The SNF2/SWI2/GAM1/TYE3/RIC1 Gene Is Involved in the Coordinate Regulation of Phospholipid Synthesis in Saccharomyces cerevisiae

Tsutomu Kodaki, Kohei Hosaka, Jun-ich Nikawa, and Satoshi Yamashita

Department of Biochemistry, Gunma University, School of Medicine, Maebashi, Gunma 371

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Genes involved in the phospholipid synthesis of Saccharomyces cerevisiae, such as PEM1, PEM2, PSS, and INO1, are coordinately repressed by myo-inositol and choline. In order to investigate this regulation, we transformed wild-type yeast with a PEM1 promoter–lacZ fusion and isolated two mutants, named ric1 and ric2 (regulation by myo-inositol and choline), exhibiting decreased PEM1 expression. The lowered PEM1 expression in the mutants was monitored in colonies in terms of their failure fully to develop blue color on 5-bromo-4-chloro-3-indolyl-β-galactopyranoside-containing agar. ric1 mutant was auxotrophic for myo-inositol, indicating that INO1 expression was also affected, whereas ric2 mutant required myo-inositol only in the presence of choline. The RIC1 gene was isolated by complementation of the Ino− phenotype of ric1 mutant and its identity was confirmed by genetic cross between the original ric1 mutant and a gene disruptant. The RIC1 gene was sequenced and found to be identical with the previously identified gene, SNF2/SWI2/ GAM1/TYE3, which is known to encode a general transcription factor required for the expression of various genes including INO1. Analysis using various lacZ fusion constructs containing promoters for genes in phospholipid synthesis revealed that the expression of myo-inositol-choline-regulated genes, PEM1, PEM2, PSS, CKI, and INO1, was markedly decreased in the snf2/swi2/gam1/tye3/ric1 background, but the expression of a constitutive gene, PIS, was not. We conclude that SNF2/SWI2/GAM1/TYE3/RIC1 is a positive regulatory gene required for the expression of not only INO1 gene, but also of myo-inositol-choline-regulated genes in general.

Key words: choline, inositol, phospholipid synthesis, regulation.

A number of enzymes in the phospholipid synthesis of the yeast Saccharomyces cerevisiae are known to be regulated by myo-inositol and choline. In order to investigate the mechanism of the regulation, we transformed wild-type yeast with a PEM1 promoter–lacZ fusion and isolated two mutants, named ric1 and ric2 (regulation by myo-inositol and choline), exhibiting decreased PEM1 expression. The lowered PEM1 expression in the mutants was monitored in colonies in terms of their failure fully to develop blue color on 5-bromo-4-chloro-3-indolyl-β-galactopyranoside-containing agar. ric1 mutant was auxotrophic for myo-inositol, indicating that INO1 expression was also affected, whereas ric2 mutant required myo-inositol only in the presence of choline. The RIC1 gene was isolated by complementation of the Ino− phenotype of ric1 mutant and its identity was confirmed by genetic cross between the original ric1 mutant and a gene disruptant. The RIC1 gene was sequenced and found to be identical with the previously identified gene, SNF2/SWI2/ GAM1/TYE3, which is known to encode a general transcription factor required for the expression of various genes including INO1. Analysis using various lacZ fusion constructs containing promoters for genes in phospholipid synthesis revealed that the expression of myo-inositol-choline-regulated genes, PEM1, PEM2, PSS, CKI, and INO1, was markedly decreased in the snf2/swi2/gam1/tye3/ric1 background, but the expression of a constitutive gene, PIS, was not. We conclude that SNF2/SWI2/GAM1/TYE3/RIC1 is a positive regulatory gene required for the expression of not only INO1 gene, but also of myo-inositol-choline-regulated genes in general.

Key words: choline, inositol, phospholipid synthesis, regulation.

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1 To whom correspondence should be addressed.

1 Present address: Department of Biochemical Engineering and Science, Faculty of Computer Science and Systems Engineering, Kyushu Institute of Technology, Iizuka, Fukuoka 820. Abbreviations: inositol, myo-inositol; UAS, upstream activation sequence.

and inositol-1-phosphate synthase (17), the changes of the enzyme activities have been correlated with changes of mRNA abundance.

