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PIK1, an essential phosphatidylinositol 4-kinase associated with the yeast nucleus

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Transmission of mitogenic and developmental signals to intracellular targets is often mediated by inositol derivatives. Here we present the cloning and characterization of a gene from *Saccharomyces cerevisiae*, *PIK1*, encoding the enzyme that catalyses the first committed step in the production of the second messenger inositol-1,4,5-trisphosphate. *PIK1* encodes a phosphatidylinositol 4-kinase (PI 4-kinase) essential for growth. Cells carrying *PIK1* on a multicopy vector overexpress PI 4-kinase activity exclusively in a nuclear fraction, suggesting that PIK1 is part of a nuclear phosphoinositide cycle. Temperature-sensitive mutations, but not a null mutation, can be suppressed by high osmolarity or an elevated concentration of Ca^{2+}. Conditional mutants have a cytokinesis defect as indicated by a uniform terminal phenotype of cells with large buds and fully divided nuclei. We suggest that PIK1 controls cytokinesis through the actin cytoskeleton.

Key words: actin/cytokinesis/nucleus/PI 4-kinase/signal transduction/*S.cerevisiae*

Introduction

Stimulation of a variety of receptors at the cell surface can activate either one of two different signalling pathways that use phosphatidylinositol (PI) derivatives as messengers. One pathway involves PI 3-kinase which phosphorylates position 3 of the inositol ring [see Downes and MacPhee (1990) and Cantley *et al.* (1991) for reviews]. In mammalian cells, PI 3-kinase is thought to mediate the transmission of the oncogenic or mitogenic signal of receptor or non-receptor tyrosine kinases (Cantley *et al.*, 1991; Valius and Kazlauskas, 1993). A PI 3-kinase and two homologues have also been detected in yeast cells, where they have been implicated in the control of membrane traffic (Herman *et al.*, 1992) and proposed to play a role in cell cycle regulation, respectively (Kunz *et al.*, 1993; Helliwell *et al.*, 1994). A second and better characterized signal transduction pathway involves the activation of a phosphoinositide-specific phospholipase C (see reviews cited above) which hydrolyses phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) to produce diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC) which in turn phosphorylates and activates a number of other protein kinases and transcription factors, leading to cell proliferation [reviewed in Nishizuka (1988)]. PKC could phosphorylate transcription factors in the cytoplasm and these could be subsequently translocated into the nucleus. Alternatively, PKC itself could be translocated into the nucleus as part of the activation process. There is some evidence for the latter possibility in *ras*-transformed cells (Chiarugi *et al.*, 1990) and in fibroblasts treated with growth factors (Divecha *et al.*, 1991). A wholly nuclear phosphoinositide signalling system has been proposed (Divecha *et al.*, 1993), based on the detection of many of the enzymes involved in phosphoinositide metabolism in isolated rat nuclear envelopes and in demembranated mammalian nuclei (Smith and Wells, 1983; Payrastre *et al.*, 1992). The other product of phospholipase C, IP_3, binds to specific receptors and induces the release of Ca^{2+} ions from intracellular stores, thus regulating the Ca^{2+}/calmodulin-dependent protein kinases and phosphatases and promoting cell division in synergy with the DAG signal [reviewed in Berridge and Irvine (1989) and Whitman and Cantley (1988)]. IP_3 receptors are found mainly in the nuclear envelope and the endoplasmic reticulum proximal to the nucleus (Ross et *al.*, 1989), and IP_3 seems to have an essential role in nuclear envelope assembly (Sullivan *et al.*, 1993). Another inositol phosphate, inositol-1,4-bisphosphate (IP_2), possibly produced by dephosphorylation of IP_3, also has a nuclear role in activating DNA polymerase α, perhaps to initiate the S phase of the cell cycle (Sylvia *et al.*, 1988). A DAG/IP_3 signalling pathway in yeast cells may play a role in the control of the cell cycle (Hawkins *et al.*, 1993 and references therein).

The first phosphorylation of the PI inositol ring in the pathway leading to IP_2 and IP_3 is accomplished by PI 4-kinase. This enzymatic activity has been described in vertebrates and yeast (Buxeda *et al.*, 1991; Flanagan and Thorner, 1992; Nickels *et al.*, 1992; McKenzie and Carman, 1993) and all, except one of the at least three yeast activities, have been found to be membrane-associated (Flanagan and Thorner, 1992). PI 4-kinase is also one of the phosphoinositide cycle enzymes described to have a nuclear form. More specifically, it has been claimed to be part of the nuclear pore–lamina fraction of hepatocytes and NIH3T3 cells (Payrastre *et al.*, 1992).

We have searched for components of the yeast nuclear pore complex with antibodies raised against nuclear envelopes. One such antibody was the monoclonal antibody QE5 that recognizes hNUP153, a component of the nuclear pore complex of HeLa cells (McMorrow *et al.*, 1994). In preparations of *Xenopus* nuclear envelopes, this antibody immunodecorates intranuclear structures of the pore complex (Pante and Aebi, 1993). In the present work, we use this

antibody to clone a yeast gene encoding an essential, nuclear PI 4-kinase (ATP:phosphatidylinositol 4-phosphotransferase, EC 2.7.1.67), which we have termed *PIK1* (**p**hosphatidylinositol **k**inase).

Results

Isolation of the essential PIK1 gene

Membrane blots containing total yeast proteins were probed with the anti-human nucleoporin monoclonal antibody QE5 (McMorrow *et al.*, 1994). The antibody recognized predominantly a protein of ~102 kDa. This protein was recovered quantitatively in a highly purified preparation of yeast nuclei, as evidenced by probing blots containing cell-equivalent amounts of protein from a total cell homogenate, a cytosolic fraction and purified nuclei (Figure 1).

The antibody was used to screen a λgt11 genomic yeast expression library and the ~500 bp insert from a positive phage was subcloned into the integrative shuttle vector YIplac211. Sequencing of the cloned DNA revealed that it was a previously undescribed sequence. A preliminary genomic disruption of the cloned sequence by targeted integration of the plasmid cut at a *Bgl*II site in the insert indicated that the cloned sequence was an internal segment of an essential gene. The gene was subsequently named *PIK1* (see below and Introduction). To obtain the complete gene, the cloned DNA fragment was used as a probe to screen a yeast genomic library constructed in the high copy number vector pSEY18. A positive clone containing a 15 kbp insert (pGB1) was identified which also overexpressed the antigen recognized by the QE5 monoclonal (Figure 2), suggesting that the genomic clone was a full-length clone encoding the yeast protein recognized by the antibody. Based on three independent measurements by densitometry on Western blots, the antigen was overexpressed 2.5- to 4-fold in strains containing pGB1.

To determine the location of *PIK1* within the 15 kbp insert of pGB1, restriction digests of pGB1 were probed with the original ~500 bp fragment. The region corresponding to the ~500 bp fragment mapped to a position within the insert designated as λ in Figure 3A. Knowing the location of *PIK1* within the 15 kbp genomic insert, a second gene disruption was constructed by substituting a *Bgl*II fragment containing the *ADE2* gene (Stoz and Linder, 1990) for the 1.2 kbp *Bgl*II fragment present in the insert (Figure 3B). This construct was used to transform diploid strain JK9-3da/α *ade2Δ/ade2Δ* to generate strain FM1 (JK9-3da/α *pik1::ADE2-1/PIK1 ade2Δ/ade2Δ*). The disruption was confirmed by Southern blotting (data not shown). When strain FM1 was sporulated and dissected, only two spores from each of the >30 tetrads analysed survived and formed colonies, all of them ade$^-$. Microscopic examination of the spores that did not form colonies revealed that they either did not germinate or germinated but arrested before completing cytokinesis. This further demonstrated that *PIK1* is a unique gene essential for growth.

