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Role of phospholipase C in *Dictyostelium*: formation of inositol 1,4,5-trisphosphate and normal development in cells lacking phospholipase C activity

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The micro-organism *Dictyostelium* uses extracellular cAMP to induce chemotaxis and cell differentiation. Signals are transduced via surface receptors, which activate G proteins, to effector enzymes. The deduced protein sequence of *Dictyostelium discoideum* phosphatidylinositol-specific phospholipase C (PLC) shows strong homology with the mammalian PLC-δ isoforms. To study the role of PLC in *Dictyostelium*, a *plc*$^-$ mutant was constructed by disruption of the PLC gene. No basal or stimulated PLC activity could be measured during the whole developmental programme of the *plc*$^-$ cells. Loss of PLC activity did not result in a visible alteration of growth or development. Further analysis showed that developmental gene regulation, cAMP-mediated chemotaxis and activation of guanylyl and adenylyl cyclase were normal. Although the cells lack PLC activity, inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] was present at only slightly lower concentrations compared with control cells. Mass analysis of inositol phosphates demonstrated the presence of a broad spectrum of inositol phosphates in *Dictyostelium*, which was unaltered in the *plc*$^-$ mutant. Cell labelling experiments with [^{3}H]inositol indicated that [^{3}H]Ins(1,4,5)P_3 was formed in a different manner in the mutant than in control cells.

Key words: cAMP/chemotaxis/inositol phosphates/mutant/signal transduction

Introduction

The cellular slime mould *Dictyostelium discoideum* is used as a model organism to study signal transduction processes during growth and development (Devreotes, 1989). It is a haploid eukaryotic micro-organism that grows on bacteria. Upon depletion of the food source a multicellular developmental programme is initiated that starts with aggregation of the individual cells. The amoebae move chemotactically towards a cAMP signal, secreted by the cells in a pulsatile manner. A multicellular slug-like structure is formed in which extracellular cAMP regulates the expression of cell type-specific genes to induce differentiation, resulting in the formation of a fruiting body.

The availability of biochemical and genetic techniques and the presence of *Dictyostelium* mutants with specific defects have made it possible to unravel parts of the signal transduction pathway, which appear to be analogous to the transmembrane signalling pathways of higher eukaryotic cells (reviewed in Van Haastert *et al.*, 1991). Extracellular cAMP applied to cells induces the transient accumulation of intracellular cAMP, cGMP and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] (Roos and Gerisch, 1976; Mato *et al.*, 1977; Wurster *et al.*, 1977; Europe-Finner and Newell, 1987; Van Haastert *et al.*, 1989), resulting from the activation of adenylyl cyclase, guanylyl cyclase and phospholipase C (PLC), respectively.

Four surface receptors that recognize extracellular cAMP have been cloned. These cAMP receptors possess seven putative transmembrane-spanning domains, thus having a topography comparable to that of mammalian G protein-coupled receptors (Klein *et al.*, 1988; Saxe *et al.*, 1991, 1992; Johnson *et al.*, 1992). They are expressed at different times during *Dictyostelium* development. Genes encoding eight G protein α subunits (Pupillo *et al.*, 1989; Hadwiger *et al.*, 1991; Wu and Devreotes, 1991) and one G protein β subunit (Lilly *et al.*, 1993) have been identified. Two structurally distinct adenylyl cyclase genes have been cloned (Pitt *et al.*, 1992). One contains the structure proposed for mammalian adenylyl cyclases, while the other contains a novel structure which resembles that of membrane-bound guanylyl cyclases.

Recently we have reported the cloning of a *Dictyostelium* phosphoinositide-specific PLC (DdPLC) (Drayer and Van Haastert, 1992). In mammalian cells three families of PLC have been identified, classified as β, γ and δ (reviewed in Rhee and Choi, 1992). The corresponding genes share two regions of homology, designated X and Y or A and B (Suh *et al.*, 1988a). In the PLC-β and δ isoforms the two conserved domains are separated by a short variable region. The PLC-γ family contains regions homologous to the nonreceptor tyrosine kinases of the *src* family located between the two conserved domains (Stahl *et al.*, 1988; Suh *et al.*, 1988b). *Dictyostelium* PLC contains the two conserved domains found in other PLC isoforms; structurally it resembles mammalian PLC-δ.

Two independent mechanisms of PLC regulation have been extensively studied. The PLC-γ isoforms are specifically activated by a tyrosine kinase-mediated pathway (Kim *et al.*, 1991; Mohammadi *et al.*, 1992; Rotin *et al.*, 1992), whereas members of the PLC-β family are activated via the α subunits of the G_q class of G proteins (Smrcka *et al.*, 1991; Taylor *et al.*, 1991; Lee *et al.*, 1992). A third pathway is suggested in which the β- and γ-subunits of G proteins are regulators of specific PLC isoforms, PLC-β and PLC-δ_1 (Camps *et al.*, 1992; Katz *et al.*, 1992; Carozzi *et al.*, 1993; Park *et al.*, 1993).

Previous observations in *D.discoideum* suggested that the regulation of PLC is important for chemotaxis and differentiation (Bominaar *et al.*, 1991a; Bominaar and Van

