The yeast *mic2* mutant is defective in the formation of mannosyl-diinositolphosphorylceramide

Andrea Leber, Petra Fischer, Roger Schneiter, Sepp D. Kohlwein, Günther Daum*

Institut für Biochemie und Lebensmittelchemie, Technische Universität Graz, A-8010 Graz, Austria

Received 7 May 1997; revised version received 26 May 1997

Abstract The *mic2* mutation dominantly blocks formation of mannosyl-diinositolphosphorylceramide, the most abundant sphingolipid of the yeast, *Saccharomyces cerevisiae*. Interestingly, lack of mannosyl-diinositolphosphorylceramide is not lethal but is compensated for by increased amounts of inositolphosphorylceramide and mannosyl-inositolphosphorylceramide in the plasma membrane and Golgi of the mutant. The level of negatively charged phospholipids in the plasma membrane of the *mic2* strain is markedly reduced; the sterol composition is not altered. In spite of dramatic changes of its lipid composition the mutant grows like wild type on complex and minimal media, under osmotic stress conditions, at low pH, and in the presence of high ionic strength. While sensitivity to several drugs is not altered, the *mic2* mutant strain becomes resistant to the polyene antibiotic nystatin.

Key words: *Saccharomyces cerevisiae*; Yeast; Sphingolipid; Plasma membrane; Nystatin; Inositol

1. Introduction

As in higher eukaryotic cells, phospholipids, sterols, and sphingolipids are the major membrane lipid components of the yeast, *Saccharomyces cerevisiae*. The sphingolipids of this microorganism contain a long-chain amino alcohol, phytosphingosine, amide-linked to a very long-chain fatty acid (mostly a C_{26} hydroxy fatty acid), and inositol phosphate or mannosylated inositolphosphate as polar head group. The three classes of yeast sphingolipids, IPC[1], MIPC, and $M(IP)_2C$ [1], are intermediates and products of a common biosynthetic pathway. IPC is produced in the endoplasmic reticulum by IPC synthase that catalyzes the condensation of inositolphosphate with ceramide [2,3]. After transport to the Golgi, IPC is converted to MIPC with GDP-mannose as the carbohydrate donor [4]. Finally, introduction of the second inositolphosphate group results in the formation of $M(IP)_2C$ in the Golgi [5].

Only few yeast mutants defective in the biosynthesis of sphingolipids are known. The phytosphingosine auxotrophic mutants, *lcb1* and *lcb2* (*l*ong-*c*hain *b*ase auxotrophic) are defective in serine palmitoyltransferase [6]. Both genes are essential, and mutants are not viable unless supplemented with phytosphingosine. An *lcb1* mutant carrying the *SLC1* (*s*phingo*l*ipid *c*ompensation) suppressor gene is viable in the absence of long-chain bases. These suppressor mutants, however, synthesize novel phosphatidylinositol derivatives with a C_{26} fatty acid in the *sn*-2 position and structurally mimic sphingolipids [7]. The *slc1* mutant cannot grow at low pH, high salt concentration, or high temperature [8] indicating that the novel phosphatidylinositol species cannot fully compensate for the lack of sphingolipids. Based on the finding that the *SLC1* gene complements the growth phenotype of an *E. coli* acyltransferase mutant *plsC*, Slc1p was suggested to be a fatty acyltransferase [9]. A Ca^{2+} hypersensitive mutation, *csg2*, was found to affect the synthesis of mannosylated sphingolipids [10]. Csg2p is required for Ca^{2+} regulation, and supplementation with phytosphingosine reverses the Ca^{2+} sensitive phenotype of the *csg2* strain. Suppressor mutants of *csg2*, *scs*, also have an altered sphingolipid metabolism [11]. Scs1p is identical to Lcb2p, the regulatory subunit of serine palmitoyltransferase. These observations led to the idea that ceramide synthesis in yeast is either regulated by Ca^{2+} and/or is required for Ca^{2+} homeostasis.

