

Rad: A Member of the Ras Family Overexpressed in Muscle of Type II Diabetic Humans

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To identify the gene or genes associated with insulin resistance in Type II (non-insulin-dependent) diabetes mellitus, subtraction libraries were prepared from skeletal muscle of normal and diabetic humans and screened with subtracted probes. Only one clone out of 4000 was selectively overexpressed in Type II diabetic muscle as compared to muscle of non-diabetic or Type I diabetic individuals. This clone encoded a new 29-kilodalton member of the Ras-guanosine triphosphatase superfamily and was termed Rad (Ras associated with diabetes). Messenger ribonucleic acid of Rad was expressed primarily in skeletal and cardiac muscle and was increased an average of 8.6-fold in the muscle of Type II diabetics as compared to normal individuals.

Non-insulin-dependent diabetes mellitus (NIDDM, or Type II diabetes) is among the most common metabolic disorders, affecting up to 6% of the population of the United States (1). The high incidence of diabetes in certain populations and among first-degree relatives of Type II diabetic patients, as well as the high concordance in identical twins, provide strong evidence that genetic factors underlie susceptibility to this disease (1, 2). Although defects in both insulin secretion and insulin action may be necessary for disease expression, in groups with a high incidence of NIDDM, offspring of Type II diabetic parents and Pima Indians, insulin resistance and decreased glucose disposal have been shown to precede and predict the onset of diabetes (3, 4). In these populations, there is also evidence of familial clustering of insulin sensitivity (4, 5). Thus, insulin resistance appears to be a central feature of Type II diabetes and may be an early and inherited marker of the NIDDM phenotype.

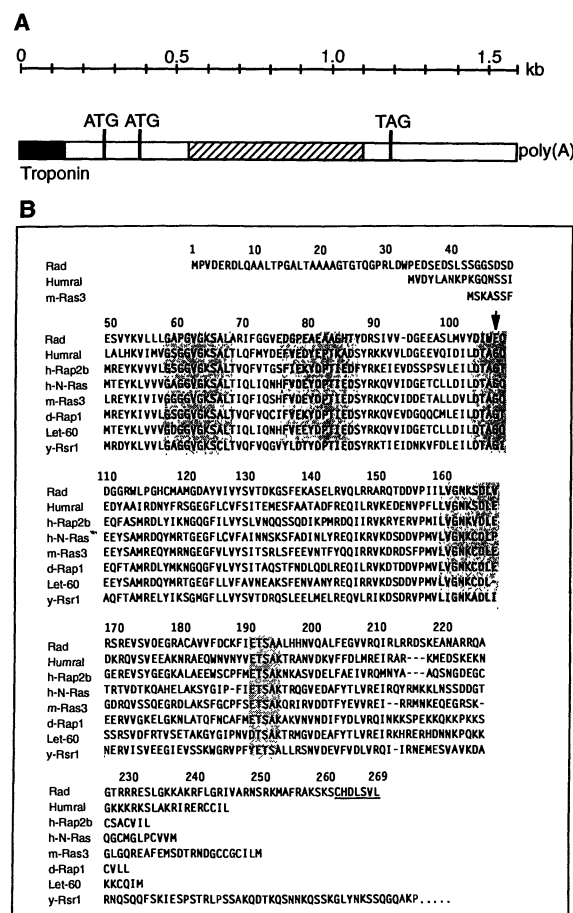
Attempts to define the specific genes involved in the pathogenesis of NIDDM have relied mainly on the study of specific candidate genes (6). In small subgroups of diabetic patients, mutations have been identified in the insulin molecule (7), in the insulin receptor (8), and in a mitochondrially encoded gene for a tRNA (9). In several families with a subtype of Type II diabetes known as maturity onset diabetes of the young (MODY), genetic defects have been found in the enzyme glucokinase (10), and in one MODY kindred a linkage has been established to a gene on chromosome 20 (11). However, the gene or genes responsible for the most common type or types of NIDDM have not yet been uncovered. We have undertaken the strategy of subtraction cloning in an attempt to iden-

tify genes that may influence NIDDM and have focused our attention on skeletal muscle, the major site of insulin resistance in the diabetic and prediabetic patient (12).

