phate with the Amersham multiprime DNA labeling system and purified with an Elutip (Schleicher & Schuell). Hybridization was performed in 50% formamide, 0.5% sodium dodecyl sulfate (SDS), 0.8 M NaCl, 20 mM Pipes (pH 6.5), and salmon sperm DNA (100 μg/ml) at 42°C. Filters were washed with 1 × standard sodium citrate (SSC) (150 mM NaCl, 15 mM sodium citrate) and 0.1% SDS at room temperature for 15 min four times, then with 0.1 × SSC and 0.1% SDS at 52°C for 30 min. The pBluescript SK⁻ plasmid containing the cDNA inserts that remained positive after two rounds of plaque purification were released from the lambda Zap II phagemid (Stratagene) by in vivo excision with the helper phage R408 as described by the manufacturer. The cDNA inserts were sequenced with the dideoxynucleotide chain termination method and a Sequenase kit (U.S. Biochemical). Both strands of the clones were sequenced with T3 and T7 primers as well as synthetic oligonucleotide primers deduced from the partially determined sequence and selected at convenient intervals. Sequences were aligned and analyzed with the EUGENE and SAM programs (Molecular Biology Computing Research Resource, Dana Farber Cancer Institute and Harvard School of Public Health, Boston, MA).

30. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
31. The filter binding assay was done as described [C. F. Albright, B. W. Giddings, J. Liu, M. Vito, R. A. Weinberg, *EMBO J.* **12**, 339 (1993)]. GST-Rad (20 pmol) or GST (20 pmol) was incubated with [α-^{32}P]-GTP (100 pmol, 3000 Ci/mmol) at room temperature in a solution containing 50 mM tris (pH 7.5), 5 mM $MgCl_2$, 10 mM EDTA, 1 mM dithiothreitol (DTT), and bovine serum albumin (BSA; 1 mg/ml). At the indicated times, 50 μl of the reaction mixture was removed, and the reaction was quenched with 50 μl of ice-cold buffer containing 50 mM tris (pH 7.5), 20 mM $MgCl_2$, 1 mM DTT, and BSA (1 mg/ml). Samples were brought to 500 μl with 50 mM tris (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT and filtered through nitrocellulose (BA85, Schleicher & Schuell). Filters were washed with 10 ml of the same solution, and the radioactivity remaining on the filters was determined.
32. Muscle samples were obtained at surgery from humans undergoing amputation above or below the knee for peripheral vascular disease or trauma. All samples were dissected from the viable margin of either the gastrocnemius or quadriceps muscle, immediately frozen in liquid nitrogen, and extracted for RNA with the RNAzol method (BIOTECX Laboratories, Friendswood, TX). Total RNA was then fractionated on a 1% agarose-formaldehyde gel, transferred onto nylon membrane (ICN) in 20 × SSC for 20 hours and cross-linked with ultraviolet light. The Rad cDNA insert, purified on an agarose gel and ^{32}P-labeled as described in (*29*), was hybridized (4 × 10^6 cpm/ml) to the RNA blots at 42°C in 50 mM Pipes (pH 6.5), 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, 5% SDS, and salmon sperm DNA (100 μg/ml) for at least 24 hours. Filters were washed with 5% SDS and 0.5 × SSC at room temperature for 15 min, twice at 65°C for 15 min, and then for 15 min with 0.1% SDS and 0.2 × SSC at 65°C. Filters were exposed on Kodak X-Omat film with an intensifying screen at 70°C.
33. We thank L. Michaelowsky, R. Taub, R. Johnson, K. Claffey, C. Albright, X.-J. Sun, and E. Araki for their many helpful discussions and useful suggestions; W. G. Kaelin Jr. for providing pGEX-2TK vector; Physicians of the New England Deaconness Hospital for making muscle samples available; and especially Dr. Bruce Spiegelman for his support and advice during the initial phases of this work. Supported by grants from the Juvenile Diabetes Foundation (C.R.), the Joslin Diabetes and Endocrinology Research Center (DK 36836), the Marilyn Simpson Family Trust (C.R.K.), and the Mary K. Iacocca Professorship (C.R.K.).

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Phosphatidylinositol 4-Kinase: Gene Structure and Requirement for Yeast Cell Viability

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Phosphatidylinositol (PtdIns) 4-kinase catalyzes the first step in the biosynthesis of PtdIns-4,5-*bis*phosphate (PtdIns[4,5]P_2). Hydrolysis of PtdIns[4,5]P_2 in response to extracellular stimuli is thought to initiate intracellular signaling cascades that modulate cell proliferation and differentiation. The *PIK1* gene encoding a PtdIns 4-kinase from the yeast *Saccharomyces cerevisiae* was isolated by polymerase chain reaction (PCR) with oligonucleotides based on the sequence of peptides derived from the purified enzyme. The sequence of the *PIK1* gene product bears similarities to that of PtdIns 3-kinases from mammals (p110) and yeast (Vps34p). Expression of *PIK1* from a multicopy plasmid elevated PtdIns 4-kinase activity and enhanced the response to mating pheromone. A *pik1* null mutant was inviable, indicating that PtdIns4P and presumably PtdIns[4,5]P_2 are indispensable phospholipids.