The boundaries of *PIK1* within the cloned fragment (Figure 3A) were determined by testing the ability of different subclones to complement the lethality of the disruption in strain FM1. The smallest subclone tested (pGB3) that complemented the *PIK1* disruption was the 4.1 kbp *Sph*I−*Sac*I fragment shown in Figure 3B. Cells containing pGB3 also overproduced the 102 kDa antigen

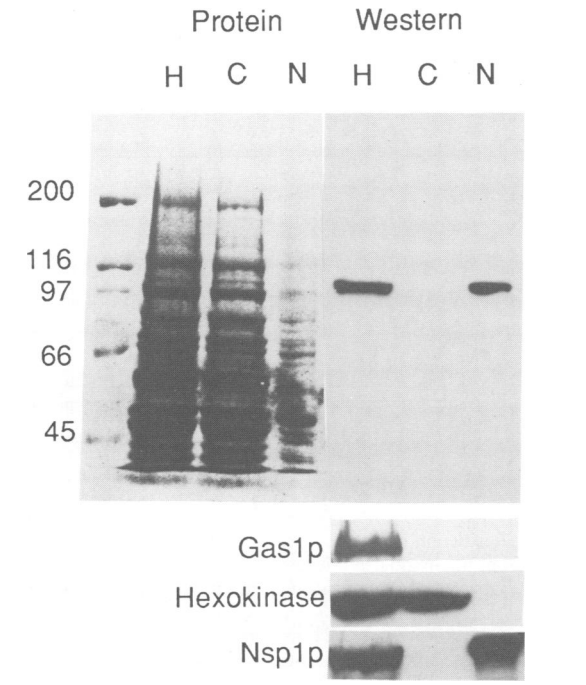

Fig. 1. Recognition of yeast proteins by the QE5 monoclonal antibody. A yeast homogenate (H) was fractionated into cytosolic (C) and nuclear (N) fractions, separated on an 8% SDS−PAGE gel and blotted on to a PVDF membrane. Cell-equivalent amounts of protein from each fraction and the homogenate were loaded. The respective amounts for homogenate, cytosol and nucleus were 68, 45 and 14 μg of protein. The left panel (Protein) shows the membrane stained for protein with amido black. The right panel (Western) shows the same membrane probed with the QE5 monoclonal (uppermost). Shown below are the signals obtained by probing with antibodies against the plasma membrane protein GAS1 (Gas1p) (Nuoffer *et al.*, 1991), the cytoplasmic protein hexokinase (Hexokinase), and the nuclear protein NSP1 (Nsp1p) (Nehrbass *et al.*, 1990). The sizes of the molecular weight standards are indicated in kDa.

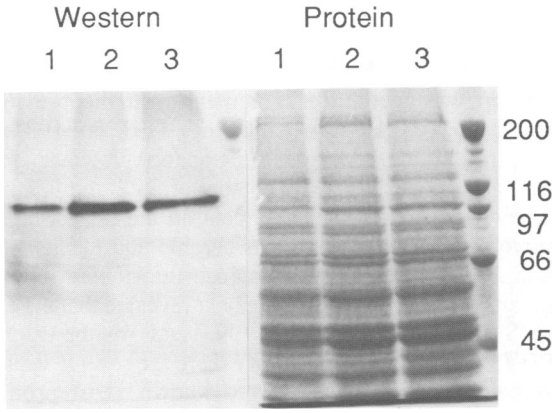

Fig. 2. Overexpression of the QE5 yeast antigen by strains containing *PIK1* on a high copy number plasmid. Approximately 30 μg of protein from total homogenates of strain JK9-3d containing no plasmid (lane 1), pGB1 (lane 2) and pGB3 (lane 3) were separated in duplicate on two halves of the same SDS−PAGE gel. One half of the gel was stained with Coomassie blue (Protein) and the other half was blotted to a membrane and probed with the QE5 monoclonal (Western). The sizes of the molecular weight standards are indicated in kDa.

recognized by QE5 (Figure 2). Furthermore, using the essential 1.2 kbp *Bgl*II fragment contained within pGB3 as a probe, it was observed that cells carrying pGB1

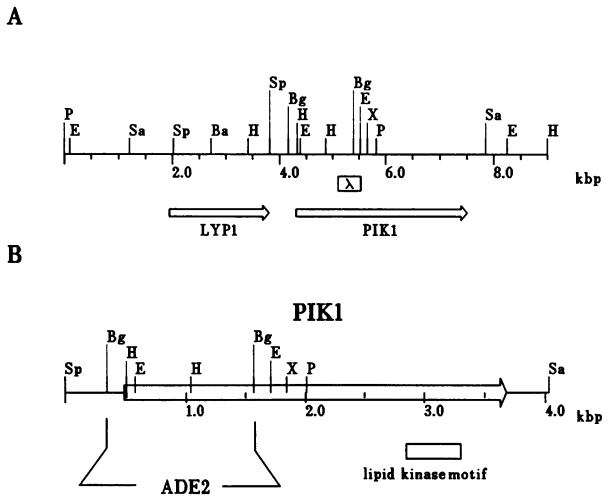

Fig. 3. Restriction maps and disruption of *PIK1*. (**A**) Segment of the insert from the original full-length clone pGB1. The boxed-in λ indicates the segment corresponding to the insert in the original λgt11 clones. *LYP1* and *PIK1* ORFs are indicated by open arrows. (**B**) Detail of the *PIK1* region and disruption. The open box marks the region of highest homology to other PI kinases. Open arrow indicates the *PIK1* ORF. Restriction sites are Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sa, *Sac*I; Sp, *Sph*I; X, *Xho*I.

overexpressed an RNA that migrated slightly slower than the yeast 3.4 kb rRNA (data not shown), indicating that the mRNA encoded by *PIK1* was between 3.5 and 4.0 kb in size. An mRNA of this size would normally encode a protein >102 kDa, but 10–20% discrepancies between calculated and observed sizes in SDS gels are not unusual. The size discrepancy between the coding capacity of the mRNA and the observed 102 kDa protein could also be accounted for by proteolysis.