Two subtraction complementary DNA

(cDNA) libraries were prepared through a modification of the procedure of Schweinfest and colleagues (13, 14). One was enriched in mRNA species preferentially expressed in skeletal muscle of normal individuals and the other in mRNA species preferentially expressed in muscle of a Type II diabetic. About 4000 colonies from these two libraries were individually screened in duplicate 96-well dot blots with probes prepared by the polymerase chain reaction (PCR) from the two subtraction libraries (15). On this initial screen, 29 clones (about 0.7% of the two subtraction libraries) appeared to be differentially expressed in these two libraries and represent potential diabetes-related changes in gene expression. These clones were then used as probes on comparative Northern blots containing RNA samples from several normal and diabetic individuals. Eleven clones (~0.3% of those initially screened) were confirmed to be consistently differentially expressed,

Fig. 1. Molecular characteristics of the Rad cDNA clone. **(A)** Schematic representation of human muscle Rad cDNA. The original Rad clone (C9D6) was identified by the screening of the diabetic subtraction library with normal- and diabetic-enriched subtraction probes. Five additional longer Rad cDNA clones were then picked from the diabetic muscle cDNA library (29). The structure of the full-length Rad cDNA is represented by a schematic diagram that shows a portion of the 5' untranslated sequence, two in-frame ATGs at bases 124 and 241; an open reading frame ending at base 1048 with a TAG and containing a region from base 400 to base 870 homologous to the Ras-GTPase cDNAs family (44 to 55% homology; hatched area), and the entire 3' untranslated region 392 bp long containing a poly(A)⁺ adenylation signal (AATAAA) followed by a poly(A)⁺ tail of 18 nucleotides. The shaded area at the 5' end represents a fragment of troponin that formed an artifactual concatamer with the longest Rad cDNA clone. **(B)** Predicted amino acid sequence of Rad and comparison with the sequences of selected Ras proteins. The numbers correspond to the amino acid positions in the Rad sequence, starting from the presumed initiating (second) methionine. Sequences were compared to those in the Genbank data base with the FASTA program (Molecular Biology Computer Research Resource, Boston, MA). The protein sequences were aligned with the BESTFIT program. Gaps have been inserted to maximize the identity between the sequences. The five conserved amino acid sequences of the GTPase-active sites (G1 to G5) of the Ras-related protein superfamily are shown by the shaded areas, and the variant glutamic acid is marked by an arrow. In addition, a possible site of lipid acylation or prenylation, represented by the seven COOH-terminal amino acids beginning with a cysteine, is underlined (30).



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whereas the remainder showed variable patterns of expression among individuals. Ten of these 11 were either overexpressed or underexpressed in parallel in both Type I and Type II diabetic muscle, that is, they represented diabetes-regulated genes (16). One clone (C9D6), however, was selectively increased in expression in muscle from patients with NIDDM as compared to muscle from normal individuals or patients with Type I diabetes, indicating that this clone might be specifically linked to the insulin resistance, the genetics of Type II diabetes, or both. The 0.4-kb insert of this clone was used to isolate several longer clones from the original diabetic cDNA library to obtain a full-length cDNA.

The full-length clone was 1440 bases in length and included a portion of the 5' untranslated region, an open reading frame with two possible start codons, and the entire 3' untranslated region followed by a polyadenylate poly(A) tail (17) (Fig. 1A). Analysis of the open reading frame revealed 45 to 55% identity at the nucleotide level with members of the Ras superfamily. The highest degree of similarity was with human Rap2b and Ral, but there was also a similarity with N-Ras, murine Ras3, and Ras-related cDNAs from *Drosophila*, *Caenorhabditis elegans*, and yeast. We termed this new member of the Ras-guanosine triphosphatase (GTPase) superfamily Rad for Ras associated with diabetes. Because the second in-frame ATG presented a better consensus Kozak sequence and two-thirds of proto-oncogene transcripts have ATG codons preceding the start of the major open reading frame, the presumptive start of translation of this cDNA is likely the second in-frame ATG, leading to an open reading frame of 807 bases.

