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A Phosphatidylinositol (PI) Kinase Gene Family in *Dictyostelium discoideum*: Biological Roles of Putative Mammalian p110 and Yeast Vps34p PI 3-Kinase Homologs during Growth and Development

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Three groups of phosphatidylinositol (PI) kinases convert PI into PI(3)phosphate, PI(4)phosphate, PI(4,5) bisphosphate, and PI(3,4,5)trisphosphate. These phosphoinositides have been shown to function in vesicle-mediated protein sorting, and they serve as second-messenger signaling molecules for regulating cell growth. To further elucidate the mechanism of regulation and function of phosphoinositides, we cloned genes encoding five putative PI kinases from *Dictyostelium discoideum*. Database analysis indicates that *D. discoideum* PIK1 (DdPIK1), -2, and -3 are most closely related to the mammalian p110 PI 3-kinase, DdPIK5 is closest to the yeast Vps34p PI 3-kinase, and DdPIK4 is most homologous to PI 4-kinases. Together with other known PI kinases, a superfamily of PI kinase genes has been defined, with all of the encoded proteins sharing a common highly conserved catalytic core domain. *DdPIK1*, *-2*, and *-3* may have redundant functions because disruption of any single gene had no effect on *D. discoideum* growth or development. However, strains in which both of the two most highly related genes, *DdPIK1* and *DdPIK2*, were disrupted showed both growth and developmental defects, while double knockouts of *DdPIK1* and *DdPIK3* and *DdPIK2* and *DdPIK3* appear to be lethal. The Δ*Ddpik1* Δ*Ddpik2* null cells were smaller than wild-type cells and grew slowly both in association with bacteria and in axenic medium when attached to petri plates but were unable to grow in suspension in axenic medium. When Δ*Ddpik1* Δ*Ddpik2* null cells were plated for multicellular development, they formed aggregates having multiple tips and produced abnormal fruiting bodies. Antisense expression of *DdPIK5* (a putative homolog of the *Saccharomyces cerevisiae VPS34*) led to a defect in the growth of *D. discoideum* cells on bacterial lawns and abnormal development. *DdPIK5* complemented the temperature-sensitive growth defect of a *Schizosaccharomyces pombe* Δ*Svps34* mutant strain, suggesting *DdPIK5* encodes a functional homolog of yeast Vps34p. These observations indicate that in *D. discoideum*, different PI kinases regulate distinct cellular processes, including cell growth, development, and protein trafficking.

Phosphoinositides represent important regulatory molecules in eukaryotes and are involved in regulating cellular responses as diverse as vacuolar protein sorting, cytoskeletal organization, cell growth, and development (see below). Phosphatidylinositol (4,5)bisphosphate [PI(4,5)P_2] acts both as an activator of protein kinases (3, 5, 11, 12, 38, 40) and as a substrate for phospholipase C, producing the two second messengers, inositol-1,4,5 triphosphate and 1,2-diacylglycerol (7, 10). Phosphatidylinositol (3,4,5)trisphosphate [PI(3,4,5)P_3], the product of phosphorylation of PI(4,5)P_2, is an activator of protein kinase Cζ (47) and may be involved in regulating other, as yet unidentified downstream pathways. Phosphatidylinositol(3)phosphate [PI(3)P], the product of phosphorylation of phosphatidylinositol (PI) by the PI 3-kinase encoded by *VPS34*, is important in vacuolar/lysosomal protein sorting in *Saccharomyces cerevisiae* (53, 56, 57).

Three groups of PI kinases convert PI into PI(3)P, PI(4)P, PI(4,5)P_2, PI(3,4)P_2, and PI(3,4,5)P_3. PI is phosphorylated by PI 3-kinase to form PI(3)P (10, 59, 70) and by PI 4-kinase to form PI(4)P (25). Vps34p from *S. cerevisiae* (ScVps34p) catalyzes the conversion of PI to PI(3)P (56), while the mammalian p110 PI 3-kinase is also capable of phosphorylating PI(4)P and PI(4,5)P_2 (7, 10). Two PI 4-kinase genes have been cloned from *S. cerevisiae* (23, 73), and one has been cloned from mammals (71). PI(4)P is converted to PI(4,5)P_2 by PI(4)P 5-kinases (4, 16, 42, 46, 52, 62). Several PI 3-kinase activities convert PI(4,5)P_2 into PI(3,4,5)P_3 and include p110-related isoforms activated by tyrosine kinases (2, 10, 13, 21, 54) or by βγ subunits of heterotrimeric G proteins (60, 67, 75). There are also reports of increased PI 4-kinase and PI(4)P 5-kinase activity following receptor stimulation (20, 50, 65, 68).

While much knowledge on the structure of p110 PI 3-kinase, its activation by tyrosine kinases in mammalian cells, and its potential role cell proliferation in mammals has been accumulated, little is known about its downstream effectors or the possible roles of PI 3-kinases other than Vps34p homologs in other cellular functions (e.g., differentiation). The mammalian p110 PI 3-kinases, in contrast to the ScVps34p, can use PI(4)P and PI(4,5)P_2 as well as PI as substrates (58). There is evidence suggesting a correlation between PI 3-kinase activity and receptor internalization (35, 37). PI 3-kinase activity also correlates with modulation of cytoskeletal (actin) structure (17, 48, 72, 74).

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Dictyostelium discoideum grows vegetatively as single cells which, upon starvation, initiate multicellular development leading to the production of a mature fruiting body. Except for a previous report identifying a developmentally regulated PI 4-kinase activity (66), little is known about PI metabolism in this organism and its relationship to growth and multicellular development. In this paper, we report the cloning and molecular genetic analysis of genes encoding four putative PI 3-kinases and one PI 4-kinase. Sequence analysis of this superfamily in *D. discoideum* and family members from other eukaryotes allowed us to establish an evolutionary relationship between these genes. Three of the PI 3-kinase genes from *D. discoideum* encode proteins most related to the mammalian p110 PI 3-kinase, while the fourth is most highly related to the *ScVPS34* gene product. Disrupting the genes encoding any of the three p110 homologs individually had no effect on growth or development; however, a knockout of two of the three caused defects in cell proliferation and multicellular development. This represents the first comprehensive molecular genetic analysis of homologs of the mammalian p110 family of PI 3-kinases in a nonmammalian system.

MATERIALS AND METHODS

Construction of plasmids for gene disruption. DdPIK1-12 is the longest partial cDNA of the *D. discoideum* (*DdPIK1*) gene and contains the 3.6-kb 3′ portion of *DdPIK1*. A *Hin*cII-*Hin*cII fragment containing the *THY1* gene (18, 26) was inserted into *Hin*cII-digested PIK1X1 to give K1T-3. For the *DdPIK1* disruption, K1T-3 was digested with *Eco*RV and *Eco*RI (located in polylinker at the 3′ end of the gene).

The partial *DdPIK2* cDNA P3 contains 2.2 kb of the 3′ portion of the *DdPIK2* gene. The 5.1-kb genomic DNA clone 8-2 of *DdPIK2* overlaps with the 5′ portion of cDNA P3. A 4.6-kb *Nde*I fragment from *DdPIK2* genomic clone 8-2 was inserted into the cDNA P3 at the *Nde*I site in the correct orientation to give plasmid PIK2N, which contains the whole open reading frame (ORF) of *DdPIK2* and 0.5 kb of the 5′ untranslated regions. The *Pst*I-to-*Xba*I region in the polylinker of PIK2N was deleted to give PIK2PX. A 2,757-bp region (*Bgl*II at 2520 to *Bam*HI site at 5277) in PIK2PX was replaced with a 2.2 kb Neo^r cassette that confers G418 resistance to give PIK2NEO-15. For the *DdPIK2* gene knockout, PIK2NEO-15 was digested with *Sna*BI and *Hin*dIII.

For disruption of *DdPIK2* with the *THY1* marker, K2D was constructed. K2D is a simple insertion of a 3.2-kb *THY1*-*Hin*cII-*Hin*cII fragment into the *Sna*BI site of cDNA PIK2-7, the longest cDNA in both orientations, to give K2D-1 and K2D-2. The vectors were digested with *Hin*dIII before transformation with the *D. discoideum thy1* null strain JH10. The whole mixture was transformed into JH10. Both vectors gave gene knockouts when transformed into *D. discoideum* cells.