Nucleotide sequence and genomic location of PIK1

The complete nucleotide sequence of both strands of the minimal complementing fragment, the 4.1 kbp *Sph*I–*Sac*I fragment (Figure 3B), was determined. A single ORF of >300 bp was found. In agreement with the previously detected 3.5–4.0 kb RNA, the *PIK1* ORF is 3198 bp in length. In addition, it includes the sequence previously found in the λgt11 clone, has a GC content of 38% and is flanked by known yeast promoter elements on the 5′ side and by putative polyadenylation signals (Wahle and Keller, 1992) on the 3′ side (Figure 4). Sequence information obtained from outside the *Sph*I–*Sac*I fragment revealed that the *LYP1* gene (Sychrova and Chevalier, 1993) ends 493 bp upstream of *PIK1*. Between *LYP1* and *PIK1* there are several sequence motifs with strong homology to known yeast promoter elements. Of several putative TATA boxes, one at −102 from the ATG codon is optimally located (Hahn *et al.*, 1985; Rudolph and Hinnen, 1987). Additionally, there are three pheromone response elements (PRE) (Kronstad *et al.*, 1987) at −441 (inverted, one mismatch), −348 (inverted, one mismatch) and −195 (direct, no mismatches). There are also two SWI4-SWI6 cell cycle boxes (SCB) (Koch *et al.*, 1993) located between the two gene-proximal PREs, at −236 (direct, one mismatch) and −212 (inverted, no mismatches). A functional SCB has been shown to promote transcription in the late G_1 phase of the cell cycle (Breeden and Nasmyth, 1987; Koch *et al.*, 1993). Also intriguingly present are the three conserved elements of yeast splicing signals (Langford *et al.*, 1984); the 5′ splice site at −351, the internal consensus sequence at +333 and the 3′ splice site at +382. We consider it unlikely that these signals are functional, however, because the theoretical 5′ splice site at −351 probably lies outside the transcribed region. Furthermore, no significant amount of the predicted spliced product was found by Northern blotting.

The genomic location of *PIK1* was investigated by probing blots of separated *Saccharomyces cerevisiae* chromosomes with the 1.2 kbp *Bgl*II fragment described above (Figure 3B). A single band corresponding to chromosome XIV was obtained. To localize the gene more precisely within chromosome XIV, the same probe was hybridized to filters containing a set of ordered λ and cosmid clones of the yeast genome (Riles *et al.*, 1993). The probe hybridized to clones corresponding to a position ~140 kbp from the left telomere of chromosome XIV, between *MET2* and *RAD50*.

PIK1 is homologous to PI kinases

Translation of the *PIK1* ORF yielded a protein of 119 922 Da with a calculated pI of 6.46. As discussed above, we do not know at present the reason for the discrepancy between this theoretical molecular weight and the observed size of 102 kDa in Western blots (Figure 1). The amino acid composition is rich in leucine and serine and has a ratio of charged to apolar residues of 0.9, suggesting that the protein is not globular (Cohen and Parry, 1986). There are no obvious secretory signal sequences or transmembrane domains but there is a conspicuous 25 amino acid acidic stretch (amino acids 335–361) with a net charge of −12 that has some resemblance to the Zn^{2+} binding site of parathymosin (Brand *et al.*, 1988). The presence of a metal binding site would be congruent with the requirement for divalent cations to stabilize the nuclear pore structures recognized by the QE5 monoclonal (Jarnik and Aebi, 1991; Pante and Aebi, 1993). Other features of the deduced sequence are a putative nuclear localization signal K491KGK in a typical context of close proline residues and a casein kinase II site (reviewed in Garcia-Bustos *et al.*, 1991a), suggesting that the protein can be translocated into the nucleus.

A search for sequence homologies using the BLAST and FASTA programs (see Materials and methods) revealed a lipid kinase sequence motif at the carboxy-terminus of PIK1 (amino acids 794–950). This motif is also found at the carboxy-terminus of all other proteins known or presumed to be PI kinases (Figure 5) and is supposedly part of the lipid kinase active site (Hiles *et al.*, 1992; Kunz *et al.*, 1993; Schu *et al.*, 1993; Helliwell *et al.*, 1994).

PIK1 is a PI 4-kinase associated with the nucleus

Because PIK1 contains a lipid kinase motif, we investigated whether it has PI kinase activity. We examined whether a strain (BJ2168/pGB1) containing *PIK1* on a high copy number plasmid overexpressed PI kinase activity; as mentioned above, strains containing pGB1 were previously shown to overexpress an antigen recognized by the monoclonal antibody QE5. The reaction products obtained by incubating total cell homogenates (data not shown) and subcellular fractions with PI and [^{32}P]ATP were assayed by thin layer chromatography (TLC) and autoradiography (see Materials and methods). Total cell homogenates displayed

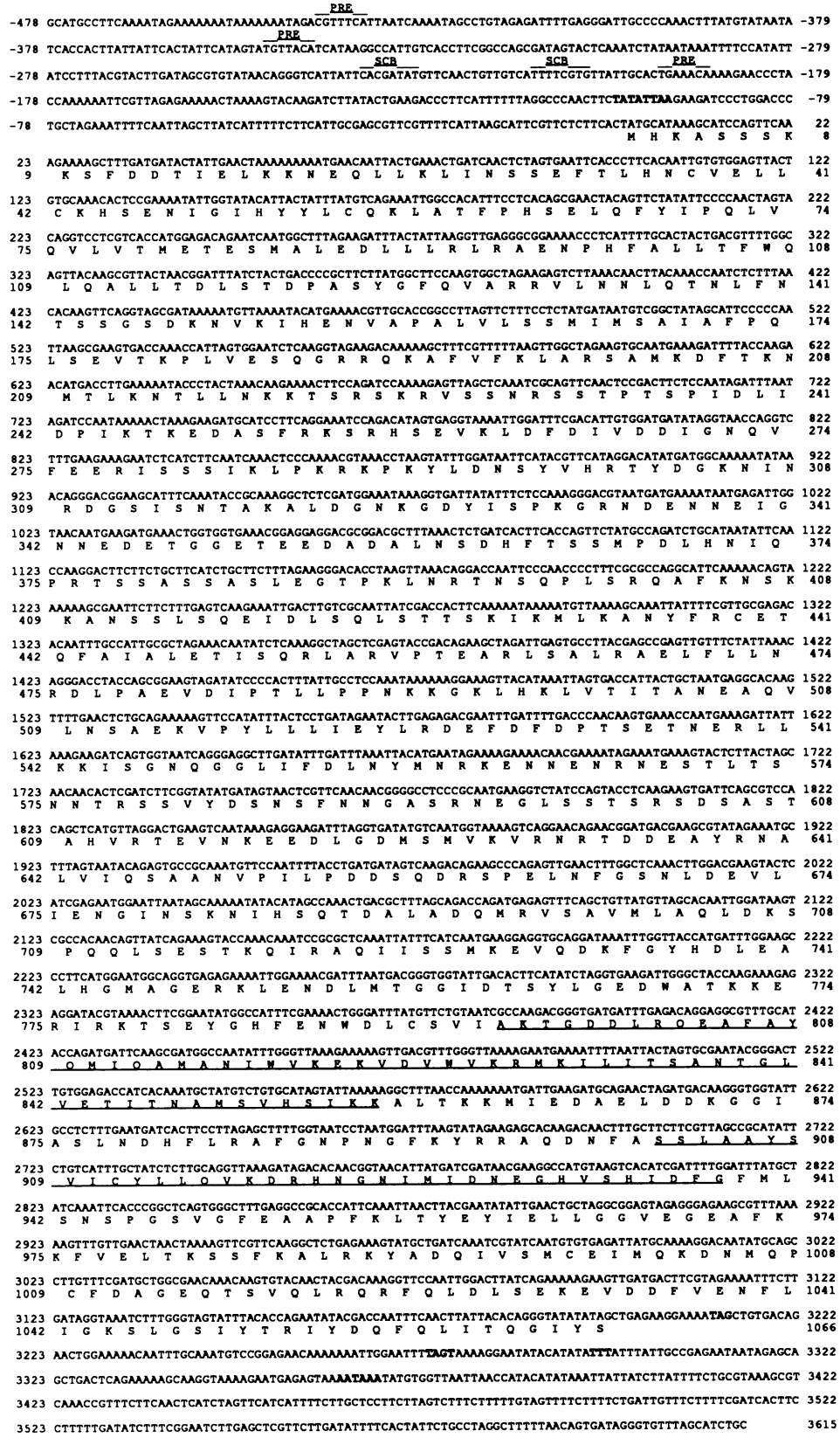

Fig. 4. Nucleotide and deduced amino acid sequence of *PIK1*. A set of putative TATA boxes and polyadenylation signals are in bold. Consensus sequences for the PRE, TGAAACA, and the SWI4-SWI6 cell cycle box (SCB), CACGAAA, in the 5′ untranslated region are indicated. The underlined amino acid sequences are the lipid kinase motif (see text and Figure 5). The EMBL Data Library accession number for the sequence reported in this paper is X76058.