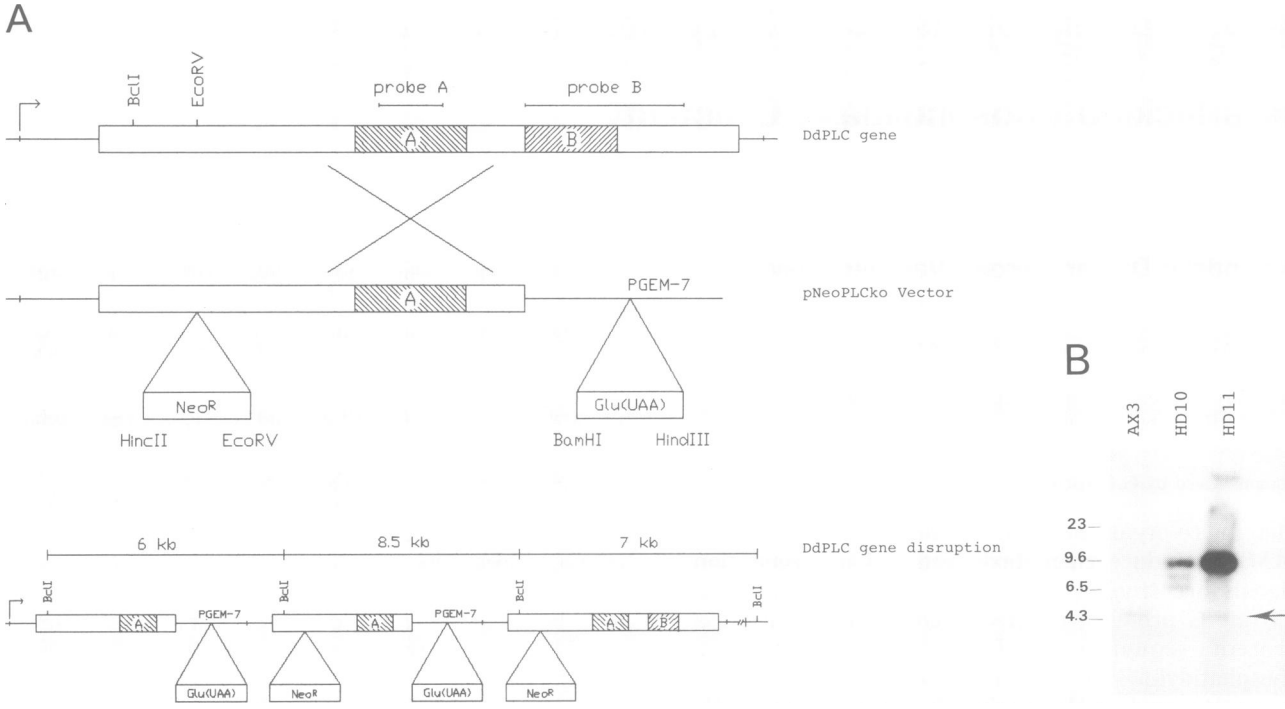

Fig. 1. Construction and genetic analysis of the DdPLC gene disruption mutant. (**A**) Scheme of the disruption of the DdPLC gene. A fusion of the actin 15 and neomycin resistance (Neo^R) genes was inserted into a unique *Eco*RV site of the DdPLC cDNA clone L9. The $tRNA^{Glu(UAA)}$ suppressor gene was inserted in between the *Bam*HI and *Sac*I sites of the pGEM-7 vector 3′ of the DdPLC cDNA. The open bar containing the A and B domains depicts the coding region of DdPLC; the arrow indicates the start of transcription. Positions of DdPLC probes used in further analysis are indicated. The lengths of the fragments generated after *Bcl*I digestion of genomic DNA of HD10 are given in the lower panel. The $tRNA^{Glu(UAA)}$ suppressor gene was included in the gene disruption vector to select for homologous recombination in the DdPLC gene. Transformed cells containing the $tRNA^{Glu(UAA)}$ gene by integration of the whole pNeoPLCko were supposed to be inviable due to incorrect protein synthesis. The results showed that no adverse effects were found in transformed cells that had incorporated the tRNA suppressor gene. (**B**) Southern analysis of DdPLC in AX3 cells (parent strain), HD10 (DdPLC gene disruption mutant) and HD11 (random integrant in AX3). Genomic DNA was digested with *Bcl*I and hybridized with a probe consisting of the conserved A domain of PLC. Numbers on the left indicate size in kb of *Hin*dIII-digested phage λ DNA. The arrow indicates the endogenous DdPLC band.

Haastert, 1993). To study the function of DdPLC in *Dictyostelium* growth and development, a plc^- mutant was made by gene disruption. The mutant we isolated no longer contained any PLC activity, but showed normal growth, chemotaxis and development. Analysis of the inositol phosphates showed that $Ins(1,4,5)P_3$ levels were only slightly reduced in the plc^- mutant.

Results

Disruption of the DdPLC gene

A plc^- mutant was constructed using gene disruption. A vector for gene disruption, named pNeoPLCko, was constructed by inserting a G418 resistance cassette into the internal *Eco*RV site of a DdPLC cDNA clone (Figure 1A). After transformation of the *Dictyostelium* AX3 strain and selection with neomycin, eight clones were further investigated by Southern analysis. The results of two, HD10 and HD11, are presented. As shown in Figure 1B, hybridization of a fragment encoding the conserved A domain of DdPLC to AX3 genomic DNA digested with *Bcl*I gave a 4.5 kb band. Transformant HD10 no longer contained this band, but gave rise to single copy bands at 6 and 7 kb and to a multicopy band at 8.5 kb. The results are consistent with a single crossover event between the pNeoPLCko construct and the DdPLC locus together with integration of multiple copies of the vector (Figure 1A). HD11 has multiple copies of pNeoPLCko integrated into its genome without recombining into the DdPLC locus. Of the eight clones analysed, only HD10 showed recombination within the DdPLC locus.

To verify further the disruption of the DdPLC gene, Northern blots were analysed using a B-domain probe, the sequence of which is not contained in the pNeoPLCko vector. In Northern blots (Figure 2A) no DdPLC transcript could be detected in HD10 when hybridized with the B-domain probe. However, a transcript slightly smaller than normal could be seen when the blot was hybridized with an A-domain probe. A transcript of the same size was detected when the $tRNA^{Glu(UAA)}$ was used as a probe (the endogenous tRNA transcripts present in all samples are smaller and not shown in this figure). We therefore conclude that this transcript in HD10 consists of part of the DdPLC mRNA and the $tRNA^{Glu(UAA)}$.