Turnover of PtdIns[4,5]P_2 is a cellular response to diverse agonists in many different cell types and is thought to be an important signal transduction mechanism (*1*). Hydrolysis of PtdIns[4,5]P_2 by several different classes of receptor-activated phospholipase C enzymes (*2*) produces two intracellular second messengers—inositol 1,4,5-*tris*phosphate, which promotes release of Ca^{2+} from intracellular stores, and diacylglycerol, which can activate various protein kinase C isotypes (*3*). It is important to clarify the contribution of PtdIns[4,5]P_2 turnover to cellular responses because receptor-mediated stimulation of PtdIns[4,5]P_2 hydrolysis occurs simultaneously with activation of other signaling pathways (*4*). Also, PtdIns[4,5]P_2 is thought to be a regulator of the activity of certain actin-binding proteins (*5*). Given these roles, synthesis of PtdIns[4,5]P_2 is likely to be as stringently controlled as its cleavage. The first committed step in biosynthesis of PtdIns[4,5]P_2 is catalyzed by PtdIns 4-kinase.

At least two different mammalian PtdIns 4-kinases have been partially characterized (*6*). One enzyme (type II) is a membrane-bound 55-kD protein whose activity is inhibited by adenosine; the other (type III) is also membrane-bound, but has a molecular size of ~200 kD and is not inhibited by adenosine (*7*). Little is known, however, about the structure, localization, or regulation of these enzymes. In fact, a mammalia complementary DNA (cDNA) thought to encode a PtdIns 4-kinase (*8*) actually encodes a long-chain fatty acyl–CoA ligase (*9*). Previous studies suggested that multiple forms of PtdIns 4-kinase exist in *Saccharomyces cerevisiae* (*10*), and two different membrane-bound PtdIns 4-kinases of 45- and 55-kD have been reported from this yeast (*11*). We identified and purified a 125-kD PtdIns 4-kinase (p125) from the soluble fraction of *S. cerevisiae* cell extracts (*12*). Here we report the isolation and characterization of the gene encoding this enzyme.

Peptides from p125 were generated in sufficiently high yield for microsequencing (*13*). One 21-residue sequence obtained (Fig. 1A) was used to design degenerate oligonucleotides for cloning by polymerase chain reaction (PCR) (*14*). Reactions containing yeast genomic DNA and primers 2 and 3 generated a single product, which was authentic on the basis of several criteria. First, it was obtained only when primer 2 (and not primer 1) was used in combination with primer 3 and only when template DNA was also provided, indicating a specific requirement for the correct Arg codon to amplify a product from genomic DNA. Second, its length [50 base pairs (bp)] was precisely that predicted from the peptide sequence. Third, after labeling with [γ-^{32}P]-adenosine triphosphate (ATP) and polynucleotide kinase, a fourth set of oligonucleotides (primer 4), which corresponded to the least ambiguous region of the peptide sequence (residues 9 to 15) (Fig. 1A), hybridized specifically to the 50-bp PCR product

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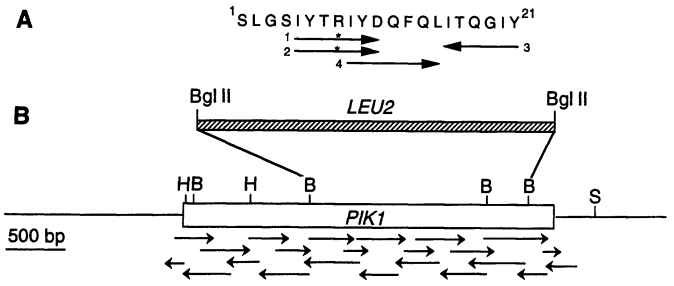

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1          10         20         30         40         50         60         70
MHKASSSKKSFDDTIELKKNEQLLKLINSSEFTLHNCVELLCKHSENIGIHYYLCQKLATFPHSELQFYI

          80         90        100        110        120        130        140
PQLVQVLVTMETESMALEDLLLRLRAENPHFALLTFWQLQALLTDLSTDPASYGFQVARRVLNNLQTNLF

         150        160        170        180        190        200        210
NTSSGSDKNVKIHENVAPALVLSSMIMSAIAFPQLSEVTKPLVESQGRRQKAFVFKLARSAMKDFTKNMT

         220        230        240        250        260        270        280
LKNTLLNKKTSRSKRVSSNRSSTPTSPIDLIDPIKTKEDASFRKSRHSEVKLDFDIVDDIGNQVFEERIS

         290        300        310        320        330        340        350
SSIKLPKRKPKYLDNSYVHRTYDGKNINRDGSISNTAKALDGNKGDYISPKGRNDENNEIGNNEDETGGE

