A Diverse Family of Inositol 5-Phosphatases Playing a Role in Growth and Development in Dictyostelium discoideum* [S]

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Inositol phosphate-containing molecules play an important role in a broad range of cellular processes. Inositol 5-phosphatases participate in the regulation of these signaling molecules. We have identified four inositol 5-phosphatases in Dictyostelium discoideum, Dd5P1–4, showing a high diversity in domain composition. Dd5P1 possesses only an inositol 5-phosphatase catalytic domain. An unique domain composition is present in Dd5P2 containing a RCC1-like domain. RCC1 has a seven-bladed propeller structure and interacts with G-proteins. Dd5P3 and Dd5P4 have a domain composition similar to human Synaptojanin with a Sac1 domain and OCRL with a RhoGAP domain, respectively. We have expressed the catalytic domains and show that these inositol 5-phosphatases have different substrate preferences. Single and double gene inactivation suggest a functional redundancy for Dd5P1, Dd5P2, and Dd5P3. Inactivation of the gene coding for Dd5P4 leads to defects in growth and development. These defects are restored by the expression of the complete protein but not by the 5-phosphatase catalytic domain.

Inositol phosphates play a role in a variety of eukaryotic cellular processes, including chemotaxis and membrane trafficking. They are regulated by a number of enzymes. The group of phosphatidylinositol 3-kinases (PI3K) phosphorylates the lipid substrates PI, PI(4)P, and PI(4,5)P2 at the 3-position of the inositol ring (1). The lipid product PI(3,4,5)P3 has been shown to mediate chemotaxis and, in particular, the localization of PH-domains. In stimulated B-cells, SHIP1 accounts for the major phosphatase activity toward PI(3,4,5)P3, and inactivation of SHIP1 leads to an increased and prolonged PI(3,4,5)P3 production (9). Other inositol 5-phosphatases have been shown to play important roles in a number of cellular processes. Mutations in the inositol 5-phosphatase OCRL are responsible for Lowe syndrome in human (10), and deletion of the presynaptic inositol 5-phosphatase Synaptotagmin leads to neurological abnormalities and early death of mice (11).

In the social amoeba, Dictyostelium discoideum motility toward folate acid and cAMP is an essential strategy for survival (12). Several observations suggest that phosphatidylinositol phosphates mediate chemotaxis and, in particular, the localization of the signal inside D. discoideum cells. The PH domains of a number of proteins involved in chemotaxis, including CRAC, Akt/PKB, and PhdA, have been shown to transiently localize at the leading edge of cells moving in a chemotactic gradient (13–15). As these PH domains bind to PI(3,4,5)P3 and PI(3,4)P2 symmetric lipid distribution is implicated by these observations. In p3k1/2-null cells, a strain with two putative PI3Ks inactivated (16), the transient localization of PhdA can no longer be observed and cells show reduced chemotaxis (15). On the other hand, in PTEN-null cells, a strain in which a putative 3-phosphatase is inactivated, the localization of PH-domains is prolonged and broadened and chemotaxis is also reduced (17, 18). Inositol 5-phosphatases may play an important role in the regulation of the phosphoinositides. As this group of enzymes leads to the degradation of PI(3,4,5)P3 and at the same time formation of PI(3,4)P2, another PH-binding molecule, they can be central players in the metabolic route of these signaling molecules.

Phosphoinositides have also been implicated in endocytosis in D. discoideum. Pichk1/2-null cells are affected with respect to pinocytosis (19), suggesting a role for PI(3,4,5)P3 in this process. The inhibitors of phospholipase C, an enzyme converting PI(4,5)P2 into Ins(1,4,5)P3 and diacylglycerol, reduce the rate of phagocytosis (20, 21). Because inositol 5-phosphatases can act on PI(4,5)P2, PI(3,4,5)P3, and Ins(1,4,5)P3, they are probably important in the endocytic pathway.