Western blots (Figure 2B) reveal that anti-DdPLC antibodies recognized a protein of ~97 kDa in HD11 cells, slightly larger than the calculated size of DdPLC, 92 kDa. A smaller protein of ~55 kDa was also detected. Proteins of the same sizes were also detected in AX3 cells (not shown). The smaller protein could represent a cleaved PLC-δ isoform, which is also found in mammalian cells (Divecha *et al.*, 1993). In order to investigate if HD10 translates the truncated DdPLC mRNA, protein samples were analysed on Western blots. The reactivity of the antiserum for the N-terminal region of DdPLC was confirmed by positive detection of a 3′ truncated DdPLC protein expressed in

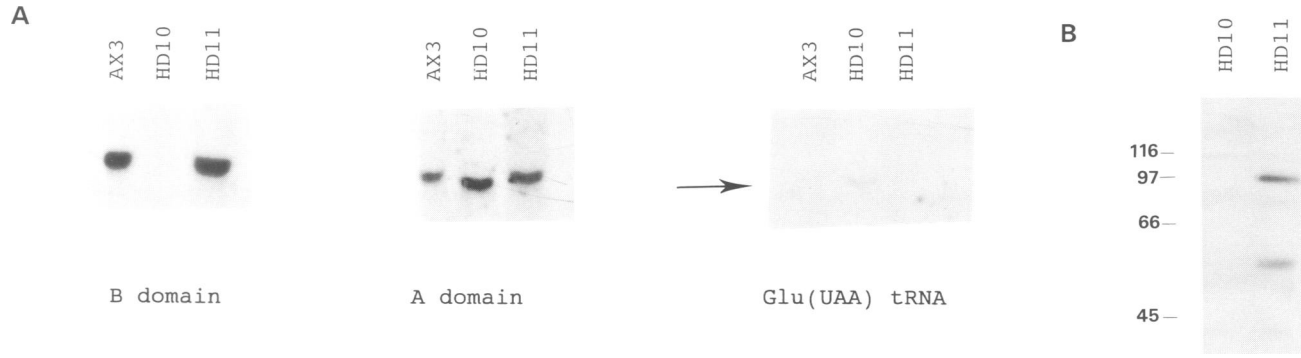

Fig. 2. Expression of DdPLC. Samples were taken from vegetatively growing AX3 and HD11 (control cells) and HD10 (DdPLC gene disruption mutant). (**A**) Analysis of total RNA. The Northern blots were probed with DNA sequences encoding the conserved B-domain of PLC (left panel), the conserved A-domain of PLC (middle panel) and the $tRNA^{Glu(UAA)}$ (right panel). (**B**) Western blot of proteins prepared from HD10 and HD11 cells using DdPLC-specific antiserum. Numbers at the left indicate the migration position of molecular weight standards in kDa.

Escherichia coli (not shown). As shown in Figure 2B, no proteins were detected in HD10 cells using this antiserum. We therefore conclude that HD10 does not express any DdPLC protein.

PLC activity during development

To examine whether DdPLC accounts for basal, receptor mediated or G protein regulated PLC activity, PLC activity was measured in HD10 cell lysates. For receptor and G protein mediated PLC activity in aggregation competent cells, cAMP or GTPγS was added to cells before lysis. The results for vegetative cells and for cells starved for 6 h are shown in Figure 3. Vegetative control AX3 cells showed a basal PLC activity of 110 pmol $Ins(1,4,5)P_3$ produced/min/mg protein. In the HD10 mutant no PLC activity could be measured in vegetative cells. In aggregation competent cells PLC activity is increased after stimulation with cAMP or GTPγS (Bominaar and Van Haastert, 1991). In HD10 cells no PLC activity was detectable with or without stimulation (see Figure 3). Under basal activity conditions the assay can detect an increase in $Ins(1,4,5)P_3$ levels of 14 pmol/min/mg protein, indicating that HD10 has $<7\%$ of the PLC activity found in wild-type cells. Stimulated and unstimulated PLC activity was measured every 2 h during the 28 h developmental programme of *Dictyostelium* plc^- cells. No PLC activity was measured during development under these conditions (data not shown).

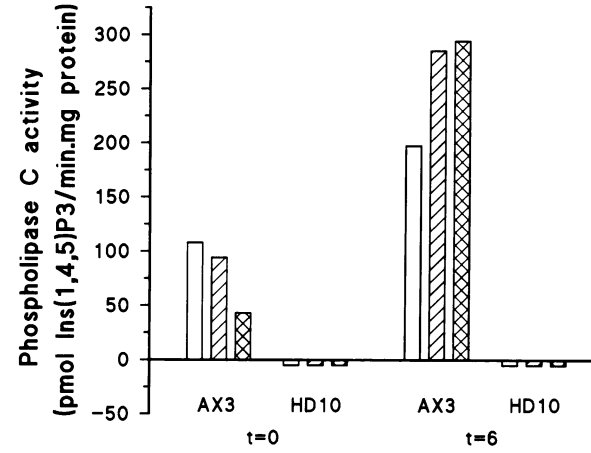

Fig. 3. Phospholipase C activity in AX3 cells and HD10 mutant. Exponentially growing cells were washed and used directly in the assay ($t = 0$) or starved for 6 h. Cells were not stimulated (open bars), or stimulated with 1.0 μM cAMP (hatched bars) or 10 μM GTPγS (cross-hatched bars).

Growth and development of the plc⁻ mutant

HD10 cells were grown axenically in HL5 medium containing G418 and on agar plates in association with *Klebsiella aerogenes*. No difference in growth rate could be detected between the plc^- mutant HD10 and the control strain. After plating the HD10 cells on non-nutrient agar, development into fruiting bodies proceeded as normal (see Figure 4).

Northern blot analysis of HD10 development showed that the expression of developmentally regulated genes was, overall, regulated as in control cells (Figure 5). In HD10 cells D14 was expressed 2 h later than in AX3. The prespore marker D19 was expressed by 8 h of development in both the mutant and the control cells.