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PIK1:  (795) AKTGDDLRQEAFAYQMIQAMAN.IWVKE....KVDVWVKRMKILITSA
p110:  (801) FKNGDDLRQDMLTLQIIRIMEN.IWQNQ....GLDLRMLPYGCLSIGD
VPS34: (623) FKVGDDLRQDQLVVQIISLM.NELLKNE....NVDLKLTPYKILATGP
TOR2: (2127) LKGHEDIRQDSLVMQLFGLV.NTLLQNDAECFRRHLDIQQYPAIPLSP
TOR1: (2123) LKGHEDIRQDSLVMQLFGLV.NTLLKNDSECFKRHLDIQQYPAIPLSP

NTGLVETITNAMSVHSIKK-(45)-SSLAAYSVICYLLQVKDRENGNIMID.NEGHVSHIDFG
CVGLIEVVRNSHTIMQIQC-(36)-RSCAGYCVATFILGIGDRHNSNIMV.KDDGQLFHIDFG
QEGAIEFIPN.DTLASILS-(31)-KSCAGYCVITYILGVGDRHLDNLLV.TPDGHFFHADFG
KSGLLGWVPNSDTFHVLIR-(70)-RSLAVMSMTGYILGLGDRHPSNLMLDRITGKVIHIDFG
KSGLLGWVPNSDTFHVLIR-(70)-RSLAVMSMTGYILGLGDRHPSNLMLDRITGKVIHIDFG
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Fig. 5. The lipid kinase motif. This motif is found in the carboxy-termini of PIK1 (1066 amino acids), the p110 catalytic subunit of bovine PI 3-kinase (1068 amino acids), VPS34 (875 amino acids), TOR2 (2474 amino acids) and TOR1 (2470 amino acids). The position of the first residue in the motif within the complete sequence is indicated in parentheses. Residues present in all the sequences are shown in bold. Note that many substitutions are highly conservative.

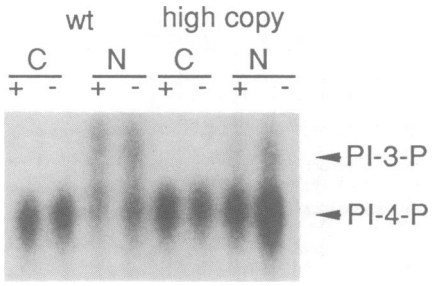

Fig. 6. Overexpression of a PI 4-kinase activity in yeast cells containing *PIK1* in a multicopy plasmid. The PI kinase activity present in the cytosolic (C) and nuclear (N) fractions of yeast cells containing a single copy of *PIK1* (wt) or the cloned gene on a multicopy plasmid (pGB1) (high copy), was assayed as described in the text. Cell-equivalent amounts of cytosolic (12 μg) and nuclear (4 μg) proteins were assayed. Plus and minus signs indicate the presence or absence of 1 mM adenosine in the assay. The apparent reduction in the intensity of the PI-3-P spot in the adenosine-treated sample from overproducing nuclei was not reproducible. It should also be noted that although approximately equal PI 3-kinase and PI 4-kinase activities are detected in total yeast extracts (Auger *et al.*, 1989), most of the PI 3-kinase activity localizes to the membrane fraction (Schu *et al.*, 1993) and is therefore not present in our assay. The R_f for PI-3-P and PI-4-P in this TLC system is indicated.

an increased capacity to phosphorylate PI and the increase in activity could be accounted for exclusively by an increase in nucleus-associated activity (Figure 6). In three independent experiments, the increase in PI kinase activity observed in nuclear fractions was 2.6-, 1.5- and 3.2-fold, compared with a non-overproducing strain and as measured by densitometry of suitably exposed autoradiograms. In the TLC system used, the overproduced product had the R_f of PI-4-phosphate (PI-4-P) (see Materials and methods) (Walsh *et al.*, 1991; Schu *et al.*, 1993). PI 4-kinase activity was also detected in cytosolic fractions but this activity was not increased by overexpression of *PIK1*.

To obtain further evidence that the increased, nuclear PI 4-kinase activity was encoded by *PIK1*, we determined whether the increased activity was lost at high temperature in nuclei overexpressing the temperature-sensitive *pik1-12* allele. A plasmid-borne *pik1-12* allele supports growth at 23 and 30°C but not at 37°C in strains containing a chromosomal disruption of *PIK1* (see below). Strain BJ2168/pCF12 containing the *pik1-12* allele on a high copy number plasmid was grown at the permissive temperature;

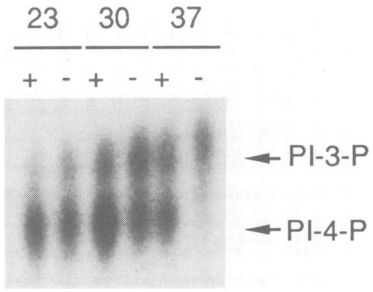

Fig. 7. The nuclear fraction of cells expressing *pik1-12* contains temperature-sensitive PI 4-kinase activity. The nuclear fraction of cells containing wild type *PIK1* (+) or the temperature-sensitive *pik1-12* allele (−) on a high copy number plasmid were assayed for PI 4-kinase activity at 23, 30 and 37°C (37). Four micrograms of nuclear protein were used in each assay. The R_f for PI-3-P and PI-4-P in this TLC system is indicated.

nuclei were isolated, equilibrated to permissive (23 and 30°C) and non-permissive (37°C) temperatures, and assayed for PI kinase activity at the same temperatures. In two independent experiments, the PI 4-kinase activity of the *pik1-12* nuclei was reduced by 2.3- and 3.7-fold at 37°C but was not significantly affected when the assay was performed at the two permissive temperatures, compared with nuclei from a strain (BJ2168/pGB1) overproducing wild type PIK1 (Figure 7). Contrary to the nuclear PI 4-kinase activity of strain BJ2168/pCF12, the cytosolic PI 4-kinase activity of the same strain was neither overexpressed or temperature-sensitive (data not shown). Thus, *PIK1* encodes a nucleus-associated PI 4-kinase.

PI 4-kinases have been classified biochemically into two different types based on sensitivity to adenosine. Type II PI 4-kinase is sensitive to adenosine, whereas type III is resistant (type I PI kinase is PI 3-kinase) (Carpenter and Cantley, 1990). We found that adenosine at 1 mM, more than 50 times the described Ki for type II PI 4-kinases (Endemann *et al.*, 1987), reduced the amount of phosphorylated inositol produced by the nuclear fraction by 40−60%, as measured by film densitometry in two independent experiments (Figure 6). This partial inhibition may reflect two different PI 4-kinases (types II and III) in the nuclear fraction. The cytosolic PI 4-kinase was insensitive to inhibition by 1 mM adenosine.