The deduced amino acid sequence of Rad contains 269 residues with a predicted molecular size of 29,266 kD (Fig. 1B). Thus

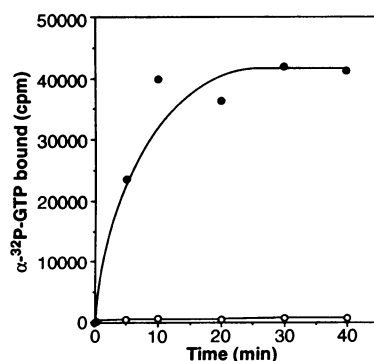


Fig. 2. Binding of $[\alpha\text{-}^{32}\text{P}]\text{-GTP}$ to GST-Rad. The amount of $[\alpha\text{-}^{32}\text{P}]\text{-GTP}$ bound to GST-Rad (filled circles) or GST (open circles) was determined in a filter binding assay at different times of incubation (31). Each data point is the average of duplicate determinations.

Rad is a member of the heterogeneous collection of small GTPases (20 to 35 kD) (18). This protein exhibits all five of the highly conserved GTPase domains (G1 to G5) (Fig. 1B) that are posited to take part in the catalytic functions of the Ras-related protein superfamily (18). However, Rad presents a number of features that are different from those of the other small GTPases. The Rad protein is longer at both the NH_2 terminus and the COOH terminus than most other members of the Ras family. The sequence of the G2 domain, the domain responsible for GTPase-activating protein (GAP) binding by the p21-Ras family members, is quite divergent from that observed for N-Ras, Rap2b, and Ral. There is a glutamic acid at position 108 in the G3 domain (equivalent to position 60 in N-Ras) that in other Ras family members is occupied by a highly conserved glycine. There is also a proline in position 61 of the G1 domain usually occupied by a glycine. Finally, the COOH terminus does not contain the typical CAAX or CCXX prenylation sites (where A is an aliphatic amino acid and X is any amino acid) present in virtually all Ras family members, although it contains a polybasic domain and a cysteine residue at position 7 from the COOH terminus (19).

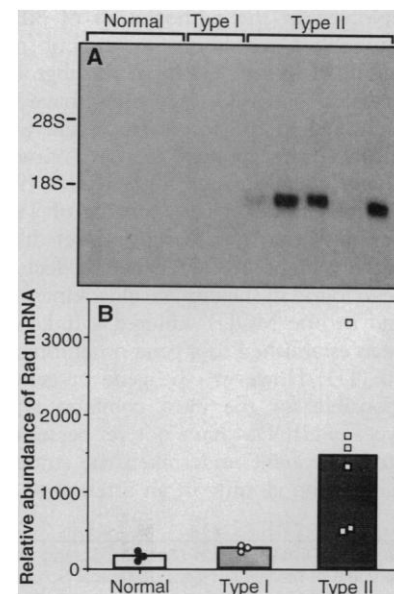
To confirm that Rad is a GTP binding protein, the terminal 217 amino acid region of Rad was expressed in *Escherichia coli* as a glutathione-S-transferase (GST) fusion protein (20). Although $[\alpha\text{-}^{32}\text{P}]\text{-GTP}$ bound to affinity-purified GST-Rad reaching a maximum by 30 min of incubation, binding to the GST protein alone was minimal (Fig. 2). Furthermore, the binding of labeled GTP to GST-Rad was

blocked by excess unlabeled GTP but not by adenosine triphosphate (ATP) (16). Thus, as predicted by the sequence similarity to the Ras-GTPase family, Rad specifically binds GTP.

On Northern blot analysis the major species of Rad mRNA was 1.6 kb (Fig. 3A). On longer exposure or with poly(A)⁺ RNA, minor species of mRNA at 3.6 and 5 kb were observed. The mRNA encoding Rad was markedly overexpressed in RNA samples of Type II diabetic muscle compared to those from nondiabetic or Type I diabetic muscle. The average amount of overexpression of Rad in the Type II diabetic as compared to the normal was 8.6-fold (range of 2.9- to 18.6-fold) (Fig. 3B). There was no correlation between the amount of Rad mRNA and age, gender, diabetes treatment, or nature of complications. In contrast, the amount of Rad expression in muscle samples from three Type I diabetic patients was similar to that of the nondiabetic controls. In normal individuals, the highest amounts of expression were in skeletal muscle, cardiac muscle, and lung, with lesser amounts in placenta and kidney (Fig. 4). Rad mRNA was also detected in adipose tissue (16) but was barely detectable or undetectable in liver, brain, and pancreas (Fig. 4).