To investigate the role of inositol 5-phosphatases in chemotaxis and endocytosis, we cloned and characterized four D. discoideum inositol 5-phosphatases. Catalytic activity was determined, indicating that they act as inositol 5-phosphatases. Single and double gene disruptants were obtained and growth, chemotaxis, and development were studied in these knock-out strains.
Inositol 5-Phosphatases in *D. discoideum*

**EXPERIMENTAL PROCEDURES**

**Identification and Sequence Analysis**—The first putative inositol 5-phosphatase sequence was obtained using degenerated primers complementary to the conserved motifs I and II found in inositol 5-phosphatases (see “Results”), and the PCR product was used to screen a cDNA library kindly provided by Dr. R. A. Firtel. The *D. discoideum* genomic (www.sdsc.edu/mpdr/dicty) and cDNA databases (www.csm. biol.tsukuba.ac.jp) were screened for other putative inositol 5-phosphatases using either the conserved motif I or II. Using Sequen from LaserGene (DNAstar), contigs were formed. Additional sequences were obtained by screening a cDNA library, kindly provided by Dr. R. H. Gomer, with a PCR fragment containing part of the catalytic domain (used primers: Dd5P2: 5P2S1 + 5P2R1; Dd5P3: 5P5S1 + 5P5R1; and Dd5P4: 5P4S1 + 5P4R1, see “Appendix A”). The longest clones obtained for Dd5P3 (4212 bp) and Dd5P4 (2795 bp) encode the complete open reading frame of 1377 and 875 amino acids, respectively. For Dd5P2, the longest clone only encoded for the amino acids 1–599. In combination with data from the genomic database, a complete open reading frame of 1794 amino acids was constructed. The longest clone obtained for Dd5P1 encodes for 411 amino acids and 1–678; the missing part of the 5′-open reading frame was obtained from the data base. A comparison of the sequence obtained from cDNA clones with the genomic database sequences revealed the presence of one intron in each inositol 5-phosphatase gene (Dd5P1: nucleotides 397–499; Dd5P2: nucleotides 53–187; Dd5P3: nucleotides 241–384; and Dd5P4: nucleotides 1357–1622) (GenBank accession numbers AY184992, AY184993, AY184994, and AY184995, respectively). BLAST, Smart, Pfam, and Expasy programs were used to analyze the obtained sequences. Alignments were made using Megalign (DNAstar) and Genedoc (Internet) and optimized by manual adjustment.

**Strains and Growth Conditions**—The *D. discoideum* strains AX3 (wild type), DH1 (uracil auxotroph wild type), and the mutant strains Y9262 (wild type), DH1 (uracil auxotroph wild type), and the mutant strains AY184995, respectively. BLAST, Smart, Pfam, and Expasy programs were used to analyze the obtained sequences. Alignments were made using Megalign (DNAstar) and Genedoc (Internet) and optimized by manual adjustment.

**Activity Measurements**—Activity toward Ins(1,4,5)P3 and Ins(1,3,4,5)P4 was determined as described previously (23). 5 μl (diluted) of enzyme was added to the reaction mixture (50 mM HEPES, pH 7.4, 2 mM MgCl2, 48 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin), containing either 100 μM Ins(1,4,5)P3 (“His” Ins(1,4,5)P3) or 10 μM Ins(1,3,4,5)P4/“His”Ins(1,3,4,5)P4. After 15 min of incubation the reaction was stopped and the products were separated on Dowex columns. Activity toward PI(4,5)P2 and PI(3,4,5)P3 was determined as described previously (24). 50 μg of phosphatidyl serine and 10 μg of [γ-32P]ATP (3000 Ci/mmol) in reaction buffer (50 mM Tris-HCl, pH 7.4, 1.5 mM dithiobis, 100 μM NaCl, 0.5 mM EDTA, 5 mM MgCl2, 100 μM ATP). After 30 min, the reaction was stopped and the products were extracted. Lipids were separated by TLC and visualized by exposure to film or PhosphorImager. In the case of the PI(4,5)P2 assay, the spots were visualized using 10% acetic acid. For the kinetic analysis, the purified enzyme was induced at 22°C using 1 mM isopropyl-1-thio-