Here we report isolation of a novel yeast mutant, termed *mic2*, which is defective in the last step of the sphingolipid biosynthetic pathway, the formation of $M(IP)_2C$. The mutant phenotype is described, and possible roles of $M(IP)_2C$ in plasma membrane function are discussed.

2. Material and methods

2.1. Yeast strains and culture conditions

Wild type strains of *Saccharomyces cerevisiae*, W303 (*MAT***a**, *ura3*, *trp1*, *leu2*, *ade2*, *his3*, *can1*) and X2180-1A (*MAT*α, *SUC2*, *mal*, *gal2*, *CUP1*), the temperature-sensitive secretory mutant *sec1* (provided by R. Schekman), and the $M(IP)_2C$ deficient strain *mic2* (*MAT*α, *ura3*, *trp1*, *leu2*, *ade2*, *his3*) were used throughout this study. Cells were grown either in complex medium (YPD) containing 3% glucose, 2% peptone, and 1% yeast extract (Difco), or in inositol-free medium [12] under aerobic conditions at 30°C or 24°C. Growth of yeast cells was determined by measuring the optical density at a wavelength of 600 nm.

The *mic2* mutation was initially detected in the genetic background of a temperature-sensitive *sec1* mutant. To isolate the *mic2* strain, *sec1* was backcrossed to W303, diploids were sporulated, and tetrads were dissected by standard procedures [13]. Tetrads were analyzed for temperature sensitivity and $M(IP)_2C$ formation (see below), and $M(IP)_2C$ deficient strains that were not temperature sensitive were isolated. The *mic2* mutant was established after four rounds of backcrossing of the mutant to the wild type strain W303.

Drug resistance of strains was tested in plate assays. Sterilized YPD agar was cooled to 50°C, and drugs were added from stock solutions and mixed with the agar. Plates were poured and used the following day to avoid loss of activity of the drugs. Yeast strains to be tested

*Corresponding author. Fax: (43) 316-873-6952.
E-mail: daum@ftug01.dnet.tu-graz.ac.at

Abbreviations: IPC, inositolphosphorylceramide; MIPC, mannosyl-inositolphosphorylceramide; $M(IP)_2C$, mannosyl-diinositolphosphorylceramide; PI, phosphatidylinositol; TMA-DPH, trimethylamino-diphenyl-1,3,5-hexatriene

This study was supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (project S-5811 to G.D., and project 11731 to S.D.K.).

PII S0014-5793(97)00692-3

were suspended in liquid YPD, and 5 μl spots were applied to plates. Plates were incubated for 1–3 days at 30°C.

2.2. Isolation and characterization of yeast subcellular membranes

Plasma membrane was isolated by the method of Serrano [14], and the Golgi-fraction was prepared as described by Leber et al. [15]. The quality of the preparations and cross-contamination with other organelle membranes were judged by immunoblot analysis and measurement of marker enzyme activities as described by Zinser and Daum [16].

Proteins were quantified by the method of Lowry et al. [17], SDS-PAGE was carried out as described by Laemmli [18], and Western blot analysis was performed by the method of Haid and Suissa [19]. Immunoreactive proteins were detected by ELISA with peroxidase- and phosphatase-conjugated goat anti-rabbit secondary antibodies.