With the premise that there must be a common nucleotide sequence responsible for the inositol-choline regulation in the upstream regions of these genes, we examined the regulatory regions of the PEM1/CHO2, PEM2/OPI3, and PIS/CH01 genes encoding phosphatidylethanolamine methyltransferase, phospholipid methyltransferase, and phosphatidylserine synthase, respectively. The octamer sequence, 5'-CATRTGAA-3' (R = A or G), was determined to play an important role in the inositol-choline regulation of these genes (18, 19). Recently, it was shown that this octamer sequence is required for the expression of choline-regulated genes of the ITR1 gene encoding one of the inositol transporters (14). The octamer motif occurs in the upstream regions of not only all inositol-choline-regulated genes (18), but also constitutive genes, PIS (20), FAS1, and FAS2 (21). Schüller et al. (21) identified the sequence TYTTCACATGY, which contained the octamer motif within its sequence, as the UAS element required for the expression of FAS1 and FAS2. They demonstrated that the TYTTCACATGY sequence did confer inositol-choline regulation upon a heterologous gene, and postulated that a cis-acting element capable of converting inositol-choline-regulated expression into constitutive expression exists in...
the upstream sequence of FAS1 and FAS2. At present, the reason why the octamer motif does not confer inositol-choline regulation on PIS, FAS1, and FAS2 is unknown.

Henry and coworkers (22-24) cloned and sequenced regulatory genes involved in the control of INO1 gene expression. INO2 and INO4 were identified as positive regulatory genes, and OPI1 as a negative regulatory gene (6, 11, 17, 25). These genes regulate the expression of not only INO1, but also other inositol-choline-regulated genes. INO2 and INO4 were both found to contain basic/helix-loop-helix, a structural feature of DNA-binding proteins (22, 24). The above octamer motif contains the CANNTG consensus binding site for helix-loop-helix DNA-binding proteins.

TABLE I. List of the yeast strains used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source (Ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X2180-1b</td>
<td>A SUC2 mal mal gal2 CUP2</td>
<td>YGSC*</td>
</tr>
<tr>
<td>D302-3C</td>
<td>A leu2-3 leu2-112 his4-519 can1</td>
<td>This laboratory (48)</td>
</tr>
<tr>
<td>D328-1B</td>
<td>A CSE1 leu2-3 leu2-112 his4-519</td>
<td>This laboratory (28)</td>
</tr>
<tr>
<td>D328-9C</td>
<td>A CSE1 leu2-3 leu2-112 his4-519</td>
<td>This laboratory (28)</td>
</tr>
<tr>
<td>D448-2</td>
<td>A uro3 can1</td>
<td>This laboratory (13)</td>
</tr>
<tr>
<td>D451-3</td>
<td>A leu2-3 leu2-112 uro3 can1</td>
<td>This laboratory (13)</td>
</tr>
<tr>
<td>D452-2</td>
<td>A leu2 his3 uro3 can1</td>
<td>This laboratory (29)</td>
</tr>
<tr>
<td>D451-14</td>
<td>A leu2-3 leu2-112 his4-519</td>
<td>This laboratory (48)</td>
</tr>
<tr>
<td>801</td>
<td>A ric1 leu2 his3 uro3 can1</td>
<td>Present study</td>
</tr>
<tr>
<td>802</td>
<td>A ric2 leu2 his3 uro3 can1</td>
<td>Present study</td>
</tr>
<tr>
<td>2035</td>
<td>A ric2::LEU2 leu2-3 leu2-112</td>
<td>Present study</td>
</tr>
</tbody>
</table>

*Yeast Genetic Stock Center, University of California, Berkeley.

MATERIALS AND METHODS

**Materials**—[α-32P]dCTP (800 Ci/mmol) was purchased from Du Pont-New England Nuclear (Boston, MA, USA). Restriction endonucleases, T4 DNA ligase, and T4 DNA polymerase were from Takara Shuzo (Kyoto) and Nippon Gene (Toyama). Calf intestinal alkaline phosphatase and Zymolyase were obtained from Boehringer Mannheim (Mannheim, Germany) and Seikagaku Kogyo (Tokyo), respectively.