**RESULTS**

Identification of Four Inositol 5-Phosphatase Genes in *D. discoideum*—Four putative inositol 5-phosphatases in *D. discoideum* were identified by performing a PCR with degenerate primers. The obtained PCR product was used as probe for cDNA library screening. The gene found using this method was called Dd5P1 (*D. discoideum* 5-phosphatase 1) and codes for a protein of 678 amino acids (see “Appendix B”). To identify other putative inositol 5-phosphatases in *D. discoideum*, we searched the *D. discoideum* database for sequences showing homology to the conserved motifs I and II, WZGDYXN/Y/R/P and P/A/S/W/C/T/DR/G/V/L, respectively, which are characteristic for inositol 5-phosphatases (6). Using the partial sequence obtained from the data base, complete sequences were obtained from cDNA library screens. Three putative inositol 5-phosphatases were identified (see “Appendix B”), coding for proteins consisting of...
The other two inositol 5-phosphatases identified in *D. discoideum* are homologous to human inositol 5-phosphatases. Dd5P3 resembles the synaptojanin-like proteins found in both human and yeast with the highest BLAST score of the catalytic domain with that of INP5P2 from fission yeast (37% identity, 59% similarity) (34). Similar to human Synaptojanin and yeast INP5P2, Dd5P3 has a SacI-like domain including the conserved RXNCXDCRTNM motif (35) in front of the inositol 5-phosphatase domain (see “Appendix E”). The SacI domains of Synaptojanin and INP5P2 have been shown to remove the phosphate group of PI(4)P, PI(3)P, and at a low rate, both phosphates of PI(3,5)P2 (36). The long C-terminal part of Dd5P3 does not have any homology with known domains and consists of poly(Q) and poly(N) repeats.

Dd5P4 is homologous to human OCRL (10) and INPP5b (37, 38), consisting of a inositol 5-phosphatase domain followed by a RhoGAP domain (see “Appendix F”). The catalytic domain has the highest BLAST score with the catalytic domain of human INP5Pb (44% identity, 60% similarity). RhoGAP domains are known to catalyze the GTPase activity of Rho proteins. The crystal structure of human RhoGAP has been solved indicating a role for two conserved amino acids, Arg-585 and Asn-194 (58) and in GAP-activated GTP hydrolysis (39). The role of these amino acids is supported by mutational analysis (39, 40). Mutational analysis has also shown that the conserved Arg is not predominantly involved in the binding of Rho proteins (40). In Dd5P4, the Arg and the Asn are substituted by an Ile and Gln, respectively. This observation may suggest that Dd5P4 does not exhibit high RhoGAP activity, but may still bind Rho proteins.

**Different Transcription Levels during Development**—To determine the transcription levels of the four *D. discoideum* inositol 5-phosphatases, Northern blot analysis was performed. Very low transcription levels were observed for the four inositol 5-phosphatases in all stages of the *D. discoideum* life cycle (Fig. 3). Dd5P1 and Dd5P3 were equally transcribed in all stages with the exception of the even lower transcription of Dd5P3 in the vegetative stage. The levels of transcription of Dd5P2 were higher during aggregation than during growth and multicellular development. The transcription of Dd5P4 was relatively high during growth, decreased during aggregation, and returned to almost vegetative levels in the multicellular stages. Furthermore, a smaller transcript of 2.5 kilobases was observed in the multicellular stages.

**Activity toward Soluble and Lipid Substrates of *D. discoideum* Inositol 5-Phosphatases**—To determine whether the four putative inositol 5-phosphatases identified in *D. discoideum* can function as inositol 5-phosphatases, the catalytic activity and specificity of the inositol 5-phosphatase domains were studied *in vitro*. The catalytic domains were expressed as His tag fusion proteins in *E. coli* and purified. The protein of the expected size could be detected by Western blot analysis for Dd5P1–3 (Fig. 4). The Western blot analysis performed for Dd5P4 showed a band at a higher position than expected (62 instead of 55 kDa) or, but the purified protein did show inositol 5-phosphatase activity. Unfortunately, because of the very low expression levels obtained for the catalytic domain of Dd5P1, no activity could be determined for this protein.