Spores from the plc^- mutant could be stored in frozen form and sporulation proceeded as normal. The disruption at the DdPLC locus was passed on stably during culture with and without selection on G418 through many cell cycles.

Second messenger responses and chemotaxis

As the plc^- mutant showed normal formation of aggregates, chemotaxis towards cAMP was expected to be normal. When measured in the small population assay, plc^- cells showed chemotaxis comparable to that shown by control cells at different cAMP concentrations (Figure 6).

To examine cAMP mediated second messenger responses in the absence of PLC activity, plc^- cells were stimulated with cAMP for measurement of cGMP and $Ins(1,4,5)P_3$ accumulation, or with 2′ deoxy cAMP for measurement of cAMP accumulation. As shown in Figure 7, plc^- cells showed an increase in cAMP and cGMP levels after stimulation. cGMP accumulation reached its peak at 8 s from stimulation, after which levels decreased to basal levels within 1 min. This increase was smaller than in control HD11 cells, but the difference was not statistically significant. In the non-axenic wild-type strain NC-4, cAMP gives a transient rise in $Ins(1,4,5)P_3$ levels at 6 s after stimulation (Van Haastert, 1989). In the plc^- cells we never saw receptor-stimulated formation of $Ins(1,4,5)P_3$ (Figure 7).

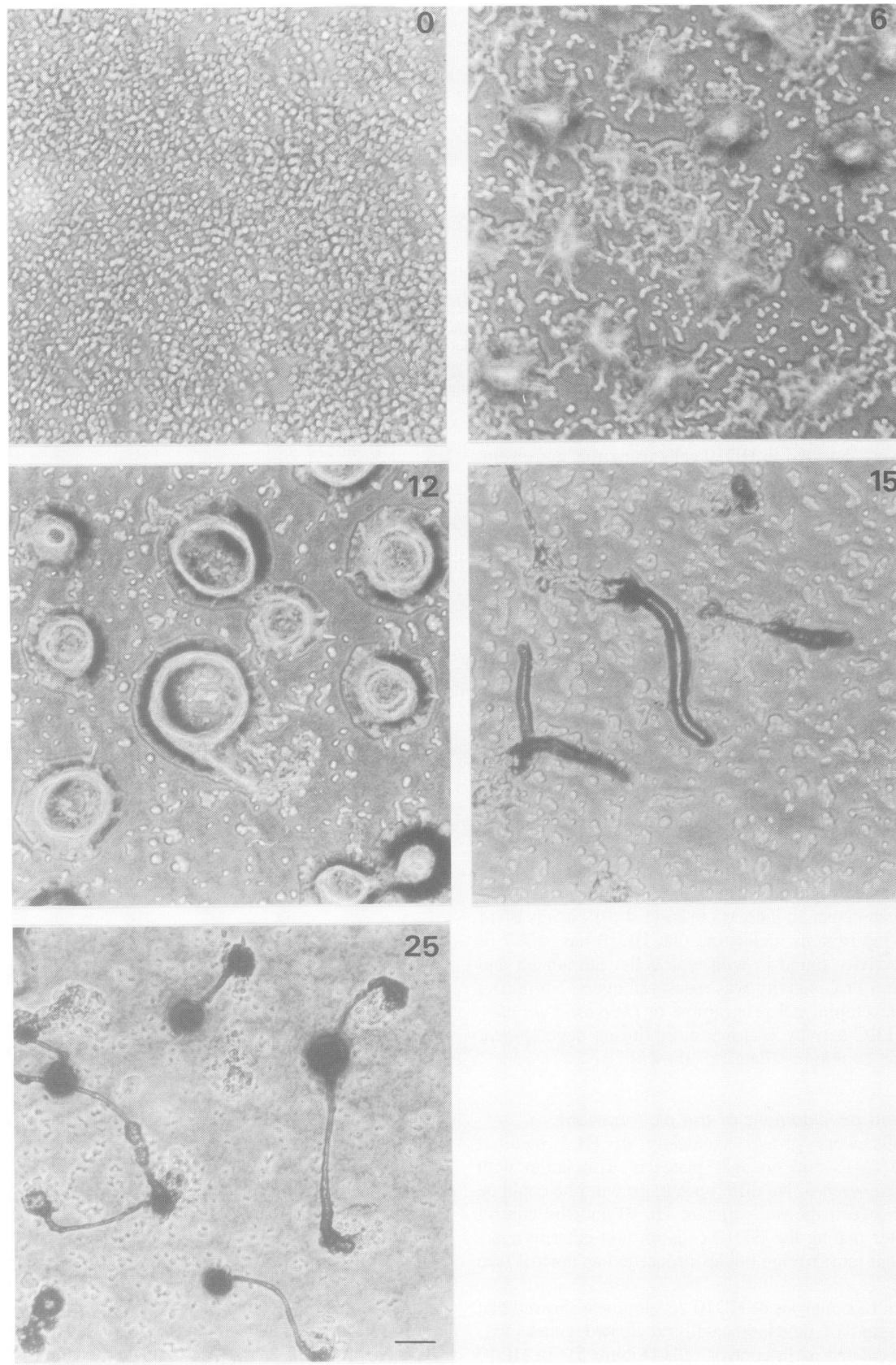

Fig. 4. Development of *plc*$^{-}$ mutant HD10. Cells were plated on non-nutrient agar plates and photographed at the indicated times (in hours). Stages shown are: vegetative cells (top left), formation of aggregates (top right), tight aggregate stage (middle left), migrating slugs (middle right) and mature fruiting bodies (bottom). The bar represents 0.1 mm.

Fig. 5. Expression of developmentally regulated genes. AX3 cells and *plc*$^-$ mutant HD10 were starved on non-nutrient agar and harvested at the indicated times (hours). Total RNA was isolated, size fractionated and transferred to filters. DNA sequences complementary to D14 and the prespore gene D19 were used as probes. The same blot was reprobed with an actin 15 DNA fragment as a control for equal loading in each lane. (Very little RNA was isolated from HD10 samples at 23 h of development, compare with the actin 15 marker.)