To disrupt *DdPIK3*, a *Bst*BI restriction site in a partial cDNA of 2.9 kb of the 3′ portion of *DdPIK3* was converted to a *Bam*HI site. Then the cDNA was cloned into the *Eco*RI and *Xho*I sites in pSP72 (Promega) to give plasmid SPN6. The 337-bp *Bam*HI fragment in SPN6 was replaced with the *Bam*HI fragment containing the *THY1* gene to give plasmid K3D. For the gene knockout of *DdPIK3*, K3D was digested with *Eco*RI and *Bgl*II.

DdVPS34 gene disruption plasmid K5D was constructed by digesting a full-length *DdVPS34* cDNA with *Hin*cII and *Bgl*II. A *Hin*cII-*Bam*HI fragment containing the *THY1* gene was then cloned into these sites. In this knockout, 802 bp of the *DdVPS34* cDNA was replaced with the *THY1* gene. The resulting plasmid, K5D, was digested with *Alw*N1 and *Apa*I before transformation into *D. discoideum* JH10 cells.

Antisense plasmid construction. A 1.8-kb *Bgl*II-*Eco*RI fragment from PIK5-9 was inserted into vector EXP4(+) (18) digested with *Bgl*II and *Eco*RI to give plasmid pAT5.

Gene disruption. The disruption plasmids were linearized with appropriate restriction enzymes as described above before transformation into *D. discoideum* cells. Transformation of JH10 selecting for *THY1* insertions has been described elsewhere (18, 26, 44). For cells transformed for selection with G418, G418 was added to about 5 μg/ml. Transformants were analyzed for their genomic structures by Southern DNA blot analysis.

PCR. Three PCR primers, SEM1 (5′-GTTTGGATCC[G/A][C/T]NAC[A/G]CA[A/G]TANCCNGC[A/G]CA-3′), SEM2 (5′-GTTTCTGCAGGNGA[T/C]GA[T/C][T/C]N[A/C]GNCA[A/G]GA-3′), and SEM3 (5′-GTTTGGATCC[T/A][A/G]NCC[A/G]AA[A/G]TCN[A/G][T/C][A/G]TG-3′) were designed for cloning PI kinase homologs in *D. discoideum*. SEM2 is the sense strand coding for GDDLRQ[D/E], while SEM1 and SEM3 are antisense primers coding for CAG YCAV[A/I] and H[I/A]DFG[HYF], respectively. *D. discoideum* genomic DNA was amplified for 40 cycles, using 37°C as the annealing temperature. (Note that *D. discoideum* genes have relatively few introns and are short [100 to 150 sets].) Agarose gel analysis of the products identified several bands clustered around 400 ± 50 bp, the expected size. The products were cloned into pSP72 (Promega) and sequenced.

Sequence analyses. Most sequence analyses were done with the DNA system, which contains the Genetics Computer Group package, the NewAT87 program, and other utility programs assembled by Doug Smith at the University of California, San Diego. Sequences were aligned with either the Pileup or NewAT87 program in the case of multiple sequence alignment. Bestfit was used for pairwise sequence alignment.

Transformation, growth, and development of *D. discoideum*. See references 18, 26, and 44 for details of the techniques.

Reverse transcription-coupled PCR (RT-PCR). Standard PCR was performed with primers from the N-terminal nonconserved regions. Reaction conditions consisted of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 3 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate (dNTP), 500 nM each primer, 0.5 μg of KAx-3 genomic DNA, and 0.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus). Following an initial denaturation step for 60 s at 94°C, 30 cycles were performed, using the following parameters: 94°C, 30 s; 45°C, 30 s; and 72°C, 30 s.

RT-PCR was performed with a GeneAmp RNA PCR kit (Perkin-Elmer Cetus). Briefly, 1 μg of total RNA was incubated with 1 U of RNase-free DNase I (Promega) in 1× RT-PCR buffer (50 mM KCl, 5 mM $MgCl_2$, 1 mM each dNTP, 10 mM Tris-HCl [pH 8.3]) for 30 min at 37°C. The DNase I was inactivated by boiling for 10 min. The tubes were then placed on ice, and the following reagents were added: 1 U of RNase inhibitor, 2.5 U of Moloney murine leukemia virus reverse transcriptase, a DdPIK1- or DdPIK2-specific primer (CGAGTTGCAA CGTATTC; 0.75 μM, final concentration), and H_2O to 20 μl. For first-strand synthesis, the samples were incubated at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min. For PCR amplication, the following reagents were added: 8 μl of 25 mM $MgCl_2$, 8 μl of 10× RT-PCR buffer, sense primer (0.15 μM, final concentration), and 0.5 U of AmpliTaq DNA polymerase. The volume was adjusted to 100 μl with H_2O, and 30 cycles of PCR were performed, using the following parameters: 94°C, 60 s; 50°C, 60 s; and 72°C, 30 s. An aliquot of each reaction was analyzed on a 1.2% agarose gel. Gels were run in duplicate, blotted, and then separately probed with *DdPIK1*- and *DdPIK2*-specific probes covering the PCR regions. Neither probe showed detectable hybridization to the other DNA.

Nucleotide sequence accession numbers. The accession numbers for the five *D. discoideum PIK* genes are as follows: *DdPIK1*, U23476; *DdPIK2*, U23477; *DdPIK3*, U23478; *DdPIK4*, U23479; and *DdPIK5*, U23480.

RESULTS

Cloning of genes encoding PI kinases. The observed sequence conservation between the *ScVPS34* gene product (28) and a mammalian catalytic subunit of PI 3-kinase (29) permitted a molecular genetic approach to examine PI 3-kinases in *D. discoideum*. Three PCR primers (SEM1, SEM2, and SEM3), based on sequence identities between the bovine p110 PI 3-kinase catalytic subunit and Vps34p, were designed and used to amplify *D. discoideum* genomic DNA (see Materials and Methods for details). Fragments were cloned and sequenced. Fragments from five distinct genes with homology to the region between the primers in p110 and Vps34p were identified. These were named *DdPIK*s because of the strong sequence identity of their products to known PI kinases (see below) and numbered sequentially according to the order of discovery (*DdPIK1*, *DdPIK2*, *DdPIK3*, *DdPIK4*, and *DdPIK5*).

The cloned PCR fragments were used to identify cDNA clones. cDNA clones for *DdPIK1*, *DdPIK2*, *DdPIK3*, and *DdPIK4* were found in cDNA libraries made from RNA isolated from vegetatively growing cells and from cells at 12 to 16 h of development, whereas *DdPIK5* clones were found exclusively in vegetative cDNA libraries. The longest *DdPIK4* and *DdPIK5* cDNAs contained the complete ORFs. The *DdPIK1*, *-2*, and *-3* cDNAs were incomplete. To obtain more of the ORFs encoded by *DdPIK1*, *-2*, and *-3*, genomic libraries were screened. Several independent clones were obtained for each gene. Overlapping clones contained the entire ORFs of *DdPIK1* and *DdPIK2*; the *DdPIK3* genomic DNAs were incomplete and lacked a portion of the 5′ coding region.

Sequence analyses. Nucleotide sequences of five *DdPIK* genes were determined, and the ORFs were deduced. *DdPIK1*, *DdPIK2*, *DdPIK4*, and *DdPIK5* were predicted to encode pro-