PI 4-kinases described so far are stimulated by Triton X-100 at concentrations above its critical micellar concentration (Endemann *et al.*, 1987 and references therein). We could not interpret the results of adding the detergent to our assays because we observed that in our reactions the endogenous inositol present in the nuclear membranes was also used as substrate, and adding detergent removed it, producing an inhibitory effect that obscured a possible activation.

PIK1 expression and function

The presence of PREs in the promoter region of *PIK1* suggested that PIK1 might play a role in some process controlled by α-factor. We tested whether *PIK1* in increased dosage affected the sensitivity of **a** cells to the growth inhibitory effect of α-factor, as measured by a halo assay. FM1-5d/pGB1 cells (disrupted for the chromosomal *PIK1* gene so as to maintain the high copy number plasmid pGB1

independently of the growth medium) showed an unchanged or very slightly increased sensitivity to α-factor compared with an isogenic strain (JK9-3da) containing a single copy of *PIK1* (data not shown). Thus, as measured by this assay, overexpression of the gene has little to no effect on sensitivity to α-factor.

To investigate whether the SCBs found in the promoter region of *PIK1* (Figure 4) conferred G_1-specific transcription, total RNA was prepared from cells synchronized using the temperature-sensitive mutation *cdc15-2* in strain RH210-3c. The *PIK1* transcript was followed during two cell cycles after the release of a temperature block. Northern blots did not reveal any significant increase in *PIK1* transcription at G_1, although the transcripts for G_1 and G_2 cyclins showed normal periodicity in the RNA preparations from the synchronized cells (data not shown). Perhaps the PREs and the SCBs function together as a hybrid promoter, conferring increased expression of *PIK1* at G_1 only in the presence of α-factor, i.e. the *PIK1* promoter may confer G_1 specificity on pheromone-induced transcription.

The antibiotic neomycin binds to PI lipids and has been proposed to inhibit signal transduction in platelets by keeping such lipids inaccessible to phosphodiesterases (Tysnes *et al.*, 1988). It is conceivable that increased synthesis of PI-4-P through overexpression of PIK1 could increase the flow through the pathway and thereby elevate the minimum inhibitory concentration for neomycin. The growth of isogenic strains with and without *PIK1* in high copy number plasmids was monitored on YPD plates containing increasing concentrations of the antibiotic. All strains displayed the same sensitivity, being completely inhibited by 6 mg/ml of neomycin. Apparently, increased dosage of *PIK1* does not alter the sensitivity of yeast cells to neomycin.

PIK1 conditional mutants are defective in cytokinesis

Because null alleles of *PIK1* are lethal, conditional mutants were isolated to facilitate the analysis of PIK1 function. Conditional alleles were obtained by chemical mutagenesis of plasmid-borne *PIK1* (pGB2, see Materials and methods). Of eight independent temperature-sensitive mutants isolated so far, *pik1-12* will be described in detail here, although all seem to have similar properties. A haploid strain with a disrupted chromosomal copy of *PIK1* and *pik1-12* on a multicopy vector (FM1-5d/pCF12) stops growing within 2 h of shifting from the permissive (23 or 30°C) to the non-permissive temperature (37°C); growth was measured as an increase in OD_{600}. Microscopic examination of the arrested cells revealed a cytokinesis defect, since most cells appeared as pairs with fully separated nuclei. Also apparent but less abundant were cell triplets, again with fully segregated nuclei (Figure 8). Flow cytometry (FACS analysis) corroborated that most cells had a 2n or higher DNA content (Figure 8). The cytokinesis defect was also observed at the permissive temperature, although at 23°C the proportion of cell triplets was significantly lower; at 23°C, the mutants grew only slightly slower than the wild type. The limited function of the *pik1-12* allele is enough to allow cells to proliferate at the permissive temperature, but it is apparently not enough to allow the cells to cope with the added stress of high temperature or, alternatively, the pik1-12 protein loses function completely at 37°C. The PI 4-kinase activity of pik1-12 is severely reduced at 37°C (see above).

By analogy with the phosphoinositide signal transduction pathway of higher eukaryotes, PKC in yeast may act downstream of PI 4-kinase. Thus, strains defective in PI 4-kinase could have phenotypic characteristics in common with strains defective in PKC. Temperature-sensitive alleles of *PKC1*, which encodes a yeast PKC, confer a cell wall defect and a terminal phenotype of cells with small buds and undivided nuclei containing 2n DNA. This temperature-sensitive phenotype, can be suppressed by increasing the osmolarity or the Ca^{2+} concentration of the growth medium (Levin and Barlett-Heubusch, 1992; Paravicini *et al.*, 1992). We tested the ability of *pik1-12* strains and of spores carrying the *pik1::ADE2-1* allele to grow on YPD plates supplemented with 1 M sorbitol, 100 mM $CaCl_2$ or 100 mM $MgCl_2$. As observed with *PKC1* mutants, sorbitol and $CaCl_2$, but not $MgCl_2$, suppressed the temperature-sensitive growth defect of *pik1-12* (data not shown). The *pik1::ADE2-1* null allele was not suppressed by any of the supplements. Furthermore, unlike the rapid lysis observed in *pkc1* mutants, *pik1-12* mutants were still viable after 6 h at the restrictive temperature. When cells were returned to 23°C and plated on YPD, ~50% of the cells counted in a Neubauer chamber gave rise to colonies.

A PIK1 disruption is not complemented by other known or presumed yeast PI kinases

Because PIK1 is structurally related to the presumed PI kinases TOR1 and TOR2, and to the PI 3-kinase VPS34 (Figure 5), we tested whether the disruption allele of *PIK1* in strain FM1 could be complemented by *TOR1* (pPW20), *TOR2* (pJK3-3) or *VPS34* (pMD21) provided on a high copy number vector. When diploid FM1 cells (*pik1::ADE2-1/PIK1*) containing pPW20, pJK3-3 or pMD21 were sporulated and dissected (14 tetrads from each transformant) only two viable spores, both carrying the non-disrupted allele of *PIK1*, were recovered from each tetrad; in each dissection, the plasmid marker (*URA3*) was detected in the viable segregants. Thus, neither *TOR1*, *TOR2* nor *VPS34* in high dosage can supply the function(s) missing in a *PIK1* disruption strain.

Discussion

We have cloned a yeast gene, *PIK1*, encoding a nucleus-associated PI 4-kinase. This study presents the first sequence of a PI 4-kinase and the first direct evidence that a PI 4-kinase is essential for growth. The carboxy-terminus of PIK1 contains a lipid kinase motif previously found in the carboxy-termini of the p110 catalytic subunit of bovine PI 3-kinase, the yeast PI 3-kinase VPS34, and the two putative PI kinases TOR1 and TOR2 (Hiles *et al.*, 1992; Kunz *et al.*, 1993; Schu *et al.*, 1993; Helliwell *et al.*, 1994), indicating that this is a general motif found in different types of PI kinases. Disruption and conditional alleles suggest that *PIK1* is required for cytokinesis.