2.3. Analysis of lipids

Labeling of yeast sphingolipids with [^{3}H]inositol, extraction of sphingolipids from subcellular fractions, thin-layer chromatographic separation and quantification were performed as described previously [20]. Phospholipids and sterols were extracted by the procedure of Folch et al. [21]. Individual phospholipids were separated by two-dimensional thin-layer chromatography on Silica gel 60 plates using chloroform/methanol/25% NH_3 (65:35:5, per vol.) as first, and chloroform/acetone/methanol/acetic acid/H_2O (50:20:10:10:5, per vol.) as second developing solvent. Phospholipids were visualized by iodine staining, scraped off the plate, and quantified by the method of Broekhuyse [22]. For ergosterol quantification samples and standards were applied to thin-layer plates, chromatographed using light petroleum/diethylether/acetic acid (70:30:2, per vol.), and scanned at 275 nm using a Shimadzu dual-wavelength chromato scanner CS-930. Individual sterols were analyzed after alkaline hydrolysis [23] of the lipid extract by gas chromatography-mass spectrometry using a Hewlett-Packard 5892 II plus and a Hewlett-Packard MSD 5972 mass selective detector. The column used was HP-5MS, injector temperature was 280°C, and detector temperature 300°C.

2.4. Anisotropy measurement

Fluidity of the plasma membrane was determined in vitro by measuring the fluorescence anisotropy of trimethylammonium diphenylhexatriene (TMA-DPH) probe. Samples containing 100 μg membrane protein were incubated with 2.7 nmol TMA-DPH for 30 min at 30°C. Fluorescence measurements were carried out using a Shimadzu RF 540 spectrofluorimeter as described previously [24].

3. Results and discussion

Labeling experiments with [^{3}H]inositol of a number of temperature-sensitive yeast secretory mutants revealed that one of these strains, namely a *sec1* mutant, had a second mutation in its genetic background that manifested itself by the absence of the major yeast sphingolipid, $M(IP)_2C$ [25]. This mutant was backcrossed to the wild type strain W303, and tetrads were screened for temperature sensitivity and $M(IP)_2C$ synthesis. This analysis revealed that the defect in the biosynthesis of $M(IP)_2C$ and the *sec1* mutation were not linked. Four additional rounds of backcrossing resulted in the isolation of the temperature-insensitive *mic2* mutant. Fig. 1 shows the pattern

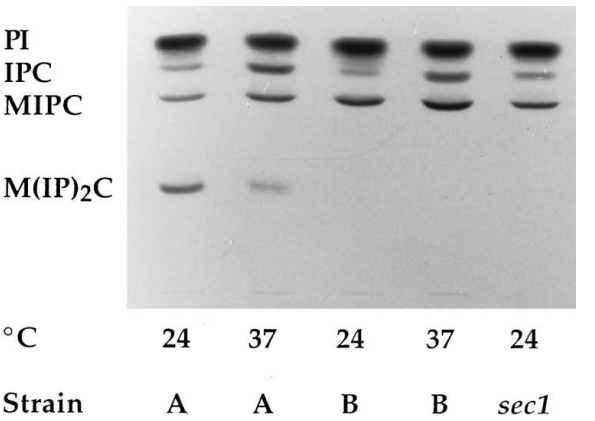

Fig. 1. The yeast mutant *mic2* is deficient in conversion of MIPC to $M(IP)_2C$. Lipids of two isogenic spores of a tetrad (A: *MIC2*; B: *mic2*) and the original *sec1* strain were labeled with [^{3}H]inositol at temperatures indicated, extracted and analyzed by thin-layer chromatography as described in Section 2.

of [^{3}H]inositol-labeled lipids of the original *sec1* strain and two spore-derived colonies of a tetrad from backcrosses with the wild type W303. One of these strains (Fig. 1, lanes B) lacked $M(IP)_2C$, whereas $M(IP)_2C$ was detectable in the other (Fig. 1, lanes A). The original *sec1* strain does not form $M(IP)_2C$. IPC and MIPC are present at comparable levels in all strains. The $M(IP)_2C^+/M(IP)_2C^-$ phenotype segregated 2:2 indicating a single chromosomal mutation. A heterozygous diploid strain *mic2/MIC2* did not produce $M(IP)_2C$ indicating that the *mic2* mutation is dominant.

