Functional Interaction of Isr1, a Predicted Protein Kinase, with the Pkc1 Pathway in Saccharomyces cerevisiae

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Staurosporine is a potent inhibitor of protein kinase C. To identify the genes that functionally interact with the Pkc1 pathway of the yeast Saccharomyces cerevisiae, we screened for the genes that cause induced staurosporine sensitivity when overexpressed from a galactose-inducible promoter. The novel gene ISR1 encodes a predicted protein kinase with the highest sequence similarity to mammalian Raf in the kinase domain. Drug sensitivity induced by ISR1 overexpression is specific to staurosporine. Although ISR1 disruption causes no obvious phenotype, it does exacerbate the phenotypes of a temperature-sensitive allele (stt1-I) of PKC1, but not of the mpk1 and bck1 mutants of the Mpkl MAP kinase pathway. These results suggest that Isr1 functions in an event important for growth in a manner redundant with a Mpkl-independent branch of the Pkc1 signalling pathways.

Key words: PKC signalling pathway; protein kinase; Saccharomyces cerevisiae; staurosporine

Introduction

Protein kinase C (PKC) is thought to play a pivotal role in the regulation of a host of cellular functions through its activation by growth factors and other agonist. These functions include cell growth and proliferation, release of hormones, and control of ion conductance channels. Nevertheless, there is far less information about the signalling components upstream and downstream of the PKC pathways. Budding yeasts have a homologue of mammalian PKC designated Pkc1, which regulates cell wall integrity through the activation of the MAP kinase cascade consisting of Bck1 (MAP kinase kinase kinase), Mkk1/Mkk2 (MAP kinase kinase), and Mpkl (MAP kinase) protein kinases. Yeast offers the possibility of using a powerful molecular genetic approach to investigate the PKC function in detail, and identify the signalling components that act in the PKC-linked pathways.

Staurosporine is a natural product that inhibits the PKC activity of mammalian cells. Staurosporine has antifungal activity and arrests the growth of Saccharomyces cerevisiae in G2 phase of the cell cycle. Previous work in S. cerevisiae has established a group of staurosporine-sensitive stt mutants as defective in the Pkc1 pathway. Among these stt mutants, stt1 was allelic to PKC1. The terminal phenotype of a temperature-sensitive stt1 mutant (stt1-I) at 37°C is G2 cell-cycle arrest resembling that caused by staurosporine treatment, indicating that Pkc1 is required for G2/M transition and it is an in vivo target of the drug. Since the stt mutants display phenotypes related to cell cycle, cell wall integrity, vacuolar protein sorting, and protein glycosylation, the staurosporine-susceptible pathways are implicated in a wide range of cellular processes.

In this paper, we describe a novel yeast gene, ISR1, the overexpression of which specifically leads to the decreased tolerance to staurosporine. The ISR1 gene encodes a protein with a protein kinase motif that is most similar in sequence to mammalian Raf. Genetic analyses suggested that Isr1 is functionally redundant with an Mpkl-independent branch of the Pkc1 pathway.

Materials and Methods

Genetic method. Standard genetic techniques are as described in Kaiser et al. All strains used in this study are derivatives of the wild-type strain W303-1A. Yeast cells were transformed by the lithium acetate method. To construct a strain with the stt1-I mutant allele of PKC1 in a W303 background, the stt1-I strain in a YS3 background was backcrossed five times with the wild-type W303 strain. The Escherichia coli strains used were NMS2 and DH5α. Standard recombinant DNA techniques were used as previously described by Sambrook et al.

Media. Yeast cells were grown in YPD (1% yeast extract, 2% polypeptone, and 2% glucose). YPGal medium is similar to YPD, except that glucose is replaced by galactose (2%). Synthetic minimal medium supplemented with 2% glucose (SD) or galactose (SG) and appropriate nutrients was used to select for plasmids and gene replacement.

Screening of a gene the overexpression of which decreases staurosporine tolerance. The W303-1A strain was transformed with a genomic DNA bank constructed in YEp51, containing S. cerevisiae genomic DNA clones under the control of the galactose inducible GAL10 promoter. The transformed cells were replicated onto an SG (Leu) agar plate containing staurosporine (7 μg/ml). Colonies that failed to grow on SG (Leu) plates containing staurosporine were picked up from the plate that does not contain staurosporine, and the cells were

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suspended in water using a toothpick. The cell suspension was inoculated on plates containing various drugs using a cell applicator. Plasmid DNA was recovered from the strain that had increased sensitivity to staurosorine and this phenotype was verified by transforming a wild-type strain with the plasmid.