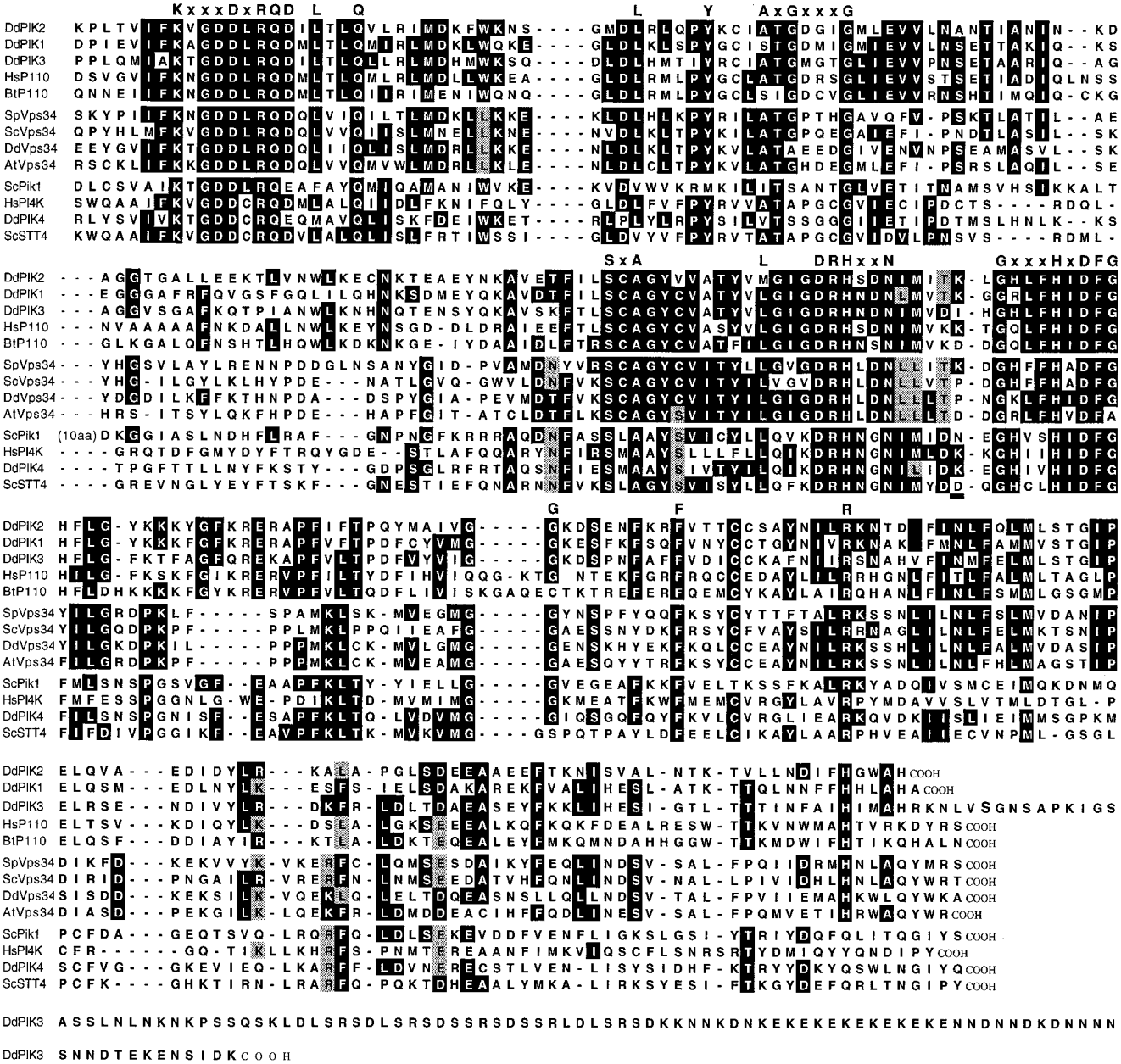

FIG. 1. Amino acid sequence comparisons of the highly conserved PI kinase catalytic domains. A progressive sequence alignment program (22) was used. The references for the sequences are given in parentheses: human p110 (HsP110; 34); bovine p110 (BtP110; 29); SpVps34p (63); *A. thaliana* VSP34 homolog (AtPI3K; 69); ScVps34, ScPI 4-kinase (28); (ScPI4K; 23); human PI 4-kinase (HsPI4K; 71); and ScSTT4 (73). Breaks have been inserted between families and subfamilies.

teins of 1,570, 1,858, 1,093, and 816 amino acids, respectively. The ORF of the *DdPIK3* gene was incomplete (no ATG was seen at the N terminus of the ORF) and encoded a protein of 1,585 amino acids. All five ORFs showed significant sequence identity to each other, with the highest identity in the C terminus of each of the predicted ORFs. A search of the nonredundant BLAST database (1) showed that all five ORFs had significant sequence identity to known PI kinases, including the PI 3-kinase Vps34p (28), ScPI 4-kinase (23), SpVPS34 (VPS34 from *Schizosaccharomyces pombe*) (63), VPS34 from *Arabidopsis thaliana* (69), bovine PI 3-kinase (catalytic subunit p110) (29), and human PI 3-kinase (catalytic subunit p110) (34). In addition, weaker sequence identity was found to *TOR1*/*DRR1* (*TOR1*) and *TOR2*/*DRR2* (*TOR2*) gene products (6, 27) from *S. cerevisiae*. Some sequence identity was also found to known protein kinases (see below). This finding is consistent with the observation that mammalian PI 3-kinase has been shown to have endogenous protein kinase activity (9, 15). The ORFs from the five *D. discoideum* genes and known PI kinases from other organisms may be members of a PI kinase superfamily (see below). The derived amino acid sequence of the conserved putative catalytic domain and the comparison with other PI kinases are shown in Fig. 1. Diagrams of the various domains are given in Fig. 2.

D. discoideum DdPIK1, -2, and -3 are most highly related to the mammalian p110 PI 3-kinase catalytic subunit, DdPIK4 is

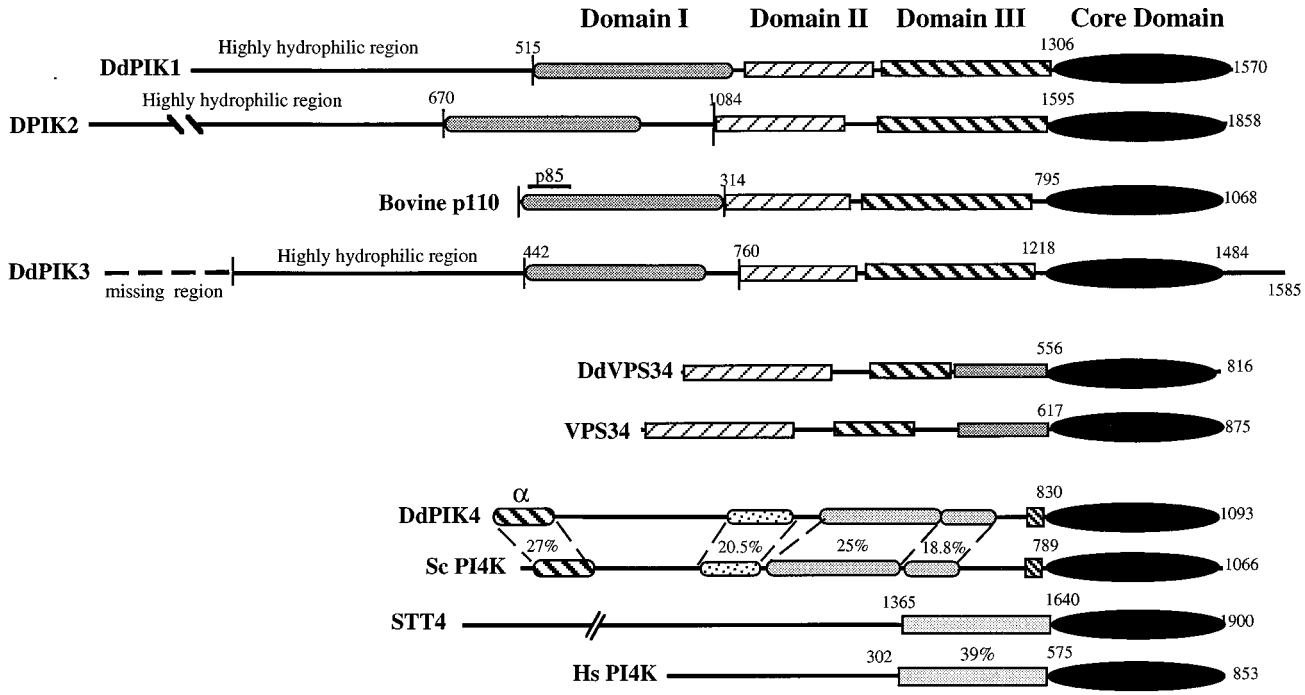

FIG. 2. Schematic of the domain structure of the PI kinase gene superfamily. The individual domains are defined in the text. Note that in the PI 3-kinase II family members (*DdVPS34* and yeast *VPS34*), domain III is split. The *D. discoideum* and yeast *PIK4* genes shown have a separate domain that is conserved between the two proteins (shown as dotted region). In addition, there is a region of ~40% conservation (shown as a dotted box) lying immediately N terminal to the core domain. There is also a domain (labeled α) that shows conservation to the middle of the PI 3-kinase domain III. The conservation between the two genes and to domain III is 27%. There is a region of the *DdPIK3* that has not been cloned and is shown as a dashed line. The lengths of the N-terminal hydrophilic domain are 670 and 515 residues, respectively, for *DdPIK1* and *DdPIK2*. Because the sequence for *DdPIK3* gene is incomplete at the N-terminal end, the exact size of this domain is uncertain, but it contains at least 442 residues. Two of the PI 4-kinases (ScSTT4 and human PI 4-kinase [Hs PI4K]) share a region of homology N terminal to the core domain that is 39% identical. The identity is weaker toward the N terminus of the barred region. The entire barred region also shares weak homology (~20% identity) to domain III of the PI 3-kinases. Sc PI4K, ScPI 4-kinase.

most highly related to the yeast and mammalian PI 4-kinases, and DdPIK5 is most highly related to the *S. cerevisiae*, *S. pombe*, and *Arabidopsis* Vps34 proteins.