What are the cell biological consequences caused by the *mic2* mutation? To answer this question two subcellular fractions were analyzed in detail: the plasma membrane, which is the compartment with the highest concentration of $M(IP)_2C$ in wild type cells [26,20], and the Golgi, the site of MIPC and $M(IP)_2C$ synthesis [5]. Comparing the sphingolipid composition of the plasma membrane and the Golgi of *mic2* and wild type (Table 1) demonstrated that in both compartments of the *mic2* mutant $M(IP)_2C$ was not detectable. The ratio of total sphingolipids to protein is markedly higher in the plasma membrane of *mic2* than in wild type (Table 2). Thus, lack of $M(IP)_2C$ in the mutant is apparently compensated for by increased amounts of IPC and MIPC.

The ergosterol concentration in the plasma membrane of the mutant was comparable to the wild type, whereas the phospholipid content in the plasma membrane of *mic2* was approximately 30% lower than in the control (Table 2). Comparison of the phospholipid composition of the plasma membrane of *mic2* and wild type revealed that the lack of $M(IP)_2C$ markedly affects the ratio of negatively charged to uncharged

Table 1
Sphingolipid composition of plasma membrane and Golgi of *mic2* and wild type

Strain	Membrane	IPC/protein (nmol/mg)	MIPC/protein (nmol/mg)	$M(IP)_2C$/protein (nmol/mg)
X2180	Plasma membrane	51	106	81
mic2	Plasma membrane	134	349	ND
X2180	Golgi	155	18	47
mic2	Golgi	−	108	ND

ND, not detectable; −, not determined. Plasma membrane and Golgi from *mic2* and the parental wild type X2180 were isolated and analyzed for their amount of sphingolipids as outlined in Section 2. Data shown are mean values of three independent experiments with a mean deviation of ±10%.

phospholipids (Table 3): the plasma membrane of wild type cells contains approximately 55%, whereas that of the *mic2* mutant contains only 41% negatively charged phospholipids. This alteration is mainly due to a decrease of phosphatidylserine and phosphatidylinositol, and an increase of phosphatidylethanolamine in the plasma membrane of *mic2*. Thus, the elevated level of negatively charged total sphingolipids in the mutant plasma membrane appears to be compensated for by a decrease of negatively charged phospholipids.

We next investigated whether the altered sphingolipid and phospholipid composition of the plasma membrane of the *mic2* mutant has an effect on membrane fluidity. Anisotropy measurements using trimethylamino-diphenyl-1,3,5-hexatriene (TMA-DPH) as a probe for lipid mobility or flexibility in the membrane demonstrated that this is not the case. The anisotropy of TMA-DPH in the plasma membrane of both mutant and wild type strain was 0.27. This result suggests that the increased levels of IPC and MIPC together with the altered phospholipid composition of the *mic2* mutant compensated for the absence of $M(IP)_2C$ in such a way as to keep the membrane fluidity constant.

The altered lipid composition of the *mic2* mutant might be expected to cause an abnormal growth phenotype. Surprisingly, the mutant grew like wild type under various conditions tested, such as on rich medium (YPD), minimal medium, on different carbon sources (glucose, ethanol, lactate), in the presence of 1 M NaCl, 1 M sorbitol plus 0.5 M NaCl, at pH 3, and on inositol-free medium. Different growth temperatures (10°C, 24°C, 30°C, and 37°C) also did not significantly affect cell viability. Similarly, the presence of 0.1 M Ca^{2+}, Mg^{2+}, or Li^{+}, and 1 mM Cu^{2+} in the growth medium did not specifically affect growth of *mic2*. In contrast to the *csg2* mutant, *mic2* is not sensitive to Ca^{2+} at concentrations of 0.1 M indicating that IPC and/or MIPC but not $M(IP)_2C$ affect Ca^{2+} homeostasis.