However, the changes of Ins(1,4,5)P_3 levels in our parent strain AX3 and the control cells HD11 were not significant either.

Mass of inositol phosphates

In the *plc*$^-$ mutant no PLC activity was measured. Surprisingly, the mutant cells contained Ins(1,4,5)P_3. The concentration was slightly lower than in HD11 control cells: 4.3 pmol Ins(1,4,5)P_3/10^7 *plc*$^-$ cells, against 5.4 pmol/10^7 control cells (see Figure 7 lower panel) as determined by isotope dilution assay.

To analyse further the distribution of the mass of inositol phosphates in these cell lysates, inositol phosphates were separated and quantified by HPLC/MDD (Table I). From the results of the determination of inositol phosphate masses shown in Table I we conclude that there are only subtle differences in the whole spectrum of inositol phosphates between the *plc*$^-$ mutant and the HD11 control cell line. In the HPLC/MDD analysis the mass of D/L-Ins(1,4,5)P_3/ D/L-Ins(2,4,5)P_3 (these isomers have not been separated yet) in HD10 cells was 80% of the mass of these isomers in HD11 cells, and comparable to the mass of D-Ins(1,4,5)P_3 found in the isotope dilution assay.

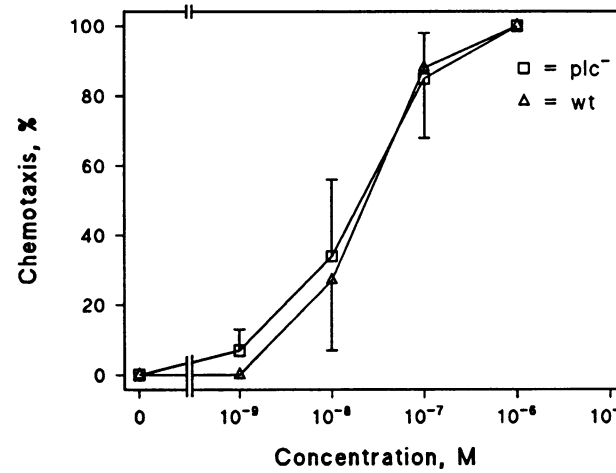

Fig. 6. Chemotaxis towards cAMP. The chemotactic response of HD11 (wt) and HD10 (*plc*$^-$) cells was measured in the small population assay at various cAMP concentrations. Values are given as the mean ± SD of three independent observations.

Cell labelling with [^{3}H]inositol

Cells were labelled with [^{3}H]inositol and the different metabolites formed during a short incubation period were analysed. Since *Dictyostelium* cells secrete large amounts

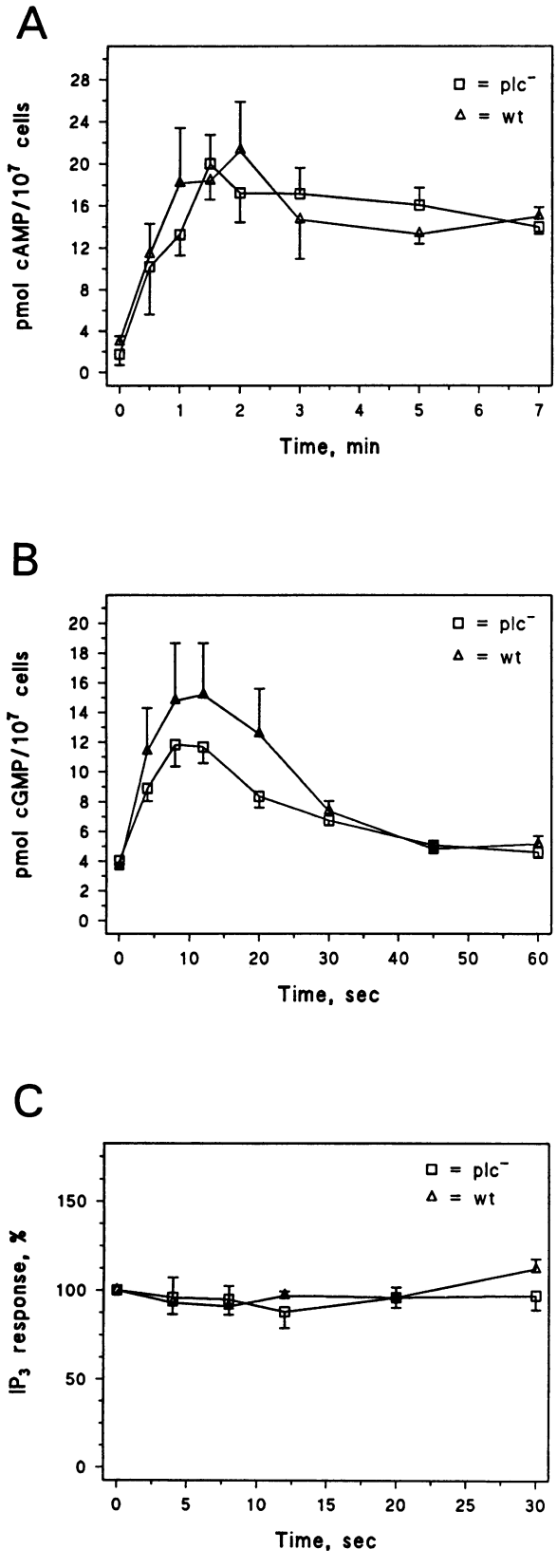

Fig. 7. cAMP induced second messenger responses. HD11 (wt) and HD10 (*plc*⁻) cells were starved for 5 h, washed and stimulated at time 0. Reactions were stopped at the indicated times by addition of perchloric acid. Values represent the mean ± SEM of two or three (lower panel) experiments performed in triplicate. In the lower panel 100% represents 4.3 pmol $Ins(1,4,5)P_3/10^7$ *plc*⁻ cells and 5.4 pmol $Ins(1,4,5)P_3/10^7$ wt cells.