**DNA sequencing.** DNA was sequenced by the dideoxy chain termination method(18) using an AFLR end sequencer (Pharmacia Biotech.) using fluorescein-labeled oligonucleotides as primers.

**ISR1 disruptions.** A deletion mutant allele of the ISR1 gene was constructed by replacing the 1.0-kb SpeI-BglII fragment within the ORF of ISR1 in the pUC19 plasmid with the 1.1-kb SalI-BamHI fragment of URA3 from pBS-URA3. The resulting plasmid was digested with EcoRI-SphI and used to transform a wild-type diploid W303 strain for chromosomal integration of the deletion allele of ISR1. Restriction mapping and hybridization analysis of genomic DNA isolated from the diploid transformants were done to confirm that disruption had occurred at the ISR1 locus.(19)

**Drug sensitivity test.** Sensitivity of yeast to drugs was assessed by streaking or spotting cell suspensions on solid medium containing various concentrations of drugs. For the spot assay, yeast cells suspended in water (2 × 10⁶ cells per ml) were applied using a cell applicator (about 4 × 10⁶ cells per spot) on YPD or YPGal solid medium containing various concentrations of drugs. The plates were incubated at 25°C for 2–3 days. To identify the minimum inhibitory concentrations (MIC), growth was examined on YPD or YPGal plates containing various concentrations of drugs, and the MIC was defined as the lowest inhibitory concentration at which cells could not grow.

**Results and Discussion**

**Isolation of the genes whose overexpression decreases staurosorine tolerance**

To isolate genes that may interact with the Pck1 pathway, we screened for a gene that, when overexpressed, led to increased sensitivity to staurosorine. Such a phenotype would arise, for example, from an overproduction of a protein that acts negatively on the tolerance to staurosorine or from an imbalance of the component of a protein complex that is required for the tolerance to staurosorine. For this purpose, wild-type yeast cells were transformed with a genomic DNA library constructed with a galactose-inducible expression vector. Out of about 10,000 yeast colonies examined, 11 failed to grow in the presence of 7 μg/ml of staurosorine on galactose medium, but not on glucose medium. The plasmid dependency of this phenotype could be confirmed with only one of these transformants. This plasmid, with a 6.0-kb insert, was designated pST11 and used for further study. The drug sensitivities of the wild-type strain harboring a control plasmid (YPE51) and pST11 were compared by plate assay. The transformant did not show induced sensitivity in galactose medium to other drugs, such as cycloheximide and fluphenazine, suggesting that staurosorine sensitivity was not due to a defect of the pleiotropic drug resistance mechanism (Fig. 1). Subclones of the genomic insert of pST11 were generated in the YEP51 vector and introduced into W303-1A. The decrease in the tolerance to staurosorine was induced only by the middle portion of the insert (Fig. 2). Nevertheless, the inserts in pST11 and pST11-1 could not lead to a staurosorine-sensitive phenotype on YEP24 vector, suggesting that the Gal promoter-mediated very high expression was required for inducing staurosorine sensitivity (data not shown).

**ISR1 encodes a novel protein kinase**

Sequence analysis of the insert of pST11-1 showed that this fragment contains a single ORF of 443 amino acids, which corresponded to the YPR106w ORF on chromosome XVI. We have named this gene ISR1 for inhibitory gene for staurosorine resistance. Protein data base searches found that Isr1 protein has amino acid se-

<table>
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<th>MIC (μg/ml)</th>
<th>strain</th>
<th>ST</th>
<th>CYC</th>
<th>FP</th>
</tr>
</thead>
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<td>9-10</td>
<td>0.15</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>wild (pST11)</td>
<td>5</td>
<td>0.15</td>
<td>50</td>
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</table>

Fig. 1. Overexpression of ISR1 Increases Staurosorine Sensitivity of Wild Type S. cerevisiae. Wild-type W303-1A cells harboring YEp51 or pST11 were streaked on YPD or YPGal solid medium containing staurosorine (ST, 7 μg/ml), cycloheximide (CYC, 0.1 μg/ml), or fluphenazine (FP, 40 μg/ml) and the plate was incubated at 25°C for 2–3 days. Minimum inhibitory concentrations (MIC) observed by the procedure described in Materials and methods are shown.
sequence similarity to members of the serine/threonine protein kinase family. Isr1 was not closely related to any known protein kinases, with the highest degree of similarity to the Raf proto-oncogene from mouse20 and to the Gcn2 protein kinase from S. cerevisiae21 (Fig. 3). The similarities were restricted to the predicted catalytic domain which resides near the carboxyl terminus of Isr1 protein. The amino terminal sequence of Isr1 was not similar to any proteins in sequence data bases.