**Strains, Culture, and Transformation**—The composition of inositol-free minimum medium was as described previously (4). L-Leucine, L-histidine, L-tryptophan, and uracil were each added to the culture medium at the concentration of 20 μg/ml. Yeast cells were grown aerobically at 30°C. The genotypes and sources of the S. cerevisiae strains used in this study are listed in Table I. Yeast cells were transformed by the lithium acetate method described by Ito et al. (41). Escherichia coli K12 strain AG1 was obtained from Stratagene (La Jolla, CA, USA) and used for the amplification of plasmids. Bacterial cells were cultured in Luria broth (42) at 37°C. Ampicillin was used at the

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concentration of 50 μg/ml. E. coli was transformed as described by Bolivar and Backman (42).

Plasmid DNA Preparation—Yeast plasmid DNA was prepared as described previously (43). E. coli plasmids were prepared as described by Sambrook et al. (44).

Plasmids—pUVBG12 (18), YCpU1 (29), and pLGΔ-178 (45) were used as the vector plasmids. pUVBG12 was derived from pUC19 and contained yeast 2-μm ori, URA3, and E. coli lacZ. YCpU1 was a derivative of YCP50 (46) containing the polycloning site. pLGΔ-178 contained UAS-deficient, but otherwise intact CYC1 promoter and the lacZ reporter, and was kindly provided by Dr. L. Guarente. pEBG343 (18), pMBG177 (18), and pSBAL239 (19) were multicopy plasmids containing the complete PEM1, PEM2, and PSS promoters in front of the lacZ reporter gene, respectively. pEBL336-299 (18), pMBL177-130 (18), and pSL239-132 (19) were multicopy plasmids containing the UASs of PEM1, PEM2, and PSS (−330 to −299, −177 to −130, and −239 to −132 relative to the A of the initial ATG) in front of the CYC1-lacZ fusion, respectively.

pEBG343 was cleaved with NcoI and StuI and the gap was filled in with the 2.2-kbp Sall/Xhol fragment containing yeast LEU2 (47) to yield pEBG343. The 0.7-kbp PstI/BgiII fragment of the CKI gene (48) and the 0.2-kbp Sall/NspV fragment of PIS (20) were inserted into the Sall site of pUVBG12 to obtain pKZ303 and pZ239, respectively. The 0.5-kbp Sall/ApalI fragment of INO1 (29, 49) was inserted into the SphI site of pUVBG12 to obtain pNZ242. Where necessary, restriction fragments were repaired with T4 DNA polymerase and ligated. The structures of plasmids constructed are shown in Fig. 1.

Isolation of ric1 and ric2 Mutants—Strain D452-2 harboring pEBG343 was mutagenized with 3% (w/v) ethyl methanesulfonate for 60 min at 30°C in 0.1 M sodium phosphate buffer, pH 7.4. The survival rate was about 20%. The mutagenized cells were grown on minimum agar supplemented, the concentrations of inositol and choline were 20 μg/ml. A, D452-2 (wild type); B, strain 801 (ric1); C, strain 802 (ric2); D, disruptive strain 2035 (ric1::LEU2).

RESULTS AND DISCUSSION

Isolation of ric1 and ric2 Mutants—We previously studied the promoter regions of PEM1, PEM2, and PSS and identified their UASs (18, 19). As expected from their common susceptibility to inositol-choline regulation, their promoters were shown to contain a common octameric sequence, 5'-CATRTGAA-3' (R = A or G). We thought that a mutant defective in the expression of one of the inositol-choline-regulated genes, for example PEM1, might also be defective in the expression of the other inositol-choline-regulated genes. Thus we decided to isolate PEM1 expression mutants. Plasmid pEBG343 (18) which carried a fusion gene comprising the PEM1 promoter and lacZ was constructed (Fig. 1). In yeast cells, the lacZ was expected to
be transcribed under the control of PEM1 promoter. Since the expressed β-galactosidase activity could be easily detected in colonies (51), transformants carrying pEBG343 are convenient parents to isolate PEM1 expression mutants. Wild-type strain D452-2 harboring pEBG343 was mutagenized with ethyl methanesulfonate and grown on minimum agar containing 1 μg/ml each inositol and choline. Since PEM1 expression mutant was considered to be also defective in the expression of INO1, the selection medium was supplemented with not only choline, but also inositol. However, their concentrations were kept at minimum levels so that phospholipid synthesis was not severely repressed. The colonies of mutagenized cells were transferred onto nylon membranes, which were then incubated with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside for the colony assay of β-galactosidase activity. Wild-type colonies showed a blue color, but PEM1 expression mutants gave only a pale blue color. Out of 40,000 colonies, two mutants were selected and designated 801 and 802.