Although we have determined the presence of PI 3-kinase and PI 4-kinase enzymatic activity in crude extracts from *D. discoideum* cells, we have not determined through expression studies and enzymatic assays that any of the five *DdPIK* genes encode PI kinases. However, the very high amino acid sequence identity in the putative catalytic domain (see below) strongly suggests that they do encode PI kinases. We henceforth refer to them as putative PI kinases.

Known PI kinases and the putative *D. discoideum* PI kinases contain a highly conserved core domain. Known PI kinases and the putative *D. discoideum* PI kinases have a common C-terminal domain, which we have designated the PI kinase core domain. We used the New AT87 program (obtained from Doug Smith, University of California, San Diego) (22) to align sequences of this domain from all known PI kinases and the DdPIKs (Fig. 1) and to derive an evolutionary tree (Fig. 3A). The length of the PI kinase core domains was well conserved (average of 265 amino acids, range from 259 to 278). Most of the PI kinase genes terminated shortly after the PI kinase core domain. The gene encoding a putative DdPI 3-kinase (*DdPIK3*) was an exception, having a C-terminal, very hydrophilic extension of 102 residues containing five imperfect repeats of five amino acids (QSKLD-LSRSD-LSRSD-SSRLD-LSRSD) and seven repeats of two amino acids (EK). Overall, the region was very rich in serine, asparagine, and basic and acidic amino acids.

When sequences from the PI kinase core domains were used to search protein sequence databases, only known PI kinase gene homologs showed extensive identity. However, significant sequence identities were also obtained with TOR family members (see above) and known protein kinases. Protein kinases with the strongest similarity to the PI kinase core domain were the serine/threonine protein kinases cdc2 (30) and ribosomal S6 protein kinase (36). The N-terminal half of the PI kinase core domain of DdPIK1 had 23% sequence identity to cdc2 protein kinase over a region of 144 amino acids. An additional 38% of the residues are similar. The C-terminal half of the PI kinase core domain from DdPIK4 showed 25% sequence identity over a 125-amino-acid region to that of cdc2. An additional 40% of the sequence showed sequence similarity. Five residues are conserved in all members of both the protein kinase and the PI kinase superfamily: $DX_4NX_{12}DFG$ for PI kinase and $DX_4NX_{13}DFG$ for protein kinases. Two aspartate residues (conserved in protein kinases) essential for protein kinase catalysis at positions 127 and 146 of cdc2 were also conserved in DdPIK1. All PI kinase genes had a histidine in the position corresponding to Lys-129 of cdc2, a residue that is conserved in all protein kinases.

The PI kinase superfamily consists of four subfamilies. The PI kinase superfamily consists of two families, PI 3-kinases and PI 4-kinases (Fig. 3). Each family consists of two subfamilies (I and II). The structure of the evolutionary tree suggests that the four subfamilies had existed before the eukaryotic organisms diverged from each other because members within each subfamily are more conserved across species than are different subfamily members of the same species. An interesting exception is the PI 3-kinase II subfamily, which contains the mam-

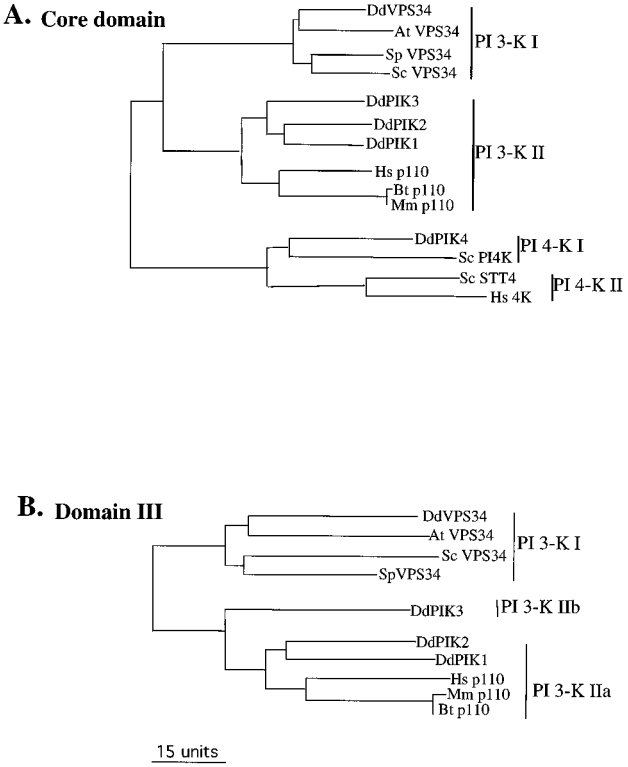

FIG. 3. Evolutionary tree analysis of the PI kinase family. (A) Tree analysis of the core domain of all PI kinase family members; (B) tree analysis of domain III of the PI 3-kinase subfamily. See text for details. At VPS34, *A. thaliana* VPS34; Hs p110, human p110; Bt p110, bovine p110; Mm p110, mouse p110.

malian p110 and the *DdPIK1*, *-2*, and *-3* genes. This subfamily was split during evolution and has not been identified in either *S. cerevisiae* or *S. pombe*, suggesting that it either evolved after yeasts or was lost. Members within the PI 3-kinase I subfamily are from 15 to 22 units away from the PI 3-kinase I ancestor. The *DdPIK5* gene (hereafter called *DdVPS34*; see below) had the shortest evolutionary distance, 15 units from the common PI 3-kinase I ancestor.

The PI 3-kinase II subfamily consists of two groups of genes from mammals and *D. discoideum*. In contrast to the PI 3-kinase I subfamily, in which only a single gene has been identified in each species, the PI 3-kinase II subfamily has multiple members. *DdPIK1* and *DdPIK2* are closely related, whereas *DdPIK3* is less related. Multiple members in this subfamily were more conserved within species.

Three regions in PI kinase core domains are highly conserved. The KDRQQ region is located in the N-terminal quarter of PI kinase core domain and characterized by five invariant residues (consensus, KX_3DXRQX_5Q). This region also has a highly conserved Gly residue that is 30 to 34 residues C terminal to the last conserved Gln. The second domain is located near the center of PI kinase core domain and has 11 invariant residues (consensus, $SXAX_{12}DRHX_2NX_{6-7}GX_3$ HXDFG). The third highly conserved region has invariant Phe and Arg residues (FR region) that are separated by 11 residues.

Sequence relationships between putative PI 3-kinases. When sequences N terminal to the PI kinase core domain were compared, substantial similarities were identified only among members of the same family. The sequences of the PI 3-kinase family members, lacking their PI kinase core domains, were aligned by using the Genetics Computer Group Pileup program. Three domains outside the core domain were recognized and are depicted in Fig. 2. The PI 3-kinase II subfamily has three common domains outside the core domain, designated domains I, II, and III. The PI 3-kinase I subfamily lacks domain I. Among members of the PI 3-kinase family, the degree of sequence conservation is lowest in the N-terminal regions of the protein. In all cases, the level of conservation is less than in the core domain. The sequence identities of PI kinase core domains are 48 to 60, 54 to 60, 40 to 45, and 36 to 88% within subfamilies PI 3-kinase II, PI 3-kinase I, PI 4-kinase I, and PI 4-kinase II, respectively (Fig. 4). Between the PI 3-kinase I and PI 3-kinase II subfamilies, the sequence identities are 30 to 41%. Sequence identities between the PI 3-kinase and PI 4-kinase families range from 24 to 31%. Within the PI 3-kinase I subfamily, sequence identity ranges from 36 to 42%, compared with 54 to 60% within the core domain. As can be seen from this analysis of the core domain and domain III, the three *D. discoideum* genes, *DdPIK1*, *-2*, and *-3*, are highly related to the mammalian p110 PI 3-kinase genes (Fig. 4). Moreover, the mammalian and bovine p110 PI 3-kinases show only slightly more sequence identity to each other than they do to the *D. discoideum* PI 3-kinase II members in these regions.