Table I. Mass of inositol phosphates as detected by HPLC/MDD analysis in HD11 control cells and HD10 *plc*⁻ mutant

Compound	Mass (pmol/10^7 cells)	
	HD11	HD10
$InsP_2$ (isomers not separated)	36	32
$Ins(1,2,6)P_3$	10	11
$Ins(1,5,6)P_3$	<1	<1
$Ins(1,2,3)P_3$	<0.8	<0.4
$Ins(4,5,6)P_3$	0.6	1.0
$Ins(1,4,5)P_3$[a]	5.4	4.3
$Ins(1,4,5)P_3/Ins(2,4,5)P_3$[b]	4.9	3.7
$Ins(1,2,4)P_3/Ins(1,2,5)P_3$[b]	13	15
$Ins(1,3,5)P_3/Ins(2,4,6)P_3$[b]	0	0
$Ins(1,3,4)P_3/Ins(1,4,6)P_3$[b]	<1	<1
$Ins(1,3,4,5)P_4$	10	8
$Ins(1,2,5,6)P_4$	18	19
$Ins(2,4,5,6)P_4$	12	12
$Ins(1,2,3,5)P_4/Ins(1,2,4,6)P_4$[b]	4	5
$Ins(1,2,3,4,6)P_5$	85	96
$Ins(1,2,3,4,5)P_5$	2.3	2.7
$Ins(1,2,4,5,6)P_5$	132	150
$Ins(1,3,4,5,6)P_5$	20	6
$InsP_6$	2584	2289
$InsP_7$[c]	389	370
$InsP_8$[c]	720	661

In no cases was discrimination between D- and L-enantiomers undertaken.
[a]Mass of $Ins(1,4,5)P_3$ determined in the isotope dilution assay.
[b]These isomers were not separated.
[c]See Mayr *et al.* (1992) and Stephens *et al.* (1993) for the putative structures of (mono)diphosphoinositolpentakisphosphate ($InsP_7$) and (bis)diphosphoinositoltetrakisphosphate ($InsP_8$) and their mass determination by HPLC/MDD.

of the lower inositol phosphates at a rate of ~10% of the intracellular amount per minute (Van Haastert, 1989; J.Van der Kaay, unpublished), the incorporation of radioactivity into the different metabolites is transient. Radioactivity in phosphatidylinositol 4,5-bisphosphate [$PtdIns(4,5)P_2$] reaches a maximum at 60 min after electroporation of NC4 cells, $Ins(1,4,5)P_3$ at 75–90 min and $InsP_6$ at more than 2 h after electroporation (Bominaar *et al.*, 1991b). As shown in Table II the amount of radioactivity of [^{3}H]inositol incorporated into $PtdIns(4,5)P_2$ was comparable in HD11 control and *plc*⁻ cells. As the mass of $PtdIns(4,5)P_2$ in the control and the *plc*⁻ cells was also comparable, the specific radioactivities (92 d.p.m./pmol) did not differ. The same was true for $InsP_6$. The specific radioactivity in the $InsP_2$ and $InsP_3$ mixtures was decreased in *plc*⁻ cells relative to the control HD11 and, interestingly, the most pronounced decrease was observed in $Ins(1,4,5)P_3$. In control cells, a 75 min incubation with [^{3}H]inositol resulted in a high specific radioactivity (168 d.p.m./pmol) of $Ins(1,4,5)P_3$. In the *plc*⁻ cells 4-fold lower levels of radioactivity were incorporated into $Ins(1,4,5)P_3$. As the mass of $Ins(1,4,5)P_3$ in the *plc*⁻ cells was only slightly lower than in control cells, this resulted in a low specific radioactivity. Longer incubation of *plc*⁻ cells (135 min) with [^{3}H]inositol did not result in an increase of incorporation of radioactivity into $Ins(1,4,5)P_3$ (data not shown).

Table II. Mass, activity and specific activity of components of the inositol cycle in HD11 control cells and HD10 *plc*$^-$ mutant cells

Compound	Mass (pmol/10^7 cells)		Activity (d.p.m./10^7 cells)		Specific radioactivity (d.p.m./pmol)		
	HD11	HD10	HD11	HD10	HD11	HD10	HD10/HD11
PtdIns(4,5)P_2	116	131	10656 ± 2230	12069 ± 3041	92 ± 19	92 ± 23	1.00 ± 0.33
InsP_2 mixture	36	32	1791 ± 389	1069 ± 206	50 ± 11	33 ± 6	0.67 ± 0.20
Ins(1,4,5)P_3	4.9	3.7	821 ± 350	215 ± 50	168 ± 72	58 ± 13	0.35 ± 0.17
InsP_3 mixture	29	30	1622 ± 290	1466 ± 262	56 ± 10	49 ± 9	0.87 ± 0.22
InsP_6	2584	2289	40430 ± 6826	38669 ± 6592	16 ± 3	17 ± 3	1.08 ± 0.26

Mass of inositol phosphates was calculated from the determinations by HPLC/MDD and by alkaline hydrolysis and subsequent determination of Ins(1,4,5)P_3 by isotope dilution assay for PtdIns(4,5)P_2. Cells were labelled with [^{3}H]inositol and incorporation into the different components was determined by reversed phase ion-pair HPLC (inositol phosphates) or TLC [PtdIns(4,5)P_2]. Values are given as the mean ± SEM of six experiments. Note: the specific radioactivity of Ins(1,4,5)P_3 in HD11 control cells was larger than that in the presumed precursor PtdIns(4,5)P_2. As samples were taken at the moment of maximum label incorporation into Ins(1,4,5)P_3 and the incorporation of radioactivity into PtdIns(4,5)P_2 has already declined to 45–75% of its maximal value, this may account for the differences in specific radioactivity between PtdIns(4,5)P_2 and Ins(1,4,5)P_3.