         360        370        380        390        400        410        420
TEEDADALNSDHFTSSMPDLHNIQPRTSSASSASLEGTPKLNRTNSQPLSRQAFKNSKKANSSLSQEIDL

         430        440        450        460        470        480        490
SQLSTTSKIKMLKANYFRCETQFAIALETISQRLARVPTEARLSALRAELFLLNRDLPAEVDIPTLLPPN

         500        510        520        530        540        550        560
KKGKLHKLVTITANEAQVLNSAEKVPYLLLIEYLRDEFDFDPTSETNERLLKKISGNQGGLIFDLNYMNR

         570        580        590        600        610        620        630
KENNENRNESTLTSNNTRSSVYDSNSFNNGASRNEGLSSTSRSDSASTAHVRTEVNKEEDLGDMSMVKVR

         640        650        660        670        680        690        700
NRTDDEAYRNALVIQSAANVPILPDDSQDRSPELNFGSNLDEVLIENGINSKNIHSQTDALADQMRVSAV

         710        720        730        740        750        760        770
MLAQLDKSPQQLSESTKQIRAQIISSMKEVQDKFGYHDLEALHGMAGERKLENDLMTGGIDTSYLGEDWA

         780        790        800        810        820        830        840
TKKERIRKTSEYGHFENWDLCSVIAKTGDDLRQEAFAYQMIQAMANIWVKEKVDVWVKRMKILITSANTG

         850        860        870        880        890        900        910
LVETITNAMSVHSIKKALTKKMIEDAELDDKGGIASLNDHFLRAFGNPNGFKYRRAQDNFASSLAAYSVI

         920        930        940        950        960        970        980
CYLLQVKDRHNGNIMIDNEGHVSHIDFGFMLSNSPGSVGFEAAPFKLTYEYIELLGGVEGEAFKKFVELT

         990       1000       1010       1020       1030       1040       1050
KSSFKALRKYADQIVSMCEIMQKDNMQPCFDAGEQTSVQLRQRFQLDLSEKEVDDFVENFLIGKSLGSIY

        1060
TRIYDQFQLITQGIYS.
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Fig. 1. Cloning, disruption, and sequence analysis of the *S. cerevisiae PIK1* gene. (**A**) PCR strategy used to generate the hybridization probe for isolation of *PIK1*. Sequence of a 21-residue peptide derived (*13*) from purified p125 (*12, 15*) and four sets of corresponding degenerate oligonucleotides (primers 1 to 4) (*14*) (length and orientation shown by arrows). (**B**) Gene disruption. Sequence (*16*) of the *PIK1* open reading frame (open box) was determined (directions and lengths shown by arrows). The indicated region was deleted and replaced by the *LEU2* gene (hatched box) (*31*). B, BclI; H, HindIII; and S, SacI. (**C**) Deduced amino acid sequence. Residues corresponding to peptides obtained from purified p125 are underlined; peptide segments used to design degenerate oligonucleotides for primers or hybridization probes are shown in italic (*39*).

blotted to a nitrocellulose filter (*15*).

The 50-bp product was labeled by the random primer method and used to screen by hybridization bacterial colonies containing a yeast genomic DNA library in a plasmid vector (*14*). A single clone with an insert of ~10 kb was isolated. Restriction fragments from the insert hybridized to oligonucleotides that corresponded to portions of two other peptide sequences (residues 31 to 39 and residues 297 to 304) (Fig. 1C) derived from p125 (*15*). The nucleotide sequence (*16*) spanning this entire region was determined (Fig. 1B) and comprised a continuous open reading frame that lacked any obvious introns. The predicted sequence contained perfect matches to 13 separate peptides (Fig. 1C) obtained by proteolytic cleavage of purified p125 (with endoproteinase Lys-C or trypsin) from two independently derived preparations (*12, 15*). Thus, the gene isolated corresponded to the enzyme that was purified, and was designated *PIK1* (for phosphatidylinositol 4-kinase).

The calculated molecular weight of the predicted 1066-residue Pik1p is 119,929, in good agreement with the apparent mass (125 kD) of the purified enzyme (*12, 15*). Pik1p contains a large number of charged and polar residues. Hydropathy analysis showed that there are no hydrophobic stretches sufficiently long to span a membrane bilayer. Thus, Pik1p is predicted to be a soluble protein, consistent with its fractionation behavior in vitro (*12, 15*). Comparison with sequences in available databases (*16*) showed similarity between Pik1p and two PtdIns 3-kinases, mammalian p110 (*17*) and yeast Vps34p (*18*). Similarities between Pik1p and the other two proteins are largely confined to two discrete domains (Fig. 2A).