Disruptions of the *PIK1* gene are lethal. This result indicates that there are no other PI 4-kinases that can substitute for PIK1, although several seemingly different PI 4-kinase activities have been described in *S.cerevisiae*. Several membrane-bound forms have been described and the molecular sizes of two of these enzymes have been estimated to be 45 and 55 kDa (Buxeda *et al.*, 1991; Nickels *et al.*, 1992; McKenzie and Carman, 1993). A partially soluble

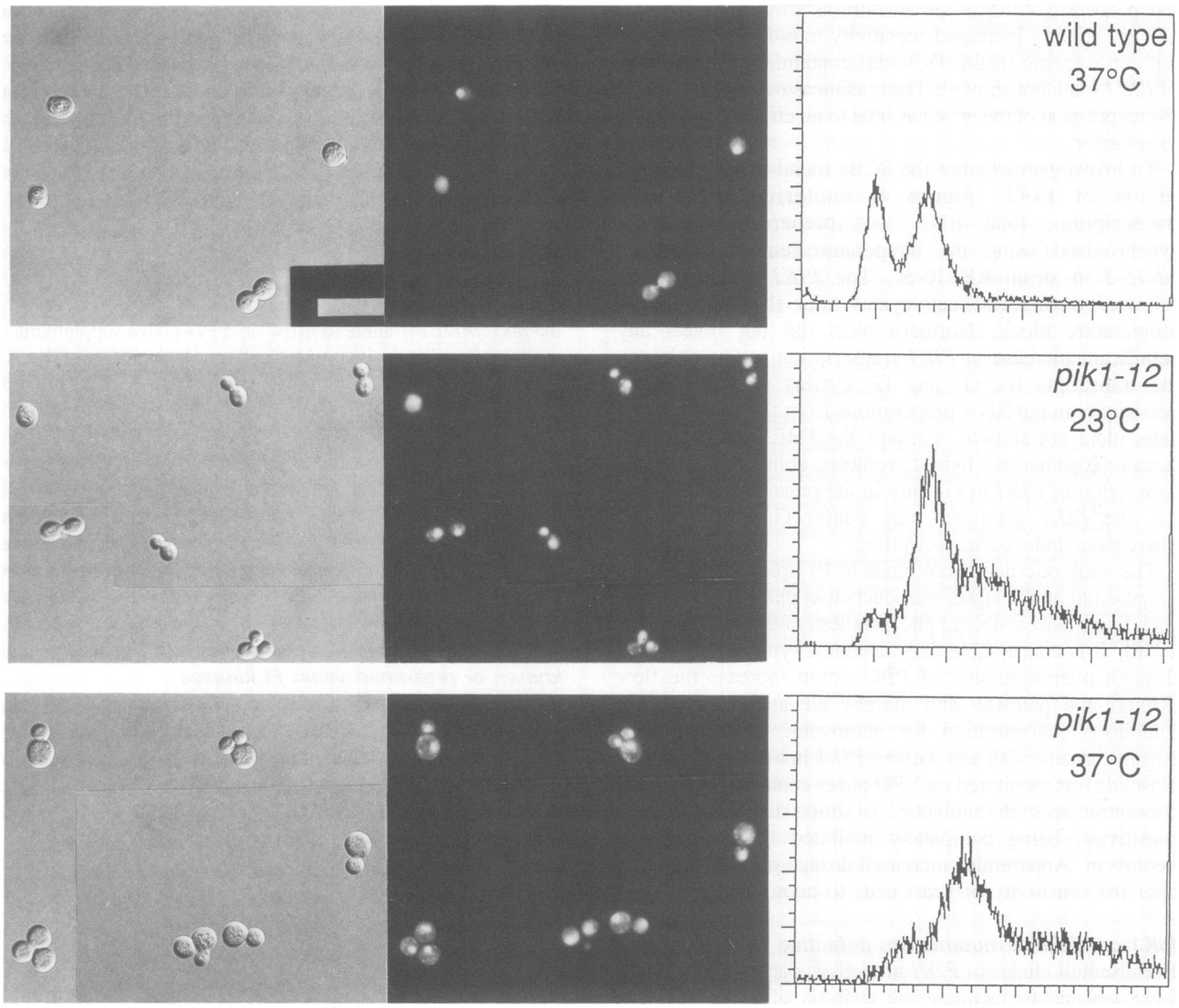

Fig. 8. Temperature-sensitive *pik1-12* mutant has a cytokinesis defect. FM1-5d/pGB2 (wild type) and FM1-5d/pCF12 (*pik1-12*) cells were grown in SD-Leu medium at 23 or 37°C. Samples were taken after 6 h and processed for FACS analysis as described in Materials and methods. A fraction of each sample was examined with Nomarski optics (left panel) and for staining with the DNA-specific fluorescent stain propidium iodide (middle panel). The results of the FACS analysis (right panel) are illustrated as the number of cells (*y*-axis) with a certain DNA content (*x*-axis). The two peaks in the wild type sample correspond to 1n (left peak) and 2n (right peak) DNA contents. The major peak in *pik1-12* at 37°C presumably represents 3n DNA content. The bar within the dark rectangle in the upper left micrograph represents 10 μm.

activity of 120 kDa has also been described (Flanagan and Thorner, 1992). Based solely on the published sizes, this last enzyme is the best candidate for the product of the *PIK1* gene. However, overexpression of PIK1 or tagging of PIK1 with a temperature-sensitive mutation causes a corresponding change only in nucleus-associated PI 4-kinase activity; cytosolic activity remains unaffected (Figures 6 and 7). This could indicate that there are two enzymes with similar sizes but different cellular locations, in agreement with the different sensitivities toward inhibition by adenosine displayed by our nuclear and cytosolic fractions.

Null mutants of *PIK1* are inviable but some spores carrying a disruption allele do germinate, only to arrest as cells with a single large bud. Cells carrying an allele of *PIK1* that confers temperature-sensitive growth display a similar morphology of cells with large buds and fully divided nuclei (Figure 8). This suggests that the product of PIK1 (PI-4-P) plays a role in controlling cytokinesis. However, even at the permissive temperature, the temperature-sensitive mutants still accumulate unseparated cells (Figure 8). The temperature-sensitive mutants arrest growth only at 37°C because the cytokinesis defect is more severe at this temperature or because, at the higher temperature, PI-4-P becomes limiting for an essential process unrelated to cytokinesis.

Mutations in another presumed phosphoinositide signalling pathway component in *S.cerevisiae*, the PKC encoded by *PKC1*, affect cell wall metabolism. Temperature-sensitive *PKC1* mutants display cell cycle-specific, rapid lysis at restrictive temperature, *PKC1* conditional alleles are suppressed by high osmolarity or an elevated Ca^{2+} concentration but null alleles are suppressed only by high osmolarity (Levin and Barlett-Heubusch, 1992; Paravicini *et al.*, 1992). Furthermore, overexpression of an endogenous PKC in *Schizosaccharomyces pombe* causes a cytokinesis defect (Mazzei *et al.*, 1993). Our observations are

reminiscent of, but not identical to, the above mentioned. Conditional *PIK1* mutations confer a growth defect that is suppressed by 1 M sorbitol or 100 mM Ca^{2+}. However, the lethality caused by *PIK1* disruptions is not suppressed by elevated concentrations of sorbitol or Ca^{2+} and the *pik1-12* mutant does not lose viability (lyse) rapidly at the restrictive temperature. Suppression of the *PIK1* conditional allele, but not the null allele, by high osmolarity or Ca^{2+} can be explained as follows. The two different supplements may be specifically suppressing the conditional allele by different mechanisms. High osmolarity could directly stabilize the mutated enzyme, as previously suggested for the suppression of missense mutations in nutritional genes (Hawthorne and Friis, 1964). The finding that high osmolarity does not suppress a null mutation suggests that the suppression is not of a cell wall defect. Increased Ca^{2+} could act as an 'effector' to stimulate the enzymatic activity of the mutated PIK1 or the activity of a compensating function; Ca^{2+} normally stimulates a number of enzymes that act on or are influenced by phospholipids (e.g. phospholipase A_2, phospholipase C and PKC). Because the normal activation of certain enzymes by Ca^{2+} is specific for this ion, this model also accounts for why Mg^{2+} does not suppress a *PIK1* conditional mutation.