Discussion

The *D.discoideum plc* gene encodes a PLC that has strong homology to the mammalian PLC-δ isoform. We have demonstrated by disrupting the DdPLC gene that the PLC is not necessary for viability and development of *Dictyostelium*. Cell division, chemotaxis, aggregation, cell type specification and germination are unaffected in *plc*$^-$ cells. No PLC activity could be measured in cell lysates from *plc*$^-$ cells during the complete developmental cycle. In the *plc*$^-$ mutant a shortened DdPLC-specific mRNA was produced as shown by Northern blot analysis. The transcript could code for a truncated protein consisting of the first 550 amino acids up to the conserved B domain. However, this fragment is most likely degraded soon after its production as no DdPLC protein could be detected in *plc*$^-$ cell lysates by Western blot analysis. These results indicate that DdPLC accounts for all measurable PLC activity, although we cannot exclude the possibility that DdPLC may be obligatory for expression of other *Dictyostelium* PLC isoforms. However, there are no genetic indications for other PLC genes in *Dictyostelium* (Drayer and Van Haastert, 1992).

The finding that disruption of the DdPLC gene in *Dictyostelium* did not affect cell growth or development is surprising since in a number of other organisms mutations in PLC genes are known to be associated with defects in growth and development. For instance, disruption of the *PLC*1 gene in the budding yeast *Saccharomyces cerevisiae* resulted in a growth defect, although the severity depended on the strain used (Yoko-o *et al.*, 1993). In *Drosophila* the *norp*A (no receptor potential A) gene encodes a PLC with sequence similarity to mammalian PLC-β (Bloomquist *et al.*, 1988). *Norp*A mutants do not contain PLC in the head region and are defective in the phototransduction process (Yoshioka *et al.*, 1985; Inoue *et al.*, 1988).

Control *Dictyostelium* cells contain PLC activity that can be stimulated by cAMP and GTPγS (Bominaar *et al.*, 1993). In the *plc*$^-$ mutant no PLC activity could be measured in the absence or presence of these stimulators. This suggests that in control cells cAMP and GTPγS stimulate the PLC that is deleted in the *plc*$^-$ cells, i.e. a PLC with strong homology to the mammalian PLC-δ isoform.

Since PLC activity was not required for chemotaxis towards cAMP, we have tested whether activation of PLC is required for cyclic AMP-induced second messenger responses in *Dictyostelium*. The presence of PLC was not necessary for activation of guanylate cyclase or adenylate cyclase; in the *plc*$^-$ mutant receptor stimulated accumulation of cGMP and cAMP proceeded as normal. The transient rise in Ins(1,4,5)P_3 after cAMP stimulation was not observed in HD11 or AX3 control cells although different batches of cells and culture conditions were tried. Unfortunately, therefore, we cannot speculate on the meaning of the absence of Ins(1,4,5)P_3 accumulation in *plc*$^-$ cells.

These results are in contrast to those of previous experiments with mutants *fgd*A and *fgd*C which suggested an important role for PLC activation in *Dictyostelium* development and chemotaxis. Mutant *fgd*A cells lacking the $G_\alpha 2$ subunit are severely defective in development, chemotaxis and signal transduction. These cells appear to be defective in the GTPγS stimulation of PLC activity (Bominaar and Van Haastert, 1993). Mutant *fgd*C cells show defects in development, cell aggregation and have reduced chemotaxis towards cAMP. In these cells the cAMP-stimulated cGMP and cAMP response are normal, but the Ins(1,4,5)P_3 response is defective (Bominaar *et al.*, 1991a). From these results it can be concluded that PLC is activated by $G_\alpha 2$ and the *fgd*C gene product. The results reported in the present paper show that these signal transducers activate, in addition, a still unknown effector enzyme which mediates development.

In the *plc*$^-$ mutant no Ins(1,4,5)P_3 or diacylglycerol was generated by *in vitro* hydrolysis of PtdIns(4,5)P_2. Nevertheless, *plc*$^-$ cells still contain Ins(1,4,5)P_3 (Table II) and diacylglycerol (A.D.Tepper, J.Van der Kaay and P.J.M.Van Haastert, unpublished). Several routes for formation of diacylglycerol from other phospholipids are known in mammalian cells. Substrates include major membrane constituents such as phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, which are also degraded by signal activated phospholipases. In *Dictyostelium* we have indications that most of the diacylglycerol is produced from a source other than phosphatidylinositols. The fatty acid composition of diacylglycerol is comparable to that of phosphatidylcholine, but not to that of PtdIns(4,5)P_2 (A.D.Tepper, J.Van der Kaay and

P.J.M.Van Haastert, unpublished). The data in Table I demonstrate the presence of a broad spectrum of inositol phosphates in *Dictyostelium*. As the mass of the different inositol phosphates analysed did not differ substantially between the *plc*$^-$ cells and control cells, we conclude that PLC activity is not necessary for generation of the wide spectrum of inositol phosphates, nor is PLC required to produce Ins(1,4,5)P_3 as a precursor for the formation of other inositol phosphates. As both our genetic and biochemical data argue against the presence of another, unidentified, PLC in *Dictyostelium*, other routes for formation of Ins(1,4,5)P_3 in *plc*$^-$ cells need to be considered. Ins(1,4,5)P_3 could be formed also by the dephosphorylation of higher inositol phosphates or by the phosphorylation of lower inositol phosphates. Short labelling of cells with [^{3}H]inositol resulted in the rapid labelling of PtdIns(4,5)P_2 and Ins(1,4,5)P_3 with high specific activity, and other inositol phosphates with lower specific activity, indicating that PtdIns(4,5)P_2 and Ins(1,4,5)P_3 rapidly incorporate inositol, whereas the incorporation in e.g. InsP_6 reaches equilibrium more slowly. The observation that the specific radioactivity of Ins(1,4,5)P_3 in *plc*$^-$ cells is much lower that in control cells suggests that Ins(1,4,5)P_3 in *plc*$^-$ cells is derived from a source with relatively slow turnover. The various inositol phosphate isomers identified in *Dictyostelium* as shown in Table I provide many potential precursors for Ins(1,4,5)P_3 in *plc*$^-$ cells.