Domain I is unique to the PI 3-kinase II subfamily, and its sequences are less conserved than domain II sequences (overall, 15% identity), with the ORFs of the bovine and human genes showing only 27% sequence identity. DdPIK1 and -2 showed 20% sequence identity in domain I, while DdPIK1 and the human PI 3-kinase II showed 14% identity. The N-terminal

	DdPIK1											
DdPIK1	100	DdPIK2										
DdPIK2	59.5	100	DdPIK3									
DkPIK3	56.6	56.1	100	Btp110								
Btp110	48.1	46.4	47.7	100	Hsp110							
Hsp110	50.8	51.3	49.6	55.2	100	ScVPS34						
ScVPS34	38.5	36.7	36.9	32.3	32.7	100	SpVPS34					
SpVPS34	38.3	33.7	34.8	31.4	34.5	59.9	100	AtVPS34				
AtVPS34	40.1	37.1	35.3	31.3	34.4	58.9	57	100	DdVPS34			
DdVPS34	39.8	35.6	37.8	31.7	34.4	53.7	56.9	58.9	100	HsPI4K		
HsPI4K	28.9	27.8	28.5	28.2	27.3	25.5	27.6	27.2	26.2	100	ScSTT4	
ScSTT4	29.1	29.6	33.5	31.9	31.4	26.5	28.2	28.6	29.6	53.3	100	ScPI4K
ScPI4K	26.7	28	30.2	25.9	24.7	27.9	27.9	24.9	25.4	36.6	40.2	100 DDPIK4
DkPIK4	27.7	28.2	30.1	23.9	25.7	27.7	28.5	30.2	31.1	42	41.6	45.8 100

FIG. 4. Percent identities in the PI kinase core domains. The genes are grouped in subfamilies as defined in the tree in Fig. 3. See the legend to Fig. 1 for gene abbreviations and references. The sequences were progressively aligned as described by Feng and Doolittle (22).

regions of the human and bovine proteins, which contain the domain that interacts with the p85 regulatory subunit (15, 31), have 37% identity. Of the three *D. discoideum* genes, the *DdPIK1* ORF is most closely related to the mammalian protein-encoding genes in this region. The N-terminal domains of DdPIK1, -2, and -3 are very hydrophilic and contain tandem repeats of single amino acids.

A putative PI 4-kinase. The PI kinase core domains of the PI 4-kinase family, including the putative PI 4-kinase, DdPIK4, are less conserved than is seen in the PI 3-kinase family. The two subfamilies, PI 4-kinase I and PI 4-kinase II, have very little sequence conservation outside the PI kinase core domain. A small region of 28 amino acids immediately N terminal to the PI kinase core domain is strongly conserved (44% sequence identity) between ScPI 4-kinase and DdPIK4. The PI 4-kinase family members have little sequence identity with the PI 3-kinases outside the PI kinase core domain. The first conserved sequence block (27% sequence identity) in the PI 4-kinase I subfamily has a similar degree of sequence identity to a portion of domain III in the PI 3-kinase family.

Developmental regulation of PI kinase genes in *D. discoideum*. To gain insight into the potential role of the PI kinase genes in regulating *D. discoideum* development, we examined the expression patterns of *DdPIK1* and *DdPIK2*. Northern (RNA) analyses did not detect *DdPIK1*, *DdPIK2*, or *DdPIK3* transcripts, suggesting that the expression level was extremely low. RT-PCR was used to quantitate the expression of *DdPIK1* and *DdPIK2*, the two most related genes. Oligonucleotides for PCR amplification were made to nonconserved regions of each gene and used to amplify cDNAs made from RNA isolated at different times during *D. discoideum* development. The gene-specific oligonucleotides were unable to amplify the other gene. Both genes were expressed uniformly throughout development (data not shown). *DdPIK3* and *-4* are also probably expressed throughout the majority of development, since clones were identified in cDNA libraries made from RNA isolated from vegetative cells and cells from 12 to 16 h of development. *DdVPS34* may be expressed predominantly in vegetative cells, as cDNA clones were found in the vegetative but not the λZAP cDNA library made from RNA isolated from 10 to 16 h, which has $>10^6$ independent clones, for which $\sim 5 \times 10^5$ plaques were screened.

Knockouts of individual PI 3-kinase II subfamily members showed no observable effect on either growth or development. To examine the possible effects of a loss of *DdPIK1*, *-2*, and *-3* function, gene disruption constructs were made by using the *THY1* marker (Materials and Methods). In addition, a *DdPIK2* disruption construct was made by using the pNEO-Act15 cassette, conferring G418 resistance. In each case, the constructs were digested with the appropriate restriction enzyme(s) and transformed into the *thy1* null strain JH10 (26, 44). Of 27 independent pK1T-3 transformants (*DdPIK1* construct), 21 were shown to have *Ddpik1* null genotype as determined by Southern blot analysis. Of 39 independent pK2NEO-Act15 (pNeo-Act15 construct of *DdPIK2*) transformants analyzed, one had the *DdPIK2* gene disrupted by a single copy of the pNEO-Act15 vector (clone KZ48) and a second had the *DdPIK2* gene disrupted by two copies of the knockout vector (clone KZ84). Genomic DNA was isolated from six K3D10-transformants (*THY1* construct of *DdPIK3*) and examined; the *DdPIK3* gene was disrupted in five of the six transformants. When the individual disruptants of *DdPIK1*, *-2*, and *-3* were checked for growth and development, no defect was detected.

Double knockouts of *DdPIK1* and *DdPIK2* given growth and developmental phenotypes. If members of the PI 3-kinase II family are redundant, it might be possible to examine function by creating double knockouts. To create a Δ*Ddpik1* Δ*Ddpik2* double knockout, the two independent pNEO-Act15 knockouts of *DdPIK2* in the *thy1* null background (strains KZ48 and KZ84, respectively) were transformed with the *DdPIK1* construct pK1T-3, carrying the *THY1* marker (19). Sixteen of twenty-seven independent transformants of the *Ddpik2* null strain KZ84 had *DdPIK1* disrupted without other detectable alterations of the genome (data not shown). One of five clones from the *Ddpik2* null strain KZ48 had *DdPIK1* disrupted (data not shown).

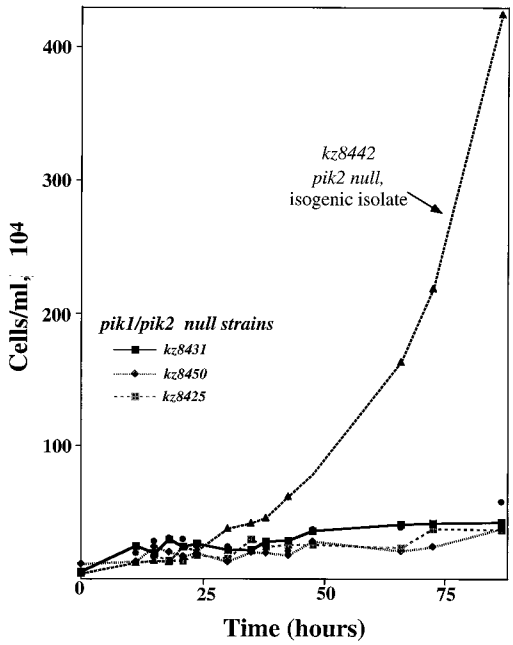

FIG. 5. Growth analysis of Δ*Ddpik1* Δ*Ddpik2* double-knockout strains in axenic suspension culture. Cells were grown on petri dishes attached to plastic in HL5 axenic medium, harvested, and inoculated into suspension cultures in HL5 medium. Cells were counted at the times indicated after inoculation. Three double-knockout clones are shown in addition to a *Ddpik2* null single-knockout clone that is isogenic except for the *PIK1* locus. This strain has the *THY1* gene complemented but insertion of the *Ddpik1* knockout vector into a different chromosomal location as determined by Southern analysis (data not shown). The *Ddpik2* null single-knockout strain shows the same growth as wild-type cells.