The purified catalytic domains of Dd5P2, Dd5P3, and Dd5P4 were incubated with the phospholipids PI(4,5)P2 and PI(3,4, 5)P3 and the water-soluble inositol phosphates Ins(1,4,5)P3 and Ins(1,3,4,5)P4. Substrate degradation was quantified using thin-layer chromatography for the phospholipids and ion-exchange chromatography for the water-soluble inositol phosphates. The results are summarized in Fig. 5 demonstrating good degradation of PI(4,5)P2 by all inositol 5-phosphatases.
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The activity of the four enzymes for each substrate indicates the specificity of inositol 5-phosphatases. PI(3,4,5)P3 is degraded predominantly by Dd5P2 and Dd5P4, but not by Dd5P3. Good degradation of the water-soluble inositol phosphates Ins(1,4,5)P3 is the catalytic domain of Dd5P2. This protein, compared with the other proteins, is poor in dephosphorylation of lipid-soluble substrates. This substrate specificity is almost opposite to the specificity of the homologue Dd5P3. This protein, compared with the other proteins, is poor in dephosphorylation of lipid-soluble substrates. Whereas Ins(1,4,5)P3 is degraded predominantly by Dd5P2 and also by Dd5P4 but not by Dd5P3. Good degradation of the water-soluble inositol phosphates Ins(1,4,5)P3 and Ins(1,3,4,5)P4 is observed for Dd5P2, Dd5P3, and Dd5P4, showing the best degradation of Ins(1,4,5)P3 by Dd5P3, whereas Ins(1,3,4,5)P4 is best degraded by Dd5P2 (Fig. 5C). Comparing the activity of the four enzymes for each substrate indicates the relatively preferred substrates (Table I). The protein with the highest activity toward Ins(1,4,5)P3 is the catalytic domain of Dd5P2. This protein, compared with the other proteins, is poor in dephosphorylation of lipid-soluble substrates. This substrate specificity is almost opposite to the specificity of the homologue INP51p, which degrades PI(4,5)P2 but does not hydrolyze Ins(1,4,5)P3. Dd5P2, in contrast, is a very good PI(3,4,5)P3- and Ins(1,3,4,5)P4-metabolizing enzyme.

Gene Inactivation Leads to Defects in Growth and Development—Upon starvation, D. discoideum wild-type cells show chemotaxis toward the cAMP secreted by other starving cells. The formed aggregate develops into a migrating slug or fruiting body. The spores of the fruiting body are resistant to severe conditions and mature into single amoeba under better conditions. To get an indication of the function of inositol 5-phosphatases in D. discoideum, the four identified inositol 5-phosphatase genes have been knocked out. The effect of gene inactivation on chemotaxis has been investigated by determining the response of cells toward different cAMP concentrations using the small-droplet chemotaxis assay. Single gene inactivation of either Dd5P3 or Dd5P4 or double gene inactivation of Dd5P2 and Dd5P3 (Dd5P2/3−) did not negatively affect chemotaxis, whereas single disruption of Dd5P1 or Dd5P2 slightly improved chemotaxis (Fig. 6). Also, the double disruption of either Dd5P1 and Dd5P2 (Dd5P1/2−) or Dd5P1 and Dd5P3 (Dd5P1/3−) resulted in slightly improved chemotaxis. Growth and development of the cells were also studied. The single gene inactivation of Dd5P1, Dd5P2, or Dd5P3 did not result in any observable difference in growth rate in axenic medium or on bacterial lawns (data not shown). The single disruptants Dd5P1−, Dd5P2−, and Dd5P3− were deposited on non-nutrient agar plates or grown on bacterial lawns to study the development of the cells. Aggregation of the cells proceeded at a rate
Comparable to wild-type cells. Slug formation was also normal as well as the formation of fruiting bodies. The spores that were formed were viable (data not shown). Also, Dd5P1Δ, Dd5P13′, or Dd5P23′ cells showed no defect in growth or development.

The single disruption of Dd5P4 did affect growth and development. The growth rate in axenic medium was significantly lower for Dd5P4 cells compared with wild-type AX3 cells (Fig. 7). Whereas the doubling time of wild-type cells is ~15 h in shaking culture, the doubling time of Dd5P4-deficient cells is 41 h. The growth rate on bacterial lawns was affected as well for Dd5P4Δ cells. Amoeba were deposited on a bacterial lawn and incubated at 22 °C. Approximately 90% wild-type amoeba formed visible plaques within 4 days, whereas <1% Dd5P4Δ cells formed visible plaques after 9 days. The development of the Dd5P4Δ cells when grown on bacterial lawns was affected as well. The inactivation of Dd5P4 lead to the formation of multiple-tipped aggregates (Fig. 8).