What are the cellular components downstream of PIK1? Obvious possibilities include the PI-specific phospholipase C (PLC1) and PKC1. As discussed above, the phenotypes of *PKC1* and *PIK1* mutants partly overlap. The same is true for *PLC1* and *PIK1* mutants as both exhibit a cytokinesis defect (Flick and Thorner, 1993; Payne and Fitzgerald-Hayes, 1993; Yoko-o *et al.*, 1993). Considering the cytokinesis defect observed in *PIK1* mutants, another candidate for a downstream component is the actin cytoskeleton. Yeast spores with a disrupted actin gene, *ACT2*, arrest as single cells with a large bud and diploid cells with only one functional copy of *ACT2* show an increase in the number of cells with multiple large buds, each having its own nucleus (Schwob and Martin, 1992). These are the same phenotypes described here for null and conditional mutations of *PIK1*. Furthermore, phosphorylated phosphoinositides have been implicated in actin rearrangement. The function of actin binding proteins is known to be modulated *in vitro* by PI-4,5-P_2. The yeast capping protein, which binds to the growing end of actin filaments, is prevented from doing so by micromolar concentrations of PI-4,5-P_2 (Amatruda and Cooper, 1992) and profilin, an essential protein in yeast which participates in the remodelling of the actin cytoskeleton, is also known to be dissociated from actin by PI-4,5-P_2 (Lassing and Lindberg, 1985; Magdolen *et al.*, 1988). In neutrophils and fibroblasts, PI-3,4,5-P_3 is believed to modulate rearrangement of the actin cytoskeleton (Eberle *et al.*, 1990; Arcaro and Wymann, 1993; Wymann and Arcaro, 1994). Thus, a strain defective in PIK1 may produce less PI-4,5-P_2 or PI-3,4,5-P_3, which could then hinder actin cytoskeleton remodelling necessary for cytokinesis.

PIK1 was identified by screening a yeast expression library with the QE5 monoclonal antibody, raised against a human nuclear pore component (McMorrow *et al.*, 1994). Although QE5 recognizes a yeast nuclear protein by Western blotting, efforts to immunolocalize the protein in intact yeast cells using the monoclonal antibody have failed to give a signal in any cellular compartment, perhaps because the epitope is inaccessible under conditions which preserve nuclear integrity. Thus, because the nuclear envelope constitutes a substantial fraction of the yeast endoplasmic reticulum, we cannot at present rigorously state that the enzyme is exclusively nuclear. Polyclonal antibodies may be required to determine more precisely the location of PIK1. The observed association of PIK1 with nuclei is in full agreement with observations of a nuclear phosphoinositide cycle distinct from the better known plasma membrane cycle (Divecha *et al.*, 1991, reviewed in Divecha *et al.*, 1993; Irvine and Divecha, 1992; Payrastre *et al.*, 1992). If PIK1 is indeed a nuclear pore component (Pante and Aebi, 1993), it could provide a mechanism to connect transport across the nuclear envelope to other cellular processes via inositol signalling. A nuclear location for PIK1 could, furthermore, link completion of nuclear division with cytokinesis.

While this manuscript was in preparation, Flanagan *et al.* (1993) presented the cloning and disruption of *PIK1*. They cloned *PIK1* using DNA probes derived from the sequence of a purified PI 4-kinase and presented evidence that PIK1 is a soluble enzyme involved in the pheromone response (Flanagan and Thorner, 1992; Flanagan *et al.*, 1993). Two significant differences between our findings and those studies are the subcellular distribution of the PI 4-kinase activity and the role of the enzyme in pheromone response. The apparent difference in localization could be attributed to different methods of cellular fractionation. Our method, consisting of manual douncing of spheroplasts followed by pelleting of nuclei onto a sucrose cushion, was designed to generate intact nuclei (Hurt *et al.*, 1988; Garcia-Bustos *et al.*, 1991b). The method used by Flanagan and Thorner (1992), consisting of breaking cells by vigorous mixing with beads (Beadbeater™) followed by extensive high speed centrifugation, was designed to produce a rich supernatant fraction. This method may break nuclei and release what might otherwise be a particulate activity; the finding by Flanagan and Thorner (1992) that ~40% of their activity was associated with the particulate fraction may indeed reflect incomplete nuclear breakage. We cannot as of yet explain why we did not detect a reproducible enhanced sensitivity to α-factor by strains overproducing PIK1 (Flanagan *et al.*, 1993), but our results do not exclude a role for PIK1 in the pheromone response.

Materials and methods

Strains, plasmids and culture media

Escherichia coli strains Y1090, used to propagate λ phages and DH5α, used to obtain DNA for sequencing, were as described (Sambrook *et al.*, 1989). Strain MH1 (*araD139* ΔlacX74 galE galK hsr rpsL) was sometimes used to isolate recombinant plasmids and strain MH4 (ΔlacX74 galE galK hsr rpsL leuB600) was used to test complementation with the yeast *LEU2* gene. *E.coli* strains were routinely grown on LB or M63 media (Sambrook *et al.*, 1989) with the antibiotics appropriate for plasmid maintenance.

Saccharomyces cerevisiae strain JK9-3d (*leu2-3,112 ura3-52 trp1 his4 GAL*$^+$ *rme1 HMLa*) or its *ade2Δ*/ade2Δ homozygous diploid derivative were used for all genetic manipulations. The protease-deficient strain BJ2168 (**a**/α *leu2/leu2 trp1/trp1 ura3-52/ura3-52 prb1-1122/prb1-1122 pep4-3/pep4-3 prc1-407/prc1-407*) (provided by E.Jones) was used for cell fractionation studies. Synchronized cultures were prepared from strain RH210-3c (*ade2-1 bar1 cdc15-2 his4 trp1*) (provided by H.Reizman), which arrests in G_2 when shifted to the non-permissive temperature. Transformation, gene disruption, sporulation and tetrad dissection were done following standard procedures (Guthrie and Fink, 1991). Yeast cells were grown on either rich medium, YPD, or synthetic minimal medium, SD, with the appropriate nutrient supplements. Growth media and sporulation

media were prepared as described (Guthrie and Fink, 1991). The following shuttle plasmids were used: pSEY18 (*URA3* 2μ amp^r) (provided by S.Emr), YEplac181 (*LEU2* 2μ amp^r), YEplac195 (*URA3* 2μ amp^r) and YIplac211 (*URA3* amp^r). The Y__plac series of plasmids has been previously described (Gietz and Sugino, 1988). pGB1, the original full-length *PIK1* clone, is pSEY18 carrying a 15 kbp insert. pGB2 is YEplac181 with *PIK1* on a *Sac*I fragment and pCF12 is the same construct with the *pik1-12^{ts}* mutant allele. pGB3 is YEplac181 containing *PIK1* in an *Sph*I−*Sac*I fragment (Figure 3B). pPW20 is pSEY18 carrying *TOR1* (Helliwell *et al.*, 1994) and pJK3-3 is the same vector with *TOR2* (Kunz *et al.*, 1993). pMD21 is YEplac195 with *VPS34* in a 4 kbp *Kpn*I−*Pst*I fragment (provided by M.Deuter-Reinhard).