The 274-residue COOH-terminal segment of Pik1p shares 30% identity with Vps34p and 29% identity with p110 (Fig. 2C). If conservative amino acid replacements are allowed, similarity among all three proteins exceeds 50% in this domain. This degree of similarity is at least as great

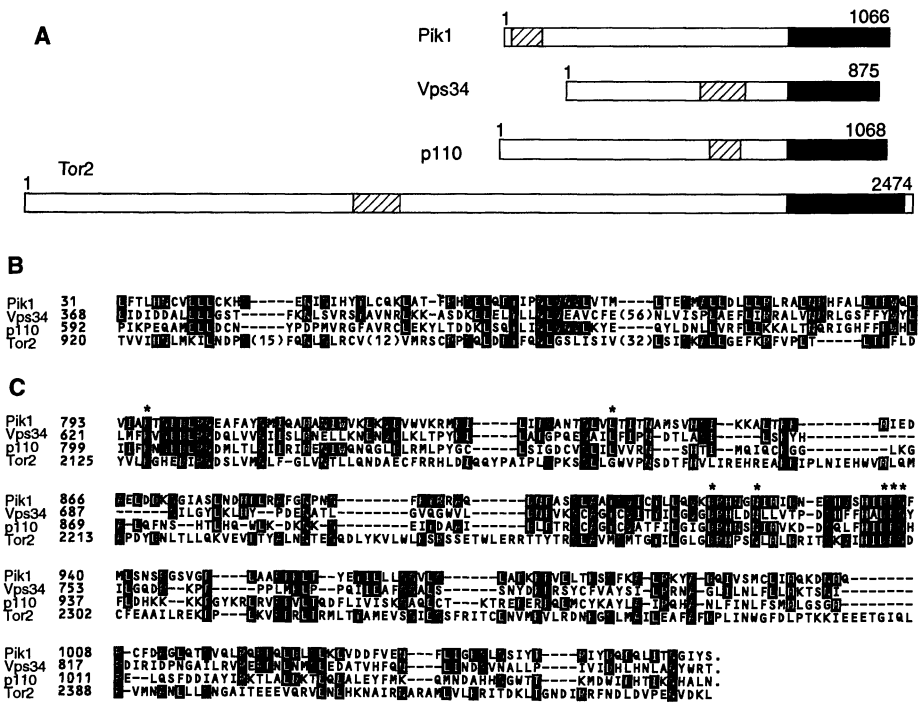


Fig. 2. Similiarity of Pik1p to PtdIns 3-kinases. (**A**) Positions of the two domains of highest similarity (hatched box and solid box) between Pik1p and *S. cerevisiae* Vps34p (*18*), bovine p110 (*17*), and *S. cerevisiae* Tor2p (*22*). Number above each bar is the length (in residues) of each protein. (**B**) NH_2-terminal domain of similarity [hatched box in (A)]. (**C**) Presumptive catalytic domain [solid box in (A)]. Residues (*39*) identical in Pik1p and one or more of the other three proteins are given as white-on-black letters. Hyphens are one-residue gaps introduced to maximize the alignment; numbers in parentheses are inserts of the indicated length. Asterisks show residues in these lipid kinases that are conserved in order and spacing in protein kinases.

as the relatedness among sugar kinases (*19*). Certain residues in this region (Lys^{796}, Glu^{843}, Asp^{918}, Asn^{923}, Asp^{936}, Phe^{937}, and Gly^{938}) (Fig. 2C) are nearly identical in order and spacing to residues that are highly conserved in protein kinases and are thought to be involved either in binding of ATP or in catalysis (*20*). The COOH-terminal segment of similarity is likely to comprise the catalytic domain in each of these enzymes (*21*).

The second region of similarity between Pik1p and the two PtdIns 3-kinases (24 and 25% identity, respectively) is situated nearer to the NH_2-terminal end of each protein (Fig. 2A); Vps34p (but not p110) contains a large insert within this region (Fig. 2B). Tor2p, another yeast protein, was presumed to be a PtdIns 3-kinase because of its similarity to Vps34p (*22*). However, Tor2p shares as much identity with Pik1p in both the COOH-terminal and NH_2-proximal domains as it does with Vps34p and p110 (Fig. 2).

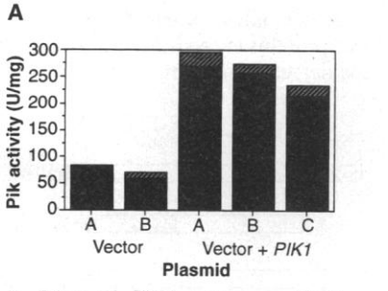
To confirm that *PIK1* encodes the enzyme we purified, we inserted the *PIK1*-containing insert into a multicopy plasmid and introduced it by DNA-mediated transformation into yeast cells. Typically, increasing the dosage of a structural gene in yeast leads to overproduction of its protein product (*23*). Yeast cells carrying the *PIK1*-containing multicopy plasmid produced five times more soluble PtdIns 4-kinase activity than the same cells carrying the vector alone (Fig. 3A).