Overexpression of Full-length Dd5P4 but Not the Separate Domains Rescues Dd5P4Δ Cells—The overexpression of the full-length Dd5P4 restored the defects of Dd5P4Δ cells in growth and development, confirming that the disruption of Dd5P4 was indeed responsible for the observed defects. Growth rate in axenic medium was comparable to the growth rate of wild-type cells (AX3) with a doubling time of 15 h (Fig. 7). When grown on bacterial plates, ~90% of the amoeba formed visible plaques within 4 days, and the multiple-tipped phenotype observed for the Dd5P4Δ cells could not be observed anymore (Fig. 8). Overexpression of the inositol 5-phosphatase catalytic domain or the RhoGAP domain of Dd5P4 in the Dd5P4 knock-out strains (Dd5P4Δ/cat5P4 and Dd5P4Δ/GAP5P4, respectively) did not restore any of the defects. As can be seen in Fig. 7, growth rate in axenic medium was not enhanced by overexpression of either one of the domains. Also, growth rate and development on bacterial lawns were not restored (data not shown).

**DISCUSSION**

The implicated role of phosphoinositide molecules in signal localization has lead to an increased interest in metabolizing enzymes such as inositol 5-phosphatases. The amount of data on mammalian inositol 5-phosphatases has expanded rapidly over the last few years, showing important functions for inositol 5-phosphatases in several processes (6).

Human inositol 5-phosphatases are divided in two groups. Type I inositol 5-phosphatases only convert the water-soluble substrates Ins(1,4,5)P3 and Ins(1,3,4,5)P4. They do not convert lipid substrates. Type II inositol 5-phosphatases do convert phosphoinositides (e.g. PI(4,5)P2 and PI(3,4,5)P3), and in most cases, they also convert water-soluble substrates. The insertions present in Type I enzymes may prevent the enzymes from binding to the membrane surface, which may explain the differences in substrate specificity between Types I and II inositol.
The four inositol 5-phosphatases in *D. discoideum* show a high diversity in domain composition compared with the evolutionary position of this organism between plants and yeast. A homologue of OCRL (*Dd5P4*) and a homologue of Synaptojanin (*Dd5P3*) were identified. Also, a unique combination of a RCC1-like domain in front of the inositol 5-phosphatase domain was identified (*Dd5P2*). This combination of domains has not been found in any other protein reported or present in the GenBank™ so far.

Knock-out strains with one or a combination of two inactive genes for *Dd5P1*, *Dd5P2*, and *Dd5P3* show no defects in growth and development, suggesting a redundancy among these proteins. Chemotaxis toward cAMP is similar or slightly improved in all of the inositol 5-phosphatase knock-out cell lines compared with wild type. Recently, it has been shown that the 3-phosphatase PTEN is the major P(3,4,5)P3/P(3,4)P2-degrading enzyme. It has been suggested that inositol 5-phosphatases add an addition layer of regulation of these molecules, fine-tuning the chemotactic signal (42). Our results support the role of the group of inositol 5-phosphatases as a minor negative regulator of chemotaxis in *D. discoideum*. It will be interesting to study the effects of either overexpression or inactivation of the inositol 5-phosphatases in a PTEN-null background.

