATPase, carboxypeptidase Y, Kex2 protease, Sec61p and BIP (Kar2 protein) were gifts from R. Serrano (Valencia, Spain); D. Wolf (Stuttgart, Germany); R. Fuller (Stanford, USA); and R. Schekman (Berkeley, USA). Antibodies against porin, the major protein of the outer mitochondrial membrane, and a 40 kDa microsomal protein were raised in rabbits as described elsewhere [15].

2.3. Enzyme analysis

Phosphatidylinositol synthase [4] and phosphatidylserine synthase [19] were analyzed as described previously. *sn*-1,2-Diacylglycerol cholinephosphotransferase was assayed by the method of Hjelmstad and Bell [20] with the modification that incubation mixtures contained neither a detergent nor exogenous phospholipids. CDP [methyl-^{14}C]choline (final concentration 0.3 mM) was incubated with 125 mg protein in the presence of 50 mM MOPS/NaOH, pH 7.5, 20 mM $MgCl_2$ in a total volume of 0.2 ml. Glycerol-3-phosphate acyltransferase was assayed by the method of Schlossmann and Bell [21]. Phosphatidylethanolamine *N*-methyltransferase and phospholipid *N*-methyltransferase were assayed essentially as reported by Kodaki and Yamashita [22] with the modifications described by Gaigg et al. [23]. Guanosine diphosphatase (GDPase) was assayed as described by Abeijon et al. [24] and LeBel et al. [25] with modifications introduced by Singer-Krüger et al. [26].

2.4. Lipid analysis

Individual phospholipids were separated by two-dimensional thin-layer chromatography using chloroform/methanol/25% NH_3 (65:35:5; v/v) as a developing solvent for the first direction, and chloroform/acetone/methanol/acetic acid/H_2O (50:20:10:10:5; v/v) for the second direction. Spots detected by iodine vapor were scraped off, and lipid phosphorus was quantified by the method of Broekhuyse [27].

Neutral lipids were separated by one-dimensional thin-layer chromatography using light petroleum/diethyl ether/acetic acid (80:20:2; v/v) as a developing solvent. Ergosterol was quantified by densitometric scanning at 275 nm on a Shimadzu dual-wavelength chromato-scanner CS 930 [5].

Inositol-containing sphingolipids, namely inositolphosphorylceramide (IPC), mannosylinositolphosphorylceramide (MIPC), and mannosyldiinositolphosphorylceramide ($M(IP)_2C$) were analyzed as described by Hechtberger et al. [28].

3. Results

Enzymatically active yeast Golgi membranes were prepared by the isolation procedure presented in section 2. As shown in Table 1, the Kex2 protein, a membrane-bound Ca^{2+}-dependent serine protease which served as a Golgi marker [29], was 150-fold enriched in our isolates over the homogenate. Another Golgi marker, GDPase, which is required for the outer-chain addition of mannose residues to yeast glycoproteins [24], was found at a lower enrichment due to the occurrence of more than one GDPases in the yeast.

In order to judge the quality of our Golgi preparations cross-contamination with other organelles was measured using various additional markers. Some microsomal markers, such as the 40 kDa microsomal protein, and NADPH-cytochrome *c* reductase were detected only at trace amounts in our Golgi preparations (see Table 1). Marker proteins of the endoplasmic reticulum, e.g. the Sec61 protein and BIP (Kar2 protein), were only slightly enriched over the homogenate. As a control, 30,000 × *g* microsomes representing the endoplasmic reticulum are 2–4-fold enriched in these markers (see Table 1). Finally, our Golgi preparations were practically devoid of plasma membrane ATPase, porin (mitochondrial marker) and carboxypeptidase Y (vacuolar marker).

Table 1
Characterization of the yeast Golgi

	Relative enrichment (-fold)[a]	
	Golgi	Endoplasmic reticulum[b]
Kex2 protease	150	ND
GDPase	21	1.8
40 kDa protein	<0.1	2.7
GDP-mannosyl transferase	1.3	3.3
Sec61 protein	1.5	4.0
BIP1 protein	1.5	2.0
NADPH-cytochrome *c* reductase	0.09	2.3
Plasma membrane ATPase	ND	1.0
Porin	ND	0.8
Carboxypeptidase Y	ND	–

GDPase = guanosine diphosphatase; 40 kDa protein = microsomal marker. ND = not detectable; – = not determined. Results are mean values from at least three experiments with a maximum mean deviation of ± 10%.

[a]The relative specific activities of marker enzymes or the relative amount of the marker proteins in the homogenate were set at 1.

[b]30,000 × *g* membrane pellet.

Two enzymes involved in phospholipid biosynthesis, namely *sn*-1,2-diacylglycerol cholinephosphotransferase (CPT) and phosphatidylinositol synthase (PIS), were found to be enriched in the yeast Golgi (Table 2). Other phospholipid-synthesizing enzymes, such as glycerol-3-phosphate acyltransferase, phosphatidylserine synthase, phosphatidylethanolamine *N*-methyltransferase, and phospholipid *N*-methyltransferase were not enriched in the Golgi; their highest specific activity was found in the endoplasmic reticulum (30,000 × *g* pellet) (see Table 2). The fact, that two enzymes involved in the synthesis of phosphatidylinositol and phosphatidylcholine, respectively, are present in Golgi may be of importance in connection to the association of the yeast phosphatidylinositol transfer protein (PITP) with Golgi membranes [11], because PITP specifically catalyzes phosphatidylinositol and phosphatidylcholine transfer in vitro [30,31].