**ISR1 is a non-essential gene**

One chromosomal copy of the *ISR1* gene was disrupted in the diploid yeast strain W303 by inserting the *URA3* gene into the *ISR1* ORF. Southern blot analysis of stable Ura’ prototrophic transformants confirmed the disruption event (data not shown). Sporulation of the diploid transformants carrying the *isr1::URA3* allele, tetrad dissection of the resulting spores on YPD and subsequent incubation at 28°C showed that all spores were able to form viable colonies, indicating that *ISR1* is a non-essential gene (data not shown). We next examined the phenotypic consequences of the null mutation under various conditions. The *Δisr1* null mutation had no obvious effect on the growth in the medium containing staurosporine or various other drugs. Furthermore, this null mutant did not have increased sensitivities to osmotic stress, low (17°C) or high (37°C) temperatures, various ionic (calcium, sodium, cadmium, manganese, potassium, and low and high pHs) or oxidative stresses (data not shown). The morphology of *Δisr1* cells was indistinguishable from that of wild-type cells. Based on these results, we concluded that the null mutation of *ISR1* by itself had no effect on normal cell growth. This raises the possibility that yeast has additional gene(s) encoding protein(s) functionally redundant with Isr1. Nevertheless, data base analysis showed that Isr1 kinase was not similar to any known protein kinases of *S. cerevisiae*.

**Genetic evidence for the functional interaction between *ISR1* and the *PKCl* signalling pathway**

We next investigated if Isr1 is functionally related to the Pkc1 signalling pathway. Because *PKCl* is an essential gene for vegetative growth, we used a temperature sensitive *stt1-1* allele of *PKCl* mutant for genetic analysis.9 The *stt1-1* mutant has sensitivities of growth to staurosporine and high temperatures.9 We first examined the effects of the *Δisr1*-null mutation on the restrictive temperature of growth and staurosporine sensitivity of the *stt1-1* mutant by comparing the growth of *stt1-1* and *stt1-1 Δisr1* strains under appropriate conditions (Fig. 4 and Table 1). The phenotypes of *stt1-1* strain were exacerbated when combined with the *Δisr1* mutation. Although the *stt1-1* mutant formed colonies at 33°C, a *Δisr1* *stt1-1* double mutant was unable to do so. This growth defect at 33°C was suppressed by the presence of 1 mM sorbitol (Fig. 4). The temperature sen-
Table 1. Analysis of the Functional Interaction of Isr1 with the Pkc1-Mpk1 MAP Kinase Pathway

<table>
<thead>
<tr>
<th>strain</th>
<th>Growth (°C)</th>
<th>MIC (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>wild type</td>
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<td>Δmpk1</td>
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</tr>
<tr>
<td>Δisr1 Δbck1</td>
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<td>+</td>
</tr>
<tr>
<td>Δisr1 Δmpk1</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Growth and MIC were observed on YPD solid medium by the spot assay as described in Materials and methods. Drug sensitivity test was done at 25°C using staurosporine (ST), chloэemide (CYC), and flufenazene (FP).

Sensitivity of stl-1 strain was exacerbated by Δisr1 mutation (Fig. 4 and Table 1). The observation that Δisr1 mutation and ISR1 overexpression causes similar effect on the stl-1 mutant phenotype may appear contradictory. The apparent discrepancy can be explained if ISR1, which is required for the tolerances to staurosporine and high temperature as suggested from the disruption phenotype, causes a dominant effect by overexpression. For example, if Isr1 functions as a protein kinase as suggested from the sequences, proteins that normally are not the substrate of Isr1 are phosphorylated by overexpression of ISR1, and the phosphorylation acts negatively on the tolerances to staurosporine and high temperature.

The Pkc1 pathway activates the Mpk1 MAP kinase cascade, and the mutants lacking a component of this cascade (Δmpk1 and Δbck1) display increased sensitivities to staurosporine and to high temperature which can be suppressed by 1 M sorbitol. The double disruption mutants Δisr1 Δmpk1 and Δisr1 Δbck1 showed similar degrees of sensitivities to high temperature and staurosporine as those of Δmpk1 and Δbck1 single disruption mutants, in a clear contrast to the additive effect of Δisr1 on stl-1 (Table 1). These results suggested that Isr1 is not functionally related to the Mpk1 pathway to a significant extent, and further suggested that Isr1 may perform a redundant function with a Mpk1-independent branch of the Pkc1 signalling pathway in an event required for the tolerances to high temperatures and staurosporine. Pkc1 is required for the events in cell wall integrity and G2/M transition. 

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References