All strains in which both genes were disrupted exhibited abnormal phenotypes (see below), while all isolates that still had the wild-type copy of the *DdPIK1* gene showed wild-type growth and developmental phenotypes. The double knockout (Δ*Ddpik1* Δ*Ddpik2* null strains) affected several aspects of *D. discoideum* growth and development. Δ*Ddpik1* Δ*Ddpik2* cells did not grow in axenic medium in suspension cultures (Fig. 5) but were able to grow slowly when grown in petri dishes in axenic medium in which cells are able to attach to the plastic (data not shown). The cells grown in axenic cultures were significantly smaller than wild-type cells. *D. discoideum* cells can also be grown on nutrient agar plates in association with nonmucoid, gram-negative bacteria that are used as a food source. When the cells were grown under these conditions in association with *Klebsiella aerogenes* as the food source, the cells grew, but the rate of growth was also reduced, as determined by the amount of time required to clear bacteria to form a plaque of similar size. In addition, the double knockout formed unusual plaques compared with wild-type cells (Fig. 6A and B). The plaques increased in diameter without fully clearing the bacterial lawn in the center, suggesting that growth was impaired on bacteria (see below).

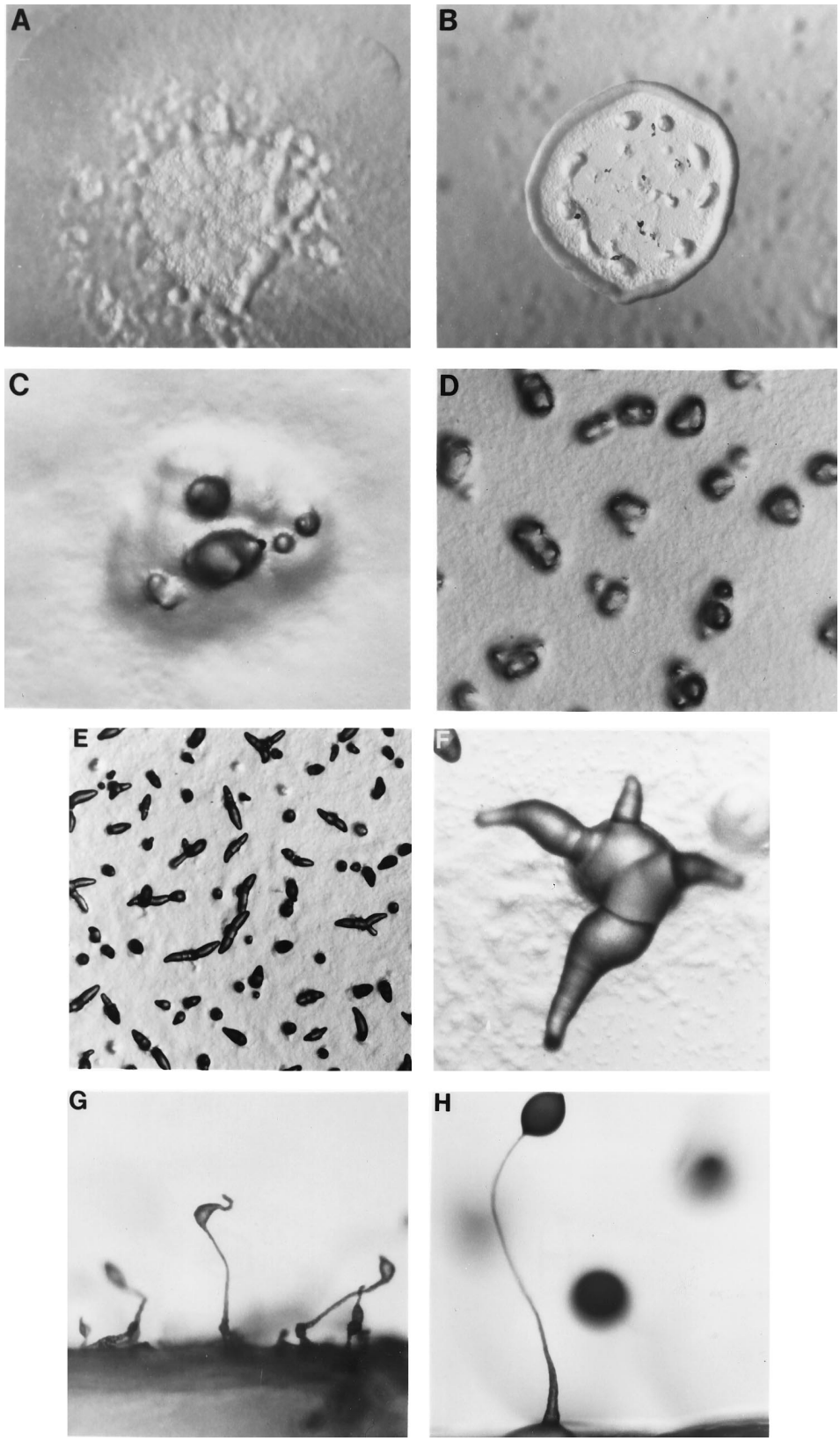

FIG. 6. Photographs of Δ*Ddpik1* Δ*Ddpik2* phenotypes. (A and B) Photographs of plaques in a bacterial lawn of the double-knockout (A) and wild-type cells (B). (C and D) Enlargements of a single developing aggregate (C) and multiple aggregates (D) after bacteria are cleared and cells start to develop. (E through H) Cells were grown attached to petri dishes, washed, and plated for development on nonnutrient agar. (E and F) Cells at the time of tip formation (~12 to 14 h) showing multiple aggregates (E) and a single aggregate (F). (G) Abnormal fruiting body that forms at low frequency. (H) Wild-type fruiting body as a comparison. Photographs of fruiting bodies were taken at the same magnification.

When wild-type *D. discoideum* cells are plated at low density in association with bacteria on nutrient agar plates, the bacteria form a confluent lawn, and the *D. discoideum* cells will form clonal plaques within this lawn as the bacteria are digested. The *D. discoideum* cells then initiate multicellular development in the center of the enlarging plaque as they starve when the bacteria are depleted. The Δ*Ddpik1* Δ*Ddpik2* null cells took 1 to 2 days longer to initiate development once the bacteria were depleted, and only a small fraction of the *D. discoideum* cells within plaques aggregated. The aggregates formed multiple tips that resolved into independent slugs that produced very abnormal fruiting bodies (Fig. 6C and D). The spores were smaller than those of wild-type cells but were fully detergent and heat resistant (data not shown). When cells grown on petri plates in axenic growth medium were washed free of the growth medium and plated on nonnutrient $NaPO_4$-KPO_4 agar plates to initiate multicellular development, the cells also formed mounds with multiple tips (Fig. 5E and F) and similar abnormal fruiting bodies (Fig. 5G). When cells were plated at a density of $>1 \times 10^6$ to 2×10^6 cells per cm^2, most cells participated in aggregate formation; however, when they were plated at lower densities, but under conditions in which wild-type cells developed normally, many of the Δ*Ddpik1* Δ*Ddpik2* null cells did not aggregate.

In addition, we attempted to make double-knockout strains of *DdPIK3* with either *DdPIK1* or *DdPIK2*. The knockouts were attempted in both directions, starting with a *Ddpik3* null strain or a *Ddpik1* or *Ddpik2* null strain. No double knockouts were identified by Southern blot analysis of isolated clones. The results suggest that the double knockouts are lethal.

Function of *DdVPS34* in *D. discoideum*. The DdVPS34 protein is most closely related to the *S. cerevisiae* and *S. pombe* Vps34 proteins (28, 63). To determine if the *D. discoideum* gene encodes a functional Vps34p homolog, we cloned the *DdVPS34* ORF into the *S. pombe* expression vector pART1 (45) and transformed this construct into an *S. pombe* strain carrying a deletion of the *VPS34* gene, which results in a temperature-sensitive growth defect (inability to grow at 37°C) (63). The transformed strain grew at 37°C whereas a control transformant did not (data not shown), indicating that *DdVPS34* can complement the *SpVPS34* deficiency and that *DdVPS34* encodes a Vps34p homolog. The *DdVPS34* gene did not complement an *S. cerevisiae* Δ*vps34* mutant; however, we could not confirm the expression of the *D. discoideum* gene product (data not shown).