Cloning, sequencing and physical mapping of PIK1

All procedures used were standard techniques previously described (Sambrook *et al.*, 1989). The mouse monoclonal antibody QE5 raised against nuclear envelopes from HeLa cells (McMorrow *et al.*, 1994) was used to screen a yeast genomic λgt11 expression library, with an average insert size of 2.3 kbp (provided by M.Altmann) (Altmann *et al.*, 1987). Plaques expressing the QE5 antigen were visualized using a peroxidase-conjugated rabbit anti-mouse antibody (Dakkopats, Denmark) and chemiluminescence detection (Amersham), as recommended by the manufacturer. Two out of 2×10^5 plaques screened gave a positive signal after four rounds of plaque purification. Restriction analysis of the corresponding phages revealed that the inserts were ~500 and 300 bp in size and that both contained a *Bgl*II site. DNA inserts from positive phages were recovered by PCR using standard techniques (Innis *et al.*, 1990). An isolated phage plaque was used as a template and custom oligonucleotides, complementary to the sequences flanking the *Eco*RI cloning site in λgt11, were used as primers. Sequence analysis of the PCR fragments indicated that the inserts of the two phages were overlapping sequences.

The full-length *PIK1* gene was obtained by using the ~500 bp phage insert to probe a yeast genomic library constructed in the pSEY18 vector, with an average insert size of 12 kbp (Helliwell *et al.*, 1994). Out of ~6000 colonies screened, 15 positives were initially picked but only two were retained after four rounds of replating. Both isolates had inserts of ~15 kbp with identical restriction patterns. One, named pGB1, was chosen for further analysis.

For sequencing, restriction fragments were cloned into plasmid pGEM7Zf(+) and sets of nested deletions were produced by exonuclease III digestion using the Erase-a-Base system (Promega), according to the manufacturer. Sequence gaps not accessible by the deletions were closed using custom oligonucleotides as primers. DNA sequencing was performed by the dideoxy chain termination method, using the T7 sequencing system from Pharmacia. Computer-assisted assembly and manipulation of the sequences was done using the GCG set of programs (Genetic Computer Group Inc.). Homology searches were conducted using the BLASTP and FASTA programs (Altschul *et al.*, 1990).

PIK1 was mapped physically by using the ~500 bp insert present in one of the original λgt11 clones to probe a membrane containing *S.cerevisiae* chromosomes separated by pulsed-field electrophoresis in a 1% agarose gel in a Rotaphor apparatus (Biometra). High resolution mapping was accomplished by probing a set of filters containing ordered λ and cosmid clones accounting for 99% of the *S.cerevisiae* genome (kindly provided by L.Riles, Washington University) (Riles *et al.*, 1993).

Isolation of conditional alleles of PIK1

Procedures for chemical mutagenesis and identification of mutated plasmids by the plasmid shuffle method have been described previously (Sikorski and Boeke, 1991). Briefly, plasmid pGB2 (*LEU2*) was treated *in vitro* for various times with 1 M hydroxylamine. The level of mutagenesis was assessed by testing the plasmids for complementation of the leucine auxotrophy of *E.coli* strain MH4. Plasmid preparations in which 3% of the *LEU2* genes had been inactivated were used to transform *S.cerevisiae* strain FM1-5d/pGB1 (*URA3*). Leu$^+$ transformants which were able to lose the pGB1 plasmid at 23°C but not at 37°C were identified by plating on 5-fluoroorotic acid-containing medium. Eight independent, recessive, plasmid-linked, temperature-sensitive mutants were obtained. The temperature-sensitive phenotype was expressed on both minimal and rich medium.

Cell fractionation and PI kinase assay

Strain BJ2168 was grown in SD medium to an OD_{600} of 0.4−0.6, spheroplasted, lysed by manual douncing in a hypotonic medium containing protease inhibitors and fractionated as described to purify yeast nuclei (Hurt *et al.*, 1988; Garcia-Bustos *et al.*, 1991b). The 100 000 *g* cytosolic fraction was obtained by centrifuging the postnuclear supernatant for 1 h at 106 000 *g* (average) in a TL-100 tabletop ultracentrifuge (Beckman). The PI kinase activity present in the different cellular fractions was assayed essentially as described (Whitman *et al.*, 1985), with the following modifications. Sonicated PI micelles at 0.25 mg/ml were used as substrate and the reactions were started by adding [γ-^{32}P]ATP (3 Ci/mmol) to a final concentration of 60 μM. Adenosine was added where appropriate to a final concentration of 1 mM. Incubations were stopped after 10 min at 30°C and the lipid reaction products were separated by TLC in the presence of boric acid, using Silicagel 60 plates (E.Merck) previously coated with *trans*-1,2-diaminocyclohexane-*N,N,N,N*,-tetraacetic acid as described (Walsh *et al.*, 1991). Cellular fractions assayed for PI kinase activity at different temperatures were pre-incubated for 2 min at the desired temperature (23, 30 and 37°C) and maintained at the same temperature for the duration (10 min) of the assay; otherwise all steps were the same as described above. Only two major phosphorylated lipid products are generated in the assay described above, PI-3-phosphate (PI-3-P) and PI-4-phosphate (PI-4-P) (Auger *et al.*, 1989). Their respective identities were established by analysing in parallel extracts from cells producing PI-4-P but lacking detectable PI 3-kinase activity (a disruption mutant of *VPS34* provided by S.Emr) (Schu *et al.*, 1993).

Miscellaneous methods

To probe for *PIK1* transcripts, total yeast RNA was prepared and analysed by Northern blotting as described (Sambrook *et al.*, 1989). The ^{32}P-labeled 1.2 kbp *Bgl*II fragment from the 5′ end of *PIK1* (Figure 3) was used as a probe.

Standard methods were used for immunological detection of proteins separated in SDS−polyacrylamide gels and transferred to PVDF membranes (Millipore). In all cases, detection was accomplished with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence detection.

Sensitivity to α-factor was tested on plates containing 20 ml of 2% agar YPD, which were overlayed with 4 ml of 0.7% agar YPD containing 50 μl of overnight cultures of the appropriate yeast strains adjusted to an OD_{600} of 3.0. α-factor (provided by E.Kübler) was serially diluted and 10 μl of each dilution were spotted onto sterile filter disks placed onto the soft agar overlay. The plates were incubated at 30°C for 24 h and the diameter of the inhibition zones around the paper disks was measured.

Flow cytometry (FACS analysis) was performed on 300 μl of cells chilled on ice, sonicated for 2 min in a water bath sonicator and diluted with 700 μl cold absolute ethanol. After shaking overnight at 4°C the cells were centrifuged briefly, washed once in 50 mM sodium citrate pH 7.4, and resuspended in 500 μl 50 mM sodium citrate pH 7.4 containing RNase A at 0.25 mg/ml. After incubating 1 h at 37°C, 500 μl of 50 mM sodium citrate and 16 μg/ml propidium iodide were added. A Becton-Dickinson FACScan machine was used to measure fluorescence; 10 000 events were analysed for each strain.

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