To determine if the increased expression of Rad in muscle of Type II diabetics resulted from gene amplification or a major chromosomal rearrangement, Southern blot analyses were done on DNA extracted from muscle samples and leukocytes of normal, Type I, and Type II diabetic individuals, and DNA was digested with Eco RI, Hind III, Kpn I, or Pst I. A single major fragment was observed in each case, and the size and

Fig. 3. Northern blot analysis of human skeletal muscle Rad mRNA. (A) Representative Northern blot of human skeletal muscle showing Rad expression. Two μg of total RNA was extracted from three nondiabetic, two Type I, and five Type II diabetic muscle samples, hybridized with the insert from clone C9D6, and autoradiographed. This blot is representative of several blots done with different amounts of RNA and with different probes (32). (B) Relative abundance of the 1.6-kb Rad mRNA. Twenty μg of total RNA was extracted from three nondiabetic, three Type I, and six Type II diabetic muscle samples and hybridized with either the 1.1-kb cDNA insert or the 1.4-kb full-length Rad cDNA. Autoradiograms from several Northern blot analyses were quantified separately by scanning densitometry. The data were normalized to the integrated intensities of signals obtained from one nondiabetic RNA sample. Each data point represents the mean of at least three determinations per RNA sample. The overlaid histograms show the average of the normalized values obtained from normal, Type I, and Type II diabetic individuals. The amount of RNA loaded on the gels was equivalent in the different individuals on the basis of the reprobing of the blots with an oligonucleotide probe to human 28S RNA.



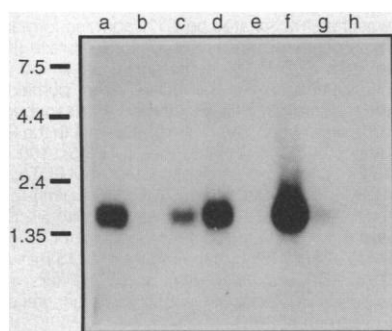


Fig. 4. Northern blot analysis of Rad expression. A multiple-tissue Northern blot (Clontech Laboratories, Palo Alto, CA) that contains 2 μ g of poly(A)⁺ RNA from a variety of normal human tissues was hybridized with ³²P-labeled Rad cDNA as described by the manufacturer and autoradiographed. The measurements are in kilodaltons. The represented tissues include (a) heart, (b) brain, (c) placenta, (d) lung, (e) liver, (f) skeletal muscle, (g) kidney, and (h) pancreas.

intensity were identical in all diabetic and nondiabetic patients, indicating that no major gene rearrangement or amplification had taken place (16).

Small GTP binding proteins related to Ras appear to be involved in controlling a diverse set of essential cellular functions including growth, differentiation, cytoskeletal organization, vesicle transport, and signal transduction between membrane-bound protein tyrosine kinases and cytoplasmic serine and threonine kinases (18, 21, 22). Proteins related to Ras have been implicated in insulin action. For example, antibodies to Ras, peptides derived from the GAP binding domain of Ras, and dominant negative mutants of Ras inhibit actions of insulin in both *Xenopus* oocytes and mammalian cells (23, 24). Insulin also increases the amount of p21-Ras in the GTP bound form (25), and this process is defective in cells expressing mutated insulin receptors (26). Recently, members of the Rab family have also been postulated to participate in glucose transporter translocation in response to insulin (27).

It is impossible to classify Rad into any of the existing subfamilies of the Ras-GTPases. Indeed, the sequence differences between Rad and the other known Ras proteins in the G2 and G3 domains suggest important differences in function because these regions are involved in GTP binding and hydrolysis. These observations suggest that Rad may have its own GAP or other effector molecule, as well as its own associated guanine nucleotide exchange factor (28). The lack of a typical isoprenylation site at the COOH terminus may indicate that Rad does not undergo either farnesylation or geranyl geranylation (19), although there is a cysteine

residue at position 263, seven amino acids from the COOH terminus.