The mutants harboring pEBG343 were cultured with different supplementation of inositol and choline and then assayed for β-galactosidase activity (Table II). As expected, the derepressed β-galactosidase level (cultured without inositol and choline) was much lower in 801 and 802 than in the parental strain, D452-2. The extent of PEM1 repression by inositol or inositol plus choline in both mutants was comparable to that of the parent. But the sensitivity to choline considerably increased in 802. As shown in Fig. 2, strain 801 required inositol for growth, but even in the presence of inositol it did not grow as well as its parent. Strain 802 grew better than strain 801. The growth of 802 was inhibited by choline, but restored by further addition of inositol. Strains 801 and 802 were then crossed with wild-type strain D451-14. Both of the resulting diploids grew normally on inositol-free, choline-supplemented minimum medium and expressed normal levels of β-galactosidase activity (Table II), indicating that both mutations were recessive. Although 801 and 802 exhibited similar phenotypes, they were different mutants affected in different loci (see below). The mutations in 801 and 802 were thus named ric1 and ric2 (regulation by inositol and choline), respectively.

### Table II. Expression of the PEM1-lacZ fusion gene in ric1 and ric2 mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>801 (ric1)</td>
<td>48</td>
</tr>
<tr>
<td>802 (ric2)</td>
<td>130</td>
</tr>
<tr>
<td>D452-2 (wild)</td>
<td>388</td>
</tr>
<tr>
<td>801 (ric1) × D451-14 (wild)</td>
<td>626</td>
</tr>
<tr>
<td>802 (ric2) × D451-14 (wild)</td>
<td>641</td>
</tr>
<tr>
<td>D451-14 (wild) × D452-2</td>
<td>503</td>
</tr>
</tbody>
</table>

**Gene Cloning**—Using the ric1 mutant, we cloned the gene capable of complementing its Ino" phenotype. Strain 801 was transformed with a yeast gene library constructed on a single copy vector, YCpU1 (29), and then Ino" Ura" transformants were selected. From the transformants, we obtained three different plasmids, pRMC1, pRMC11, and pRMC13, containing overlapping 9.2-, 10.5-, and 8.2-kbp inserts, respectively (Fig. 3). These three clones were introduced into strain 801 harboring pEBGL343, and the transformants were assayed for β-galactosidase activity. The activities were comparable to that in the wild-type strain D452-2 harboring pEBGL343 (data not shown). The cloned gene was named RIC1 since it corresponded to the ric1 mutation as revealed by genetic analysis of the disruptant (see below).

**Subcloning**—In order to locate the RIC1 gene within the cloned inserts, five subclones, pRMCA through pRMCE, were constructed (Fig. 3) and tested for their abilities to complement the Ino" phenotype of strain 801. Only pRMCA gave positive result. Consistently, this subclone restored β-galactosidase activity in strain 801 harboring pEBGL343, but the others did not (data not shown). Thus the RIC1 gene was localized within the 6.9-kbp XbaI

![Fig. 3. pRMC1, pRMC11, pRMC13, and subclones. + and − indicate the ability and inability of the plasmids to complement the ric1 mutation, respectively. Restriction enzymes sites are: Bg, BgII; Ec, EcoRI; Hi, HindIII; Ps, PstI; Pv, PvuII; Sa, SacI; Xb, XbaI; Xh, XhoI.](http://jb.oxfordjournals.org/Downloaded from http://jb.oxfordjournals.org/ at Penn State University Library on May 12, 2016)
fragment of pRMCl.