Drug sensitivity of *mic2* was tested with cycloheximide (0.1–0.3 μg/ml; inhibitor of cytosolic protein synthesis), chloramphenicol (10–50 μg/ml; inhibitor of protein synthesis in mitochondria), valinomycin (250 μg/ml; ion carrier destroying membrane potential), Brefeldin A (50 μg/ml; disassembling Golgi in mammalian cells and yeast strains defective in certain steps of sterol metabolism [27]), and terbinafine (10–70 μg/ml; inhibitor of fungal squalene epoxidase) [28]. None of these drugs specifically affected growth of the mutant. In contrast, nystatin, a polyene antibiotic [29], at a concentration of 10 μg/ml inhibited growth of the wild type strain but not that of *mic2*. Tetrad analysis confirmed that nystatin resistance coseggregated with the block in $M(IP)_2C$ synthesis (data not shown).

The antimycotic drug nystatin has been described to interact with sterol of the plasma membrane forming pores, causing leakage of cellular constituents and ultimately cell death [29]. Some sterol mutants such as *erg3* and *erg6*, which have an altered sterol composition of the plasma membrane, are resistant to nystatin [30,31], whereas others, such as *erg4*, remain nystatin sensitive (D. Zweytick, unpublished results). These findings may be explained by similar interaction of nystatin with sterols different from ergosterol, such as ergosta-5,7,22,24(28)-tetraenol accumulating as the end-product in the *erg4* mutant. On the other hand, Kerridge [29] reported that strains with apparently similar sterol composition exhibited markedly different sensitivity to polyene antibiotics. The author suggested that additional factors may be responsible for sensitivity to these drugs. Sphingolipids may be a target for nystatin similar to or in combination with ergosterol. Interaction of nystatin with $M(IP)_2C$ in combination with ergosterol is compatible with the idea that membrane domains enriched in the two lipid classes may exist in the plasma membrane. Such detergent insoluble membrane domains of the yeast plasma membrane have recently been described [32]. Studies on lipid sorting in epithelial cells [33] suggest that sphingolipids and cholesterol aggregate in the lumenal leaflet of Golgi membranes. These aggregates may then be preferentially included into anterograde vesicles which transport sphingolipids and sterols to the cell periphery. In yeast, transport of sphingolipids from their site of synthesis to the plasma membrane has been shown to follow the secretory pathway [5,25]. Enrichment of ergosterol in secretory vesicles [34] suggests participation of the secretory machinery in ergosterol transport to the plasma membrane. Thus, a sterol/sphingolipid co-transport may also function in yeast and contribute to the formation of ergosterol/$M(IP)_2C$-rich domains in the plasma membrane. If nystatin preferentially interacts with these domains, lack of one component may lead to resistance against the drug.

Table 2
Amounts of phospholipids, ergosterol, and total sphingolipids in the plasma membrane of the *mic2* mutant and the parental wild type strain

Strain	Phospholipid/protein (mg/mg)	Ergosterol/protein (mg/mg)	Sphingolipid/protein (mg/mg)
X2180	0.23	0.21	0.28
mic2	0.16	0.23	0.52

Data shown are mean values of at least three experiments with a mean deviation of ±12%.

Table 3
Phospholipid composition of the plasma membrane of *mic2* and the parental wild type strain

Phospholipids	% of total phospholipid	
	mic2	X2180
Phosphatidylcholine	10.8/10.8	16.8
Phosphatidylethanolamine	36.0/33.1	20.3
Phosphatidylinositol	13.9/13.3	17.7
Phosphatidylserine	19.8/21.6	33.6
Phosphatidic acid	4.7/ 6.6	3.9
Cardiolipin	ND	0.2
Others	14.8/14.6	6.9

ND, not detectable. Data shown for the *mic2* mutant are from two independent experiments. Data of the wild type X2180 are mean values from at least four experiments (SD≤5%).

Acknowledgements: The authors are grateful to R. Schekman, Berkeley, CA, for providing yeast secretory mutants, and to C. Hrastnik for technical assistance.