The molecular basis and pathophysiological significance of Rad overexpression in muscle of Type II diabetics are unclear. Because Southern blot analysis of the *rad* gene does not reveal any evidence of gene amplification or rearrangement, a detailed analysis of the *rad* promoter is required to determine if some regulatory element could play a role in the overexpression of Rad in Type II diabetics. Whatever the mechanism, overexpression is specific to Type II diabetic patients. In this context, Rad may be acting as an inhibitory Ras, interfering with the function of normal Ras, Rab, or Rap proteins. Alternatively, Rad may be an active member of the Ras-GTPase family that is increased to compensate for the blockade in muscle of the insulin-signaling pathway that leads to insulin resistance.

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14. Oligo-dT primed cDNA libraries were prepared into Lambda-Zap II (Stratagene) with the use of poly(A)⁺ RNA extracted from skeletal muscle of one nondiabetic and one Type II diabetic human undergoing lower limb amputation. Single-stranded DNA (ss-DNA) was prepared from each cDNA library with R408 helper phage, isolated by polyethylene glycol precipitation, and purified by repeated chloroform extractions [J. R. Drugguid *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5738 (1988)]. To prepare a library enriched for genes preferentially expressed in normal muscle, ss-DNA from normal muscle was mixed with 10-fold excess biotinylated ss-DNA from the diabetic muscle [prepared with Photoprobe biotin (Vector Laboratories)]. Hybrids between normal and diabetic DNA and unhybridized biotinylated DNA were removed with streptavidin and Vectrix avidin. The normal-enriched ss-DNA was then subjected to a second round of subtraction. Two rounds of subtraction was expected to produce around 95% depletion of cDNA species common to both libraries and a 20-fold enrichment of selected cDNAs. Diabetic-enriched ss-DNA was similarly prepared with ss-DNA from diabetic muscle and biotinylated ss-DNA from normal muscle. Both subtracted ss-DNAs were then converted to double-stranded DNAs by incubation with DNA polymerase and ligase in the presence of T3 primer and a mixture of oligonucleotides.
15. Portions of each subtraction library were used to transform competent XL-1 Blue cells (Stratagene), which were plated on a medium containing ampicillin. Individual colonies were then picked and grown in 96-well dishes. A replicate of each 96-well archive plate was made with a replicator beaded lid (FAST system, Falcon, Becton Dickinson, Lincoln Park, NJ), and the archive plate was stored in 20% glycerol at -70°C. Duplicate dot blots were prepared on Biotrans nylon membrane (ICN Biochemicals, Cleveland, OH) from the copy plate of the normal-enriched and the diabetic-enriched colonies and hybridized with subtracted probes prepared by PCR with the two subtraction libraries as templates and with SK and KS primers.
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29. About 1.5 \times 10⁶ plaques from the original diabetic muscle cDNA library (14) were plated at a density of 50,000 plaques per 150-mm plate, transferred to nitrocellulose filters (Schleicher & Schuell) and screened with the C9D6 cDNA insert that had been purified on agarose gel. The probe was labeled with ³²P deoxycytidine 5'-triphos-

- 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 -³²P]-GTP (100 pmol, 3000 Ci/mmol) at room temperature in a solution containing 50 mM Tris (pH 7.5), 5 mM MgCl₂, 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 MgCl₂, 1 mM DTT, and BSA (1 mg/ml). Samples were brought to 500 µl with 50 mM Tris (pH 7.5), 10 mM MgCl₂, 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 (BIO-TECH 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 ³²P-labeled as described in (29), was hybridized (4 × 10⁶ 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 Deaconess 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-bisphosphate (PtdIns[4,5]P₂). Hydrolysis of PtdIns[4,5]P₂ 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 *pi1* null mutant was inviable, indicating that PtdIns4P and presumably PtdIns[4,5]P₂ are indispensable phospholipids.

Turnover of PtdIns[4,5]P₂ 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]P₂ by several different classes of receptor-activated phospholipase C enzymes (2) produces two intracellular second messengers—inositol 1,4,5-trisphosphate, which promotes release of Ca²⁺ 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]P₂ turnover to cellular responses because receptor-mediated stimulation of PtdIns[4,5]P₂ hydrolysis occurs simultaneously with activation of other signaling pathways (4). Also, PtdIns[4,5]P₂ is thought to be a regulator of the activity of certain actin-binding proteins (5). Given these roles, synthesis of PtdIns[4,5]P₂ is likely to be as stringently controlled as its cleavage. The first committed step in biosynthesis of PtdIns[4,5]P₂ 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 mammalian 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 -³²P]-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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