**Identification of the RIC1 Gene**—The nucleotide sequence of the 6,918-base pairs XbaI/XhoI fragment of pRMCl was determined by the dideoxy-chain termination method (52) (data not shown). Within this sequence, there was a large open reading frame capable of encoding a 1,703-amino acid protein with a calculated molecular weight of 194,048. We performed a computer search for proteins with homology to the RIC1 product using the FASTA program (53). The RIC1 product was found to be identical with the SNF2/SWF2/GAM1/TYE3 gene product (35, 36), which is a general transcription factor and known to be required for the expression of many genes subject to different regulation (39). Hereafter, we refer to this gene as SNF2/RIC1 for simplicity.

**Gene Disruption**—To investigate the role of SNF2/RIC1 gene in the inositol-choline regulation further, we constructed a gene disruptant using the cloned gene as described in "MATERIALS AND METHODS." The disruptant strain 2035 was crossed with the original ric1 mutant, 801. Random spore analysis was carried out on the cross between ric2 mutant 802 and CSE1 mutant D328-1B. ric2 was also shown to be non-allelic to CSE1.

**Expression of PEM1, PEM2, PSS, CKI, and INO1 in the snf2/ric1 Disruptant**—The effect of snf2/ric1 disruption on the expression of the inositol-choline-regulated genes was studied using hybrid genes composed of PEM1, PEM2, PSS, CKI, or INO1 promoters fused to the E. coli lacZ reporter (Fig. 1). Plasmids pEBG343, pMBG177, pSBAL239, pKZ303, and pINZ422 carrying such fusion genes were introduced into strains 2035 and D451-3. The transformants were cultured in the presence and absence of inositol and choline, and assayed for β-galactosidase activity. The derepressed β-galactosidase activities obtained in the absence of inositol and choline were 2 to 8 times lower than that in the wild-type cells 2 and 9 times lower in disruptant 2035 than in wild-type D451-3 (Table III). Similar results were obtained when the fusion genes were recloned into single-copy plasmid YCp and expressed in the same strains (data not shown). These results indicate that RIC1 is involved in the expression of not only INO1, but also the other inositol-choline-regulated genes, PEM1, PEM2, PSS, and CKI. In contrast, the expression of the PIS-lacZ fusion, a gene insensitive to inositol-choline regulation, was only 20% lower than that in the wild-type cells, showing that ric1 is not involved in the expression of the PIS gene.

To confirm these findings, different fusion genes were constructed using the UAS detection plasmid pLGΔ-178 of Guarente et al. (45, 54) which contains the UAS-less, but otherwise complete CYC1 promoter connected to the lacZ coding region. We inserted the UAS of PEM1, PEM2, or PSS at the XhoI site in front of the CYC1 TATA box of CYC1-lacZ in pLGΔ-178 to construct pEBLG336-299 (18), pMBG177-130 (19), or pSL239-132 (19), respectively. When compared under the derepressing conditions, the expression of all these fusion genes in the disruptant was 2 to 8 times lower than that in the wild-type cells (Table IV). Taken together, these results indicate that the SNF2/RIC1 product is involved in the expression of the gene.
inositol-choline-regulated genes in general. SNF2/RIC1 encoding a general transcription factor is highly conserved among eukaryotes and defines a family of proteins with similarity to helicases and nucleic acid-dependent NTPases (55, 56). Indeed, the SNF2/RIC1 protein has DNA-stimulated ATPase activity (57). The SNF2/RIC1, together with SNF5, SNF6, SWI1, and SWI3, functions as the transcriptional activator of many differently regulated genes (37, 58). Recently, a multienzyme complex containing the SNF2/RIC1, SWI1, SWI3, SNF5, and SNF6 products was isolated from the extracts of yeast cells (59). Several genes including INO2, INO4, OPI1, and CSE1 are known to be involved in the inositol-choline regulation of phospholipid synthesis (6, 11, 17, 25, 29). Among them, the INO2 and INO4 products were shown to bind to the INO1 promoter which contains the octamer sequence mediating the inositol-choline regulation (26, 27). The present findings raise the interesting possibility that not only the INO2, INO4, and OPI1 products, but also general transcription factors, SNF2/RIC1, SWI1, SWI3, SNF5, and SNF6 could be involved in the inositol-choline regulation of yeast phospholipid synthesis.

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