References

[1] Smith, S.W. and Lester, R.L. (1974) J. Biol. Chem. 249, 3395–3405.
[2] Ko, J., Cheah, S. and Fischl, A.S. (1994) J. Bacteriol. 176, 5181–5183.
[3] Becker, G.W. and Lester, R.L. (1980) J. Bacteriol. 142, 747–754.

[4] Abeijon, C., Orlean, P. and Hirschberg, C.B. (1989) Proc. Natl. Acad. Sci. 86, 6935–6939.
[5] Puoti, A., Desponds, C. and Conzelmann, A. (1991) J. Cell Biol. 113, 515–525.
[6] Wells, G.B. and Lester, R.L. (1983) J. Biol. Chem. 258, 10200–10203.
[7] Lester, R.L. and Dickson, R.C. (1993) Adv. Lipid Res. 26, 253–274.
[8] Patton, J.L., Srinivasan, B., Dickson, R.C. and Lester, R.L. (1992) J. Bacteriol. 174, 7180–7184.
[9] Nagiec, M.M., Wells, G.B., Lester, R.L. and Dickson, R.C. (1993) J. Biol. Chem. 268, 22156–22163.
[10] Beeler, T., Gable, K., Zhao, C. and Dunn, T. (1994) J. Biol. Chem. 269, 7279–7284.
[11] Zhao, C., Beeler, T. and Dunn, T. (1994) J. Biol. Chem. 269, 21480–21488.
[12] Klig, L.S., Homann, M.J., Carman, G.M. and Henry, S.A. (1985) J. Bacteriol. 162, 1135–1141.
[13] F. Sherman, G.R. Fink, and C.W. Lawrence, Methods in Yeast Genetics, Cold Spring Harbor, NY, 1988.
[14] Serrano, R. (1988) Methods Enzymol. 157, 533–544.
[15] Leber, A., Hrastnik, C. and Daum, G. (1995) FEBS Lett. 377, 271–274.
[16] Zinser, E. and Daum, G. (1995) Yeast 11, 493–536.
[17] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Haid, A. and Suissa, M. (1983) Methods Enzymol. 96, 192–205.
[20] Hechtberger, P., Zinser, E., Saf, R., Hummel, K., Paltauf, F. and Daum, G. (1994) Eur. J. Biochem. 225, 641–649.
[21] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–500.
[22] Broekhuyse, R.M. (1968) Biochim. Biophys. Acta 260, 449–459.
[23] Lewis, T.A., Rodriguez, R.J. and Parks, L.W. (1987) Biochim. Biophys. Acta 921, 205–212.
[24] Sperka-Gottlieb, C.D.M., Hermetter, A., Paltauf, F. and Daum, G. (1988) Biochim. Biophys. Acta 946, 227–234.
[25] Hechtberger, P., Zinser, E., Paltauf, F. and Daum, G. (1994) NATO ASI Series, Membr. Dynam. Biogene. 82, 23–31.
[26] Patton, J.L. and Lester, R.L. (1991) J. Bacteriol. 173, 3101–3108.
[27] Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S. and Klausner, R.D. (1989) Cell 56, 801–813.
[28] Ryder, N.S. (1991) Biochem. Soc. Trans. 19, 774–777.
[29] Kerridge, D. (1986) Adv. Microb. Phys. 27, 1–73.
[30] Smith, M.S. and Parks, L.W. (1993) Yeast 9, 1177–1187.
[31] Gaber, R.F., Copple, D.M., Kennedy, B.K., Vidal, M. and Bard, M. (1989) Mol. Cell. Biol. 9, 3447–3456.
[32] Kübler, E., Dohlman, H.G. and Lisanti, M.P. (1996) J. Biol. Chem. 271, 32975–32980.
[33] Van Helvoort, A. and van Meer, G. (1995) FEBS Lett. 369, 18–21.
[34] Zinser, E., Paltauf, F. and Daum, G. (1993) J. Bacteriol. 175, 2853–2858.