Mutations that prevent PtdIns synthesis in *S. cerevisiae*, such as *ino1* (*myo*-inositol 1-phosphate synthase-deficient) (*24*) or *pis1* (PtdIns synthase–deficient) (*25*), are lethal, indicating that PtdIns is an essential phospholipid (*26*). In addition to serving as a component of yeast cell membranes, PtdIns also is required for synthesis of PtdIns-containing sphingolipids (*27*), for attachment of certain membrane proteins by means of PtdIns-linked glycan anchors (*28*), and as the precursor both of PtdIns4P and its derivative, PtdIns[4,5]P_2, and of PtdIns3P and its derivatives (*29*). PtdIns3P phospholipids are not cleaved by phospholipase C and serve in a different signal transduction pathway (*30*). Cloning of the *PIK1* gene allowed us to assess the cellular requirement for PtdIns4P and PtdIns[4,5]P_2 per se. For this purpose, a *pik1* null mutation (*pik1Δ1::LEU2*) was constructed (Fig. 1B) and used to transform a *leu2/leu2* diploid recipient strain (*31*). After sporulation of the resulting heterozygous *pik1Δ1::LEU2/PIK1* diploids, ascus dissection revealed that spore viability segregated 2:2 in every tetrad examined (on rich medium with or without 1 M sorbitol) (Fig. 3B) and that all of the viable spores were Leu^-, indicating that *PIK1* is an essential gene. In contrast to the unconditional lethality of the *pik1* null mutation, cells carrying a *vps34* null mutation can survive at 30°C, but are inviable at higher temperatures (*18*).

When the heterozygous *pik1Δ1::LEU2/PIK1* diploids also harbored a centromere-containing plasmid carrying the *PIK1* gene and the *HIS3* gene, viable Leu^+His^+ spores were readily recovered, confirming that the *pik1Δ1::LEU2* allele is a typical recessive mutation. Overexpression of *VPS34* from a multicopy plasmid was unable to rescue the lethality of the *pik1Δ1::LEU2* mutation (*32*).

The simplest interpretation of the lethality of the *pik1* null mutation is that the product of the reaction catalyzed by Pik1p, namely PtdIns4P, is an indispensable cellular intermediate. Because PtdIns4P is converted (via phosphorylation by PtdIns4P 5-kinase) to PtdIns[4,5]P_2 in a single step, the inviability of the *pik1* mutant suggests that yeast cells require PtdIns[4,5]P_2 for their survival. PtdIns4P and PtdIns[4,5]P_2 may be essential for viability of yeast cells because agonist-stimulated hydrolysis of PtdIns[4,5]P_2 is necessary to generate signals needed for some aspect of yeast cell growth or cell division, or both. Indeed, a PtdIns[4,5]P_2-specific phospholipase C (Plc1p) has been isolated from *S. cerevisiae* that closely resembles in sequence and biochemical properties the PLC δ isotypes found in animal cells (*33*). Moreover, the genes for components capable of propagating the signals generated by PtdIns[4,5]P_2 turnover, including a protein kinase C, calmodulin, and several calmodulin-regulated enzymes, also exist in yeast and in some cases are vital for cell survival (*34*).

Because PtdIns4P and PtdIns[4,5]P_2 bind to and modulate the activity of profilin and other actin-binding proteins in vitro (*5*), loss of PtdIns4P and PtdIns[4,5]P_2 in yeast may prevent proper coordination between signaling at the plasma membrane and reorganization of the cytoskeleton. In this regard, a yeast gene (*PFY1*) encoding a profilin has been shown to be essential (*35*).



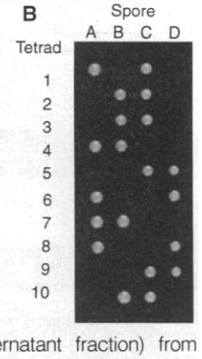


Fig. 3. Effect of *PIK1* overexpression and *PIK1* loss of function. (**A**) Overproduction of soluble PtdIns 4-kinase activity. The 10-kb insert containing *PIK1* was inserted into a multicopy (2-μM DNA-containing) vector, a derivative of YEp352 in which *URA3* was replaced by *TRP1* (*15*), and introduced into yeast strain CFY13 (*12*). Extracts (100,000*g* supernatant fraction) from three independently isolated transformants and from two control transformants carrying the vector alone were assayed for PtdIns 4-kinase activity (*12*). A unit was defined as 1 pmol of PtdIns 4-P formed per min; protein concentration was determined by standard methods. Specific activity (solid bar) is given as the average of three separate determinations done with three different amounts of each extract (standard deviation of each mean is the hatched bar). (**B**) *PIK1* is an essential gene. The *pik1Δ1::LEU2* null mutation (Fig. 1B) (*31*) was introduced by one-step gene transplacement (*40*) into an appropriate recipient. A resulting *pik1Δ1::LEU2/PIK1* diploid was subjected to sporulation and ascus dissection.