A detailed analysis of lipids present in yeast Golgi membranes is shown in Table 3. In comparison to other subcellular membranes of the yeast, *Saccharomyces cerevisiae* [5], the phospholipid-to-protein ratio in Golgi membranes is low. Phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine are the major glycerophospholipids in our isolates. The amount of phosphatidylserine in Golgi membranes is higher as compared to yeast bulk membranes. The concentration of ergosterol in Golgi membranes is similar to that of other internal membranes [5].

Yeast Golgi membranes harbor high quantities of inositol-containing sphingolipids [28] (Table 3). This observation can be explained by the fact that sphingolipid biosynthesis and transport are striktly linked to the protein secretory pathway in yeast

Table 2
Localization of phospholipid-synthesizing enzymes in the yeast Golgi

	Relative specific activity					
	GAT	PSS	PEMT	PLMT	PIS	CPT
Homogenate	1	1	1	1	1	1
Endoplasmic reticulum (30,000 × *g* pellet)	7.9	6.5	1.1	1.4	2.2	3.1
Golgi	0.7	0.8	0.4	0.4	2.35	6.0

GAT = glycerol-3-phosphate acyltransferase; PSS = phosphatidylserine synthase; PEMT = phosphatidylethanolamine *N*-methyltransferase; PLMT = phospholipid *N*-methyltransferase; PIS = phosphatidylinositol synthase; CPT = *sn*-1,2-diacylglycerol cholinephosphotransferase. Results are mean values from at least three experiments with a maximum mean deviation of ±12%.

[9,10]. The concentration of inositolphosphorylceramide (IPC), which is converted to mannosylinositolphosphorylceramide (MIPC) and mannosyldiinositolphosphorylceramide($M(IP)_2C$) in the Golgi, was found to be remarkably higher in this compartment than in other subcellular membranes [28].

4. Discussion

The present paper describes the isolation and characterization of Golgi membranes of the yeast, *Saccharomyces cerevisiae*. In contrast to the original procedure by Cleves et al. [11] we employed sucrose instead of sorbitol as a density gradient centrifugation medium. The time of centrifugation (2 × 15 h) in our protocol was markedly reduced which resulted in a much better preservation of enzyme activities. Kex2 protein, which was used as a Golgi marker [29], was found to be 150-fold enriched in our preparations. This result is in good agreement with data presented previously by Cleves et al. [11]. Contamination of the Golgi with other organelles was marginal as demonstrated by the low abundance of the respective markers (see Table 1).

The subcellular distribution of phospholipid-synthesizing enzymes in the yeast, *Saccharomyces cerevisiae*, became of renewed interest in connection with the role of the yeast phosphatidylinositol transfer protein (PITP) [30,31], which was shown to be identical to the Sec14 gene product [32] and to associate with Golgi membranes. PITP can translocate phosphatidylinositol and phosphatidylcholine in vitro, and it was tempting to speculate that this protein may fulfill a similar function in vivo. Indeed, McGee et al. [12] presented evidence that the PITP, which acts as a component of the protein secretory machinery at the stage of Golgi, regulates the phosphatidylcholine to phosphatidylinositol ratio in this compartment. Very recently, Skinner et al. [13] suggested that the phosphatidylcholine-bound form of PITP may depress the CDP-choline pathway activity by inhibiting choline-phosphate cytidylyltransferase. The observation that *sec14* dysfunction can be overcome by defects in the CDP-choline pathway of phosphatidylcholine biosynthesis [11,12] is in line with this theory. Our finding, that another enzyme of this biosynthetic sequence, namely cholinephosphotransferase, resides in Golgi membranes (see Table 2) supports this theory. However, phosphatidylinositol synthase, the enzyme producing the second and preferential substrate of PITP, is also found in Golgi membranes at a specific activity which cannot be attributed to contamination with other organelles. The localization of the latter enzyme may also influence the process of PITP-dependent regulation of choline-phosphate cytidylyltransferase activity, because the phosphatidylinositol-bound form of the PITP does not inhibit this enzyme [13].

Table 3
Lipid composition of yeast Golgi membranes

Phospholipid:protein (mg/mg)	0.20
Ergosterol:protein (mg/mg)	0.01
Ergosterol:phospholipid (mol/mol)	0.11
IPC:protein (mg/mg)	0.15
MIPC:protein (mg/mg)	0.02
$M(IP)_2C$:protein (mg/mg)	0.06
	% of total phospholipids
Phosphatidylcholine	47.0
Phosphatidylethanolamine	15.0
Phosphatidylinositol	21.0
Phosphatidylserine	11.3
Dimethylphosphatidylethanolamine	5.7

IPC = inositolphosphorylceramide; MIPC = mannosylinositolphosphorylceramide; $M(IP)_2C$ = mannosyldiinositolphosphorylceramide. Results are mean values from at least three experiments with a maximum mean deviation of ±10%.

The phospholipid composition of our Golgi preparation (see Table 3) is different from that described by McGee et al. [12]. These authors detected almost equal amounts of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine (approximately 20–25%, each) in their preparations of yeast Golgi, whereas in our isolates phosphatidylcholine is the predominant phospholipid. Differences in the preparation method may account for these discrepancies. The pattern of inositol-containing sphingolipids (Table 3) with its high level of inositolphosphorylceramide (IPC) is characteristic of yeast Golgi membranes. The fact that IPC synthesized in the endoplasmic reticulum is the substrate for mannosylation in the Golgi leading to the formation of mannosylinositolphosphorylceramide (MIPC) and mannosyldiinositolphosphorylceramide ($M(IP)_2C$) could explain this subcellular accumulation. Golgi as a branching point of sphingolipid traffic with a preferential migration of mannosylated species to the plasma membrane, and of IPC to the vacuole has been suggested before in our laboratory [28].

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