To examine the function of *DdVPS34* in *D. discoideum*, we attempted to create a gene knockout by homologous recombination. The disruption plasmid pK5D (63) was linearized either with *Hin*dIII and *Eco*RI or with *Alw*NI and *Apa*I for transformation of the *D. discoideum* JH10 (see above), and thymidine auxotrophs were selected. Genomic DNA was isolated from 30 independent isolates and examined by Southern blot analysis to identify potential *DdVPS34* gene disruptants (Fig. 7A). The analysis showed that the *DdVPS34* gene and surrounding genomic region is duplicated, as has been seen for several *D. discoideum* genes. Fourteen single gene disruptants were obtained, while no strains showed disruptions of both copies. These strains showed no growth or developmental abnormalities compared with wild-type cells.

For several duplicated genes that have been examined, double knockouts have been obtained with a frequency that is about equal to the frequency of obtaining single-gene knockouts (32, 41, 61), while in another case, only a single copy could be disrupted. Further examination of this gene by antisense analysis suggested that the gene was essential for growth (8). To see if this was indeed the case for *DdPIK5*, the 1,825-bp *Bgl*II-*Eco*RI fragment from the *DdPIK5-9* cDNA was ligated into EXP4(+) (18), which drives transcription of the inserted DNA fragment from the *ACT15* promoter in the antisense direction. When the wild-type strain was transformed with pAT5 and selected on nutrient agar plates containing G418 in association with *Escherichia coli* B/r Neo^r cells, a large number of isolates were obtained. Eleven isolates were randomly selected, and their total RNA was isolated for Northern analyses using *DdVPS34* as a probe. Three representative isolates are shown in Fig. 7B. Two antisense transcripts were detected; one was slightly shorter than the endogenous *DdVPS34* mRNA. [This was the only band that was detected in cells transformed with vector EXP4(+) alone.] The other antisense RNA was much shorter. Isolates 1 and 3 were more drug resistant than isolate 2. Clone 2 grew at 10 μg of G418 per ml but not 40 μg/ml like strains 1 and 3. The expression levels of the antisense transcripts correlated with drug resistance. The endogenous *DdVPS34* mRNA was detectable in all three clones, but the level was significantly lower in isolates 1 and 3 than in clone 2.

All isolates overexpressing *DdVPS34* antisense RNA had abnormal growth and developmental phenotypes. The colonies of antisense isolates grown on bacterial pates (grown on *E. coli* B/r, in the presence or absence of G418, or grown on *K. aerogenes* without G418) were severalfold smaller than those of strains transformed with vector alone for the same amount of time, indicating that the antisense transformants grow more slowly on bacteria (data not shown). *D. discoideum* cells initiate development on bacterial plates when the bacteria in the immediate area of the *D. discoideum* cells are exhausted (see above). When development was examined, the cells developed abnormally, showing defects in stalk morphology. The phenotypes were more severe when cells were grown in the presence of *E. coli* B/r and G418 selection (Fig. 7C). In contrast, isolates grew normally in suspension culture in the presence of G418 and developed normally when plated on nonnutrient $NaPO_4$-KPO_4 agar plates, suggesting that the cells were impaired in the ability to grow on bacteria but not liquid medium.

Analysis of *DdPIK4*. We attempted but were unable to make gene disruptions of *DdPIK4*, suggesting that it might be essential for growth.

DISCUSSION

We have reported the first molecular genetic analysis of putative PI kinases from *D. discoideum*, three of which are more closely related to mammalian p110 PI 3-kinase family members than to any other protein in the databases. Although we have not biochemically shown that the genes that we have cloned encode bona fide PI kinases, the very high amino acid sequence homology to known PI kinases within the expected catalytic domain (see below) suggests that the *D. discoideum* proteins have enzymatic activities similar to those of related proteins found in yeasts and mammals. We further present, using molecular genetic approaches, evidence for a role of these *D. discoideum* genes in growth and developmental processes. Our sequence analysis suggests that DdPIK1, -2, and -3 are homologs of the mammalian PI 3-kinases, which include p110 PI 3-kinases that can use PI(4)P, PI(4,5)P_2, and PI as substrates (58). This class of mammalian PI 3-kinases has been shown to be activated through interactions with receptor tyrosine kinases and G proteins (see the introduction). At present, there is no evidence for the presence or absence of a p85 homolog (comparable to the p85-p110 complex in mammalian cells) or other interacting, regulatory protein in *D. discoideum*. The only evidence that *DdPIK1*, *-2*, and *-3* encode PI 3-kinase II type PI 3-kinases is from sequence comparison.