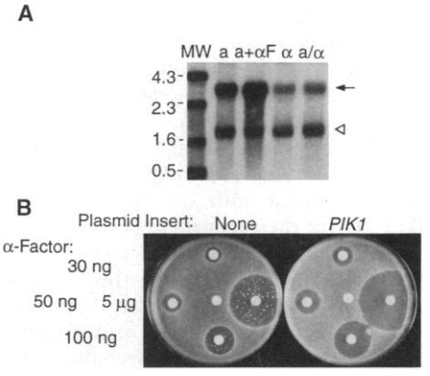


Fig. 4. *PIK1* expression and mating pheromone response. (**A**) *PIK1* mRNA (arrow). Poly(A)$^+$ RNA from *MAT***a** cells (lane **a**), *MAT***a** cells treated for 30 min with 10^{-7} M α-factor (lane **a**+αF), *MAT*α cells (lane α), and *MAT***a**/*MAT*α diploids (lane **a**/α) was fractionated by agarose gel electrophoresis, transferred to a filter, and hybridized (*14*) in 6× SSPE, 5× Denhardt's solution, 0.5% SDS, and carrier DNA (0.1 mg/ml) at 65°C to a *PIK1*-specific probe (*31*). Quantitation by densitometry of a control mRNA (triangle), corresponding to an unknown gene adjacent to *CNA1* (*34*) and not regulated by pheromone (*41*), indicated that lanes **a** and **a**/α had approximately equivalent amounts of RNA, whereas the **a**+αF and α lanes had about half as much RNA. Markers (MW) were denatured fragments of pBR322. (**B**) Increased apparent sensitivity to pheromone-induced growth arrest. Strain DK499 (MAT**a** *sst1*) carrying either a multicopy plasmid containing *PIK1* (Fig. 3A) or empty vector was tested for sensitivity to the growth-inhibitory action of the mating pheromone α-factor (*36*) by agar diffusion bioassay (*37*), with the amounts of pheromone indicated. The *sst1* mutation prevents degradation of α-factor by *MAT***a** cells (*36*), so pheromone concentration remains constant during the bioassay.

One extracellular stimulus in yeast that initiates a signal transduction cascade and results in remodeling of the cytoskeleton is the peptide mating pheromone, α-factor (36). Expression of many of the genes involved in the pheromone response is pheromone-inducible. Analysis of polyadenylated [poly(A)$^+$] RNA demonstrated that the 3.9-kb *PIK1* transcript is expressed in all three yeast cell types (*MAT***a** and *MAT*α haploids and *MAT***a**/*MAT*α diploids), but is present in amounts at least three times higher in *MAT***a** haploids exposed to α-factor for 30 min than in cells not treated with pheromone (Fig. 4A). We used a bioassay (37) to examine the effect of *PIK1* overexpression on the ability of cells to respond to and recover from pheromone. As compared with cells carrying vector alone, cells overproducing Pik1p appeared to be more sensitive to the growth-inhibitory effects of pheromone (Fig. 4B). This result suggests that in cells overproducing this PtdIns 4-kinase, the signal generated in response to pheromone is more efficient or more sustained (or both), despite a report that ^{32}P incorporated into PtdIns4P and PtdIns[4,5]P_2 showed no detectable turnover after pheromone treatment (38).

The relation between Pik1p and the 45- and 55-kD PtdIns 4-kinase activities in yeast (11) is unclear. Perhaps these species represent proteolytic fragments of p125. If the 45- and 55-kD proteins are distinct from Pik1p, these enzymes cannot substitute for Pik1p, because *pik1* null mutations are lethal. If multiple PtdIns 4-kinases exist in yeast, each may be specialized, perhaps through targeting to a particular subcellular compartment or through specific mechanisms for regulation of expression or activity, so that different pools of PtdIns[4,5]P_2 can be generated that may have different functions in cellular physiology.