Inactivation of *Dd5P4* resulted in defects in growth and development. The reduced growth rate, either in axenic culture or grown on bacterial plates, suggests a role for *Dd5P4* in endocytosis. Development is also affected as the cells form multiple-tipped aggregates. The relative high transcription levels of *Dd5P4* at vegetative and multicellular stages support the role of *Dd5P4* in growth and development. Knock-out strains of PI3K1 + 2 are defective in chemotaxis, growth, and development (15, 16). The cells grow slowly on bacterial lawns and in axenic medium and form multiple-tipped aggregates resembling the phenotype of *Dd5P4*. No enzyme activity measurements have been reported for the PI3K in *D. discoideum*, but they have been suggested to catalyze the formation of P(3)P, P(3,4)P2, and P(3,4,5)P3 on basis of sequence homology. Our results support a role for P(3,4,5)P3 and/or P(3,4)P2 in growth and development; *Dd5P4* regulates the levels of these phospholipids by degradation of P(3,4,5)P3 and production of P(3,4)P2. On the other hand, the effect of inactivation of *Dd5P4* could also be assigned to its action on either P(3,5)P2 or Ins(1,4,5)P3, two signaling molecules also implicated in endocytosis (20, 21). In addition, the RhoGAP domain could be responsible for the function of *Dd5P4* in endocytosis. Rho proteins have been shown to play a role in both development and growth. The defects in growth and development of *Dd5P4* cells can be rescued by overexpression of the full-length protein. These defects can not be restored by transfection of *D. discoideum* cells with an expression vector containing either the inositol 5-phosphatase catalytic domain or the RhoGAP domain. Although we cannot exclude that the domains are not properly expressed or folded, the fact that the inositol 5-phosphatase catalytic domain expressed in *E. coli* is catalytically active would suggest that inositol 5-phosphatase activity is not sufficient to restore the defects of *Dd5P4* cells. It is possible that the RhoGAP and inositol 5-phosphatase catalytic domain act together to perform its function in growth. The binding of a Rho protein to the RhoGAP domain could affect the inositol 5-phosphatase activity. This would lead to a direct interaction between the Rho and phosphoinositide pathways. It would be interesting to see if and which Rho protein binds to the GAP domain of *Dd5P4*.

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**Fig. 7. Growth rate of wild type and mutant strains.** Doubling times were 15 h for wild-type AX3, 41 h for *Dd5P4*–, 15 h for *Dd5P4*/Dd5P4, 47 h for *Dd5P4*/cat5P4, and 48 h for *Dd5P4*/GAP5P4.

**Fig. 8. Development of wild-type AX3, *Dd5P4*–, and *Dd5P4*/Dd5P4 on *K. aerogens*.** Pictures were taken of tipped aggregates using ×40 magnification.

5-phosphatases (28). The insertions present in Type I inositol 5-phosphatases are not present in either one of the *D. discoideum* inositol 5-phosphatases. As expected by this sequence analysis, the *D. discoideum* 5-phosphatases *Dd5P2*, *Dd5P3*, and *Dd5P4* catalyze the dephosphorylation of both water-soluble and lipid substrates, which classify them as Type II inositol 5-phosphatases. The *D. discoideum* genome has been sequenced to near completion, making it unlikely that more inositol 5-phosphatases containing the motifs WXGDXN/V/F/R and P(A/S)/W(C/T)/DR/(I/V)/L are present. Therefore, probably no Type I and only four Type II inositol 5-phosphatases are present in *D. discoideum*.

Type II inositol 5-phosphatases can be divided in three subgroups on the basis of domain composition. SHIP1 represents the group of SH2 domain containing inositol 5-phosphatases Synaptotagmin, the Sac1 domain containing enzymes, and OCRL, the RhoGAP domain containing inositol 5-phosphatases (Fig. 1). Mammals contain all three groups of proteins. Six non-mammalian organisms containing putative inositol 5-phosphatases have been sequenced completely so far. The metazoa *C. elegans* and *D. melanogaster* lack inositol 5-phosphatases containing a SH2 domain, but both contain one Synaptotagmin-like protein ([41]) and Q9W296, respectively) and one OCRL-like protein (O17590 and O46049, respectively). The diversity in the plant *Arabidopsis thaliana*, the yeast strains *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and the microsporidia *Encephalitozoon cuniculi* is even less. The inositol 5-phosphatases of *A. thaliana* contain only WD40 repeats as additional domains (e.g. Q9SKB7 and O80560), whereas in yeast only Synaptotagmin-like proteins are present (28, 34). The one inositol 5-phosphatase of *E. cuniculi* (CAD25856) only contains the inositol 5-phosphatase catalytic domain. In prokaryotes, no inositol 5-phosphatases have been identified.
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