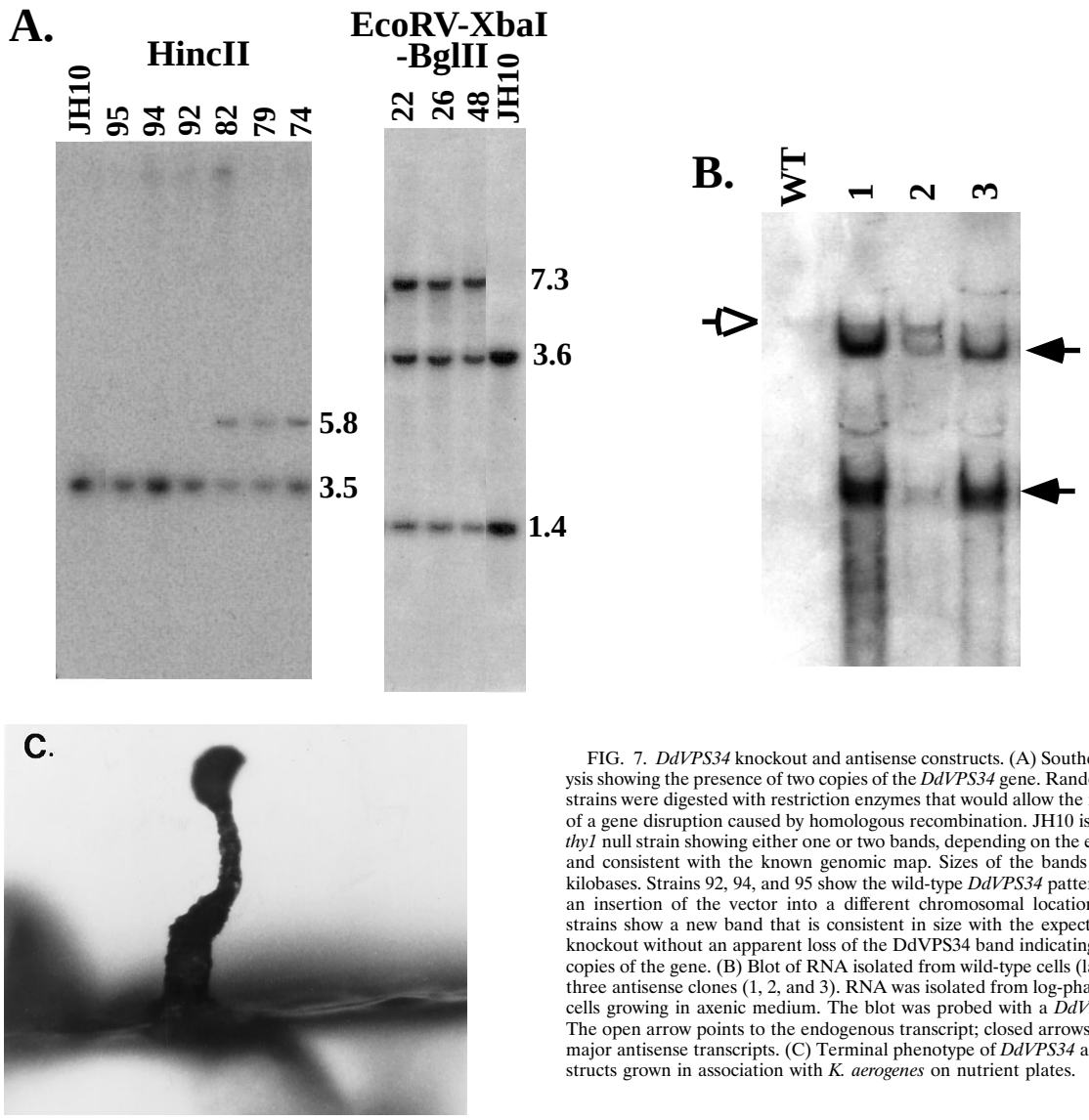

FIG. 7. *DdVPS34* knockout and antisense constructs. (A) Southern blot analysis showing the presence of two copies of the *DdVPS34* gene. Randomly isolated strains were digested with restriction enzymes that would allow the identification of a gene disruption caused by homologous recombination. JH10 is the parental *thy1* null strain showing either one or two bands, depending on the enzymes used and consistent with the known genomic map. Sizes of the bands are given in kilobases. Strains 92, 94, and 95 show the wild-type *DdVPS34* pattern, indicating an insertion of the vector into a different chromosomal location. The other strains show a new band that is consistent in size with the expected sizes in a knockout without an apparent loss of the DdVPS34 band indicating at least two copies of the gene. (B) Blot of RNA isolated from wild-type cells (lane WT) and three antisense clones (1, 2, and 3). RNA was isolated from log-phase vegetative cells growing in axenic medium. The blot was probed with a *DdVPS34* cDNA. The open arrow points to the endogenous transcript; closed arrows point to two major antisense transcripts. (C) Terminal phenotype of *DdVPS34* antisense constructs grown in association with *K. aerogenes* on nutrient plates.

However, homology between the *D. discoideum* and mammalian proteins extends significantly beyond the putative PI kinase core domain, which we expect is the catalytic domain, since changes in this region of the *ScVPS34* gene were shown to decrease catalytic activity (53). Our sequence analysis also suggests that *DdPIK4* encodes a PI 4-kinase. *DdVPS34* appears to encode a Vps34 PI 3-kinase homolog, as determined by both sequence comparison and the fact that it partially complements an *S. pombe* Δ*vsp34* mutant.

It is interesting that mammalian p110 PI 3-kinase homologs, as determined by sequence comparison, are found in *D. discoideum*, but not in either *S. pombe* or *S. cerevisiae*, by either biochemical or molecular analysis (53, 55, 64). *D. discoideum* has a complex life cycle with independent vegetative growth and multicellular developmental phases. Moreover, the development and growth of *D. discoideum* are regulated by complex signaling pathways involving both G protein-coupled receptor pathways and a growth response-activated tyrosine kinase that may have functions similar to those of growth factor receptors in mammalian cells (14, 33). It is possible that the p110 family members are important for this increased complexity compared with *S. pombe* or *S. cerevisiae* proteins. Protein sequence comparisons have suggested that *D. discoideum* is more closely related to metazoans than is either *S. pombe* or *S. cerevisiae* (43). It is probable that the p110 family may have evolved before both *Dictyostelium* species and metazoans branched from other organisms. Assuming that *DdPIK1*, *-2*, and *-3* encode PI 3-kinases, one might expect that they could use PI, PI(4)P, and PI(4,5)P_2 as substrates, like mammalian p110 (7, 10, 58), although Vps34p uses only PI as substrate both in vitro and in vivo (39, 53, 56). In *D. discoideum* extracts, no activity against PI(4)P and PI(4,5)P_2 was detected in crude extracts (unpublished observation); however, *DdPIK1*, *-2*, and *-3* are all expressed at very low levels, as determined by the inability to detect transcripts by using RNA blot hybridization, and therefore the biochemical activity may be difficult to assay in crude extracts.

Cellular functions of PI 3-kinases in *D. discoideum*. We used a combination of molecular genetic approaches to examine the possible functions of PI kinase I family members during growth

and development in *D. discoideum*. *DdVPS34* is most closely related to *VPS34* genes in *S. pombe* and *S. cerevisiae*; the *S. cerevisiae* gene is essential for protein sorting to the vacuole. It is reasonable to expect that homologs function in protein targeting to lysosomes in other species (63). A complete *DdVPS34* gene knockout was not possible in *D. discoideum*, but antisense expression of *DdVPS34* caused a growth defect in bacteria but not in axenic medium. The growth defect on bacteria may indicate the inability to properly metabolize bacteria as a food source. Growth on axenic medium may be less affected if partial activity is present, since the medium contains high concentrations of metabolites that would not require breakdown in lysosomes. *DdVPS34* may be responsible for sorting digestive enzymes into the *D. discoideum* lysosome. The remarkable sequence conservation within the PI 3-kinase I subfamily, which we expect has common enzymatic functions, also suggests that mechanisms of regulation of PI 3-kinase I may be very well conserved. The abnormal developmental morphology caused by antisense expression of *DdVPS34* is specific to bacterially grown cells and thus may be a consequence of the inability of such cells to effectively use bacteria as a food source.

The ability to use knockout and antisense techniques in *D. discoideum* also has given us some insight into the functions of genes that encode proteins which are likely to have enzymatic activities similar to those of mammalian p110 and PI 3-kinase II family members. The analysis in mammalian cells has depended on examining the correlation of p110 activation with cellular responses and examining cells unable to activate the enzyme in response to growth factor/tyrosine kinase receptor-activated pathways by expressing excess p85 that does not associate with p110 and therefore blocks PI 3-kinase activation by receptor tyrosine kinases (15). Our analysis with *D. discoideum* tested the effect of loss of constitutive as well as possible ligand-activated enzyme through the construction of null mutants. The analysis of the function of PI 3-kinase II family members is more complex because of the multiple members that have been identified in *D. discoideum* and metazoans. As indicated above, the proteins encoded by the *DdPIK1* and *DdPIK2* genes are more closely related to each other and to the mammalian PI 3-kinase II family members than is the protein encoded by the *DdPIK3* gene. We also note that the *DdPIK3* ORF has an extension of the protein sequence C terminal to the kinase domain, in contrast to other known PI 3-kinase genes. *DdPIK1* and *DdPIK2* may be functionally redundant, since a knockout of either gene has no detectable phenotype, whereas a double knockout shows both growth and developmental defects. The knockout of *DdPIK3* had no phenotype. We tried without success to obtain Δ*DdPIK1* Δ*DdPIK3* and Δ*DdPIK1* Δ*DdPIK3* double knockouts by using several selections (data not shown). It is possible *DdPIK1*/*2* and *DdPIK3* encode different but overlapping functions and that a loss of either *DdPIK1* or *DdPIK2* in the absence of *DdPIK3* is lethal.

The Δ*Ddpik1* Δ*Ddpik2* double-knockout cells showed an unusual plaque morphology when grown on nutrient agar plates in association with bacteria. They were unable to grow axenically in suspension culture but could grow slowly when cells were grown axenically attached to plastic petri plates, although the cells were small. In addition, the cells had a striking developmental phenotype, producing mounds with multiple tips and abnormal fruiting-body morphology. We have shown that mutations in a number of known genes result in a multiple-tip phenotype. These mutations include a RasD (G12T)-activating mutation (51) and overexpression of the phosphotyrosine phosphatase PTP1 (32). The downstream pathways requiring PI 3-kinase II activities that result in these phenotypes are not known. It is interesting, however, that cytoskeletal changes correlate with stimulation of PI 3-kinase activity in neutrophils (48, 49) and that PI 3-kinase may be involved in platelet-derived growth factor receptor internalization (35, 37) and antibody secretion in B cells (24). However, direct genetic evidence for a role of PI 3-kinase activity in these functions is lacking. Whether the morphological phenotypes in *D. discoideum* are related to these functions is not known.

The presence of the growth and developmental phenotypes should allow further functional analysis of the PI kinase family members, including examination of redundancy through expression studies in the Δ*Ddpik1* Δ*Ddpik2* null background, structure-function studies of the *D. discoideum* PI 3-kinase family members, and examination of whether metazoan PI 3-kinase gene family members can complement the *D. discoideum* mutations. The cloned PI 3-kinase II family members may be regulated through tyrosine kinases. Recently a Gβγ-activated PI 3-kinase enzymatic activity also has been identified (60). Sequence comparison of this enzyme to the *D. discoideum* putative PI 3-kinase II genes will be of interest, as G-protein-coupled receptor-mediated signaling pathways play essential roles in regulating *D. discoideum* development (14, 26). Further analysis of the growth and developmental phenotypes of the PI 3-kinase II family members in *D. discoideum* should identify the potential signaling pathways regulating their activity. This determination may give further insight into the overall functions of these enzymes in other eukaryotes.

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