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13. Purified PtdIns 4-kinase (~50 μg) (12) was denatured and reduced by incubation at 50°C for 15 min in the presence of 2% SDS and 5 mM dithiothreitol. After cooling to ambient temperature, the Cys residues were alkylated by incubation for 15 min in the presence of 35 mM iodoacetic acid. The sample was then subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) in a 5% gel. The region of the gel containing p125 was excised, crushed in a tissue homogenizer, and eluted by incubation for 12 hours in 50 mM Na-PO_4 (pH 7.8), 10 mM NH_4HCO_3, and 0.02% SDS. Acrylamide pieces were removed by filtration of the mixture through glass wool in a microcolumn. Endoproteinase Lys-C (50 ng) was added to the clarified eluate, and the mixture was incubated at 37°C for 12 hours, after which a second portion (50 ng) of the proteinase was added and incubation was continued for another 12 hours. Complete digestion was verified by comparison of samples after SDS-PAGE in a 5 to 20% gradient gel. The proteolyzed sample was injected into a C_4 reversed-phase column (300 Å, 2.1 by 150 mm, Vydac) on a microbore high-pressure liquid chromatography (HPLC) apparatus (Applied Biosystems 130A). Peptides were eluted with a gradient from 4.5 to 90% acetonitrile in 0.1% trifluoroacetic acid, 1% isopropanol. Sequence analysis of peptides present in well-resolved peaks was done by sequential Edman degradation with an automated gas phase sequenator (Applied Biosystems 477A) coupled online to another microbore HPLC system (Applied Biosystems 120A).
14. Two sets of mixed-sequence sense primers [20 nucleotides (nt) long with one inosine at position 9] were synthesized corresponding to the codons specifying the NH_2-terminal portion (residues 5 to 11) of the 21-residue peptide. One set (288-fold degenerate) contained the four possible CGX Arg codons (where X is any base) (primer 1), whereas the other set (144-fold degenerate) contained the two alternative AG(A/G) Arg codons (primer 2). A 144-fold degenerate antisense primer (18 nt long with one inosine at position 7) was synthesized corresponding to the complement of the codons specifying the COOH-terminal portion (residues 16 to 21) of the peptide (primer 3). Parallel PCR reactions (50 μl final volume) were done under mineral oil with Ampli-Taq DNA polymerase and reaction buffer supplied by the manufacturer (Perkin-Elmer–Cetus) and contained either primer 1 or primer 2 paired with primer 3 (2 nmol each), with yeast genomic DNA (50 ng) from strain YPH499 [R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989)] as the template. For controls, one or more of these components was omitted. Amplification was done in a thermal cycler (Eppendorf, Microcycler E) for 35 cycles according to the following program: denaturation, 94°C (1 min); annealing, 40°C (2 min); and synthesis, 72°C (0.5 min). Reaction products were analyzed by PAGE in a 10% gel. The specific 50-bp PCR product was eluted from a crushed gel slice, labeled by random priming (with both [α-^{32}P]deoxyadenosine triphosphate and [α-^{32}P]deoxycytidine triphosphate) [A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983)], and used to screen bacterial colonies containing a library (insert size 10 to 12 kb), generated by partial digestion with Sau 3A of genomic DNA from *S. cerevisiae* strain S288C, in the centromere vector, pSB32, which is a derivative of YCp50 [M. D. Rose, P. Novick, J. H. Thomas, D. Botstein, G. R. Fink, *Gene* **60**, 237 (1987)] in which the *URA3* gene is replaced by *LEU2*. Hybridization was done at 45°C in 4× hybridization buffer [saline-sodium phosphate-EDTA (SSPE); J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989)] containing 2× Denhardt's solution, 0.1% SDS, and carrier DNA (0.1 mg/ml).
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16. DNA sequence determination was done by the dideoxy chain termination method with the Sequenase DNA sequencing kit (U.S. Biochemicals) according to the manufacturer's instructions. The entire coding sequence was determined on both strands with a combination of subclones, exonuclease III–generated deletions, and custom-made primers. Any areas of ambiguity were resolved by re-sequencing with Taq DNA polymerase (Perkin-Elmer–Cetus) at high temperature. Computer analysis of the *PIK1* DNA sequence was done with programs supplied by IntelliGenetics, Inc. (Palo Alto, California), and sequence comparisons were done with the FASTP and BLAST algorithms [S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, *J. Mol. Biol.* **215**, 403 (1990)] provided by the National Center for Biotechnology Information of the National Library of Medicine. The DNA sequence of *PIK1* has been deposited in GenBank (accession number L20220).
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31. A 1.9-kb Bcl I–Bcl I fragment encoding two-thirds of the *PIK1* sequence (including the presumed catalytic domain) was replaced with a 3.1-kb Bgl II–Bgl II fragment containing the *LEU2* gene (Fig. 1B). This construction (*pik1Δ1::LEU2*) was excised by digestion with Hind III and Sac I, and the resulting linear fragment was used to transform a *leu2/leu2* diploid recipient (YPH501). Successful production of heterozygous *pik1Δ1::LEU2/PIK1* diploids was confirmed by restriction and hybridization analysis. The *PIK1* locus was assigned to chromosome 14 by hybridization at 65°C of a 2.3-kb–labeled probe [generated by PCR with the original plasmid isolate as the template, primer 3 (14) (Fig. 1A) as the antisense primer, and an oligonucleotide corresponding to codons 297 to 304 (Fig. 1C) as the sense primer] to a blot of intact yeast chromosomes separated by pulsed-field gel electrophoresis. This assignment was

confirmed by hybridization of the same chromosome to a probe (*KEX2*) known to reside on chromosome 14 and by hybridization of a different nearby band to a probe (*CYC1*) known to reside on chromosome 10.
32. Multicopy plasmid pPHY52 (*18*), which carries the *VPS34* gene and a *URA3* marker, was introduced into the *pik1Δ1::LEU2/PIK1* heterozygous diploid strain, which was then subjected to sporulation. No Leu$^+$Ura$^+$ spores were recovered from 20 tetrads dissected. Conversely, neither of two multicopy plasmids carrying the *PIK1* gene, YEp-*PIK1/URA3* and YEp*PIK1/TRP1* (*15*), rescued the temperature-sensitive lethality of a *vps34* mutant [J. H. Stack and S. D. Emr, personal communication].
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39. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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42. We thank G. Anderson for the chromosome blot; P. Hieter and F. Spencer for the yeast genomic library in the centromere vector, pSB32; M. Moore and J. Onuffer for oligonucleotides; and J. Brabson and J. Flick for careful reading of the manuscript. Supported by NIH predoctoral traineeships GM07232 and CA09041 (to C.A.F. and E.A.S.); by a salary from the Howard Hughes Medical Institute (to A.A.); by NIH research grant GM21841 (to J.T.); by funds provided from the Lucille P. Markey Charitable Trust through the Berkeley campus Program in Biomolecular Structure and Design; and by facilities provided by the Berkeley campus Cancer Research Laboratory.

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The Influence of Antigen Organization on B Cell Responsiveness

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The influence of antigen epitope density and order on B cell induction and antibody production was assessed with the glycoprotein of vesicular stomatitis virus serotype Indiana [VSV-G (IND)]. VSV-G (IND) can be found in a highly repetitive form in the envelope of VSV-IND and in a poorly organized form on the surface of infected cells. In VSV-G (IND) transgenic mice, B cells were unresponsive to the poorly organized VSV-G (IND) present as self antigen but responded promptly to the same antigen presented in the highly organized form. Thus, antigen organization influences B cell tolerance.

Several mechanisms to prevent autoantibody production have been proposed: autoreactive B cells may be clonally deleted (*1*), functionally silenced (*2–5*), or they may simply ignore self antigen (*6–8*). It is not clear why some self antigens are ignored and others lead to clonal deletion of B cells or induce B cell anergy. Possibly, membrane-bound self antigens [for example H-2K^k (*1*), CD8 (*9*), and membrane-associated hen egg lysozyme (*10*)] induce clonal deletion, whereas soluble hen egg lysozyme (*2*) or single-stranded DNA (*4*) usually induce clonal anergy. These findings have been tentatively correlated with receptor cross-linking (*11–13*) and obviously impinge on the old debate regarding the role of density and the order of antigenic epitopes for the induction of B cells (*14*).

The antibody response to vesicular stomatitis virus [VSV serotype Indiana (VSV-IND)] has been analyzed extensively. All antibodies that neutralize VSV-IND infectivity in vitro bind to the glycoprotein VSV-G (IND) (*15, 16*). The early response (days 3 to 5) is of immunoglobulin M (IgM) isotype and can be induced in the absence of T cells (*17*), whereas the switch to IgG (days 6 to 8) is dependent on help from T cells (*18*). In addition, VSV has no mitogenic effects on B cells in vivo or in vitro (<30 μg/ml) (*19*).

To determine whether neutralizing IgM antibodies were induced predominantly by VSV-G (IND) in a highly organized form, as found in the envelope of the viral particle, or after fusion of virus and infection of cells, we performed the following experiment. Peritoneal macrophages (1×10^7) were infected with VSV-IND particles that had been inactivated with ultraviolet (UV) radiation (*20*). UV-inactivated VSV-IND infects cells to the extent that viral antigens are detectable by immunofluorescence on the cell surface but no progeny are produced. Separate groups of mice were injected with either the supernatant containing unadsorbed UV-inactivated virus or with abortively infected cells (*20*). Injected cells were barely able to induce neutralizing IgM antibodies, whereas the supernatant containing unadsorbed viral particles induced a considerable response (Fig. 1A). VSV-IND particles inactivated with formaldehyde, which apparently fail to fuse with target cells (*20*), also induced a good response (Fig. 1B). Thus, inactivated VSV-IND particles that displayed highly organized VSV-G (IND) were alone able to induce a T cell–independent IgM response, apparently even better than poorly organized VSV-G (IND) on infected cells (Fig. 1, A and B).

To evaluate the influence of antigen organization on B cell tolerance, we generated transgenic mice expressing VSV-G (IND) under the control of the *H-2K^b* promotor (KINDG mice). The VSV-G (IND)–specif-

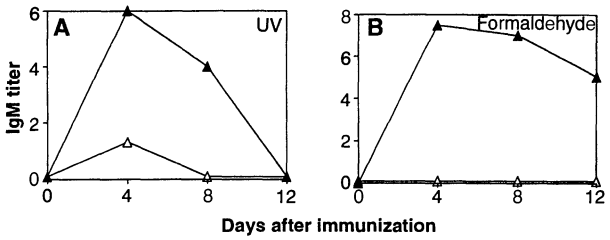

Fig. 1. More efficient induction of B cells by VSV-IND virions than by VSV-G (IND) expressed on infected cells. Peritoneal macrophages were incubated with VSV-IND inactivated by either (**A**) UV or (**B**) formaldehyde. Supernatant containing unadsorbed virus (closed triangles) or 10^7 virus-adsorbing cells (open triangles) were injected into the spleens of ICR mice (*20*), and neutralizing IgM antibody titers (*34*) were determined thereafter (*35*). Mean neutralizing IgM titers of three mice are shown; individual titers differed less than ± 1 dilution step of 2. Titers are indicated as $\log_2$ of 40-fold prediluted sera.

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