In summary, DdPLC is not essential for *Dictyostelium* growth and development, and DdPLC is not essential to generate Ins(1,4,5)P_3. Apparently, the inositol cycle in *Dictyostelium* contains degenerative routes in order to sustain a broad spectrum of inositol phosphates.

Materials and methods

Vector constructs

All DdPLC DNA sequences used in these studies as probes or for making new vector constructs have been described (Drayer and Van Haastert, 1992). Plasmid pNeoPLCko was obtained by insertion of a G418 resistance cassette (provided by J.Williams, ICRF Clare Hall Laboratories, Potters Bar, UK) and the tRNA$^{Glu(UAA)}$ suppressor gene (provided by T.Dingermann, Johann Wolfgang Goethe Universität, Frankfurt, Germany) in a DdPLC cDNA sequence as shown in Figure 1A.

A full-length DdPLC sequence was used to construct a vector for expression of DdPLC in *E.coli* for antibody production (see below). A *Bam*HI site was created at position 289 at the first ATG by oligonucleotide-directed mutagenesis (Kunkel *et al.*, 1987). The *Bam*HI–*Bam*HI fragment consisting of the coding region and 3′ untranslated region was cloned in-frame in the *Bam*HI site of the pET-3b vector (Studier *et al.*, 1990). A 3′ truncated protein expressing DdPLC up to the conserved A domain was constructed by removal of the DdPLC sequence after the internal *Nco*I site. DdPLC was expressed in *E.coli* after induction with isopropyl-β-D-thiogalactopyranoside.

Culture conditions and transformation of Dictyostelium

D.discoideum strain AX3 was grown axenically in HL5 medium (Watts and Ashworth, 1970). A calcium phosphate precipitate of plasmid pNeoPLCko was used to transform AX3 cells (De Lozanne and Spudich, 1987). Transformants were selected and cloned in HL5 medium containing G418 at 10 μg/ml.

DNA and RNA analysis

Genomic DNA and total RNA from AX3 and transformants were isolated according to Nellen *et al.* (1988). Samples were sized on a denaturing gel and transferred to Genescreen filters (DuPont) as described (Sambrook *et al.*, 1989). Probes were ^{32}P-labelled using the random priming method and hybridization conditions were as described previously (Drayer and Van Haastert, 1992).

For the analysis of expression of developmentally regulated genes, axenically grown cells were washed in 10 mM KH_2PO_4/Na_2PO_4, pH 6.5 (PB), and starved on non-nutrient agar. Samples for RNA isolation were taken at different stages of development. cDNA clones encoding the genes D14 and D19 were used as probes. D14 is expressed during aggregation and later in the anterior and posterior regions of the slug, with a slight preference for the anterior prestalk cells, while D19 recognizes a prespore specific mRNA (Barklis and Lodish, 1983).

Antibodies and Western blot analysis

DdPLC expressed in *E.coli* was excised as a 97 kDa protein band from an SDS–polyacrylamide gel and used for generating anti-DdPLC antibodies in rabbit serum. For Western blots *D.discoideum* cells were washed once in PB, pelleted and resuspended in sample buffer (Sambrook *et al.*, 1989). 40 μg protein samples were separated by SDS–PAGE and transferred to nitrocellulose (Towbin *et al.*, 1979). Blots were incubated with 1:1000 diluted antiserum against DdPLC for 2 h, and 1 h with horseradish peroxidase labelled second antibody diluted 1:50.000. Specific bands were visualized using the ECL detection kit (Amersham).

Second messenger responses in vivo

Cells were starved in PB for 5 h at a density of 10^7 cells/ml. Cells were harvested and resuspended at a density of 5×10^7 cells/ml in 40 mM HEPES/NaOH buffer, pH 6.5 (HB). For cGMP and Ins(1,4,5)P_3 responses the stimulus was 0.5 and 1.0 μM cAMP, respectively. For cAMP response cells were stimulated with 5 μM 2′-deoxy-cyclic AMP and 5 mM dithiothreitol. Cells were lysed by the addition of an equal volume of perchloric acid (3.5% v/v). The levels of second messenger were measured in neutralized cell lysates by isotope dilution assays (Kesbeke *et al.*, 1988; Van Haastert, 1989).

Chemotaxis assays

Chemotaxis towards different concentrations of cAMP was determined using the small-population assay (Konijn, 1970).

Assay of PLC activity

PLC activity was determined as described by Bominaar *et al.* (1993). Briefly, *Dictyostelium* cells at different stages of development were washed, resuspended in HB, and stimulated in the presence of 5.9 mM EGTA with 1 μM cAMP or 10 μM GTPγS. Cells were lysed and PLC activity in cell lysates was determined as the amount of Ins(1,4,5)P_3 produced after the addition of 5.9 mM $CaCl_2$. Reactions were terminated by addition of an equal volume of 3.5% perchloric acid. Ins(1,4,5)P_3 levels were determined by isotope dilution assay as described above.

Determination of mass of inositol phosphates

Cells were starved for 4 h in PB, washed twice and resuspended in 10 mM MES buffer, pH 6.5 at 5×10^7 cells/ml. Cells were lysed by addition of an equal volume of trichloric acid (20% w/v) and frozen in liquid N_2. Cell debris was removed by centrifugation at 3000 *g* for 15 min. The supernatant was extracted five times with an equal volume of water-saturated diethylether and subsequently lyophilized.

To obtain mass measurements of bis- to poly-phosphorylated compounds, isomers were quantified by HPLC combined with post-column metal-dye detection (MDD) as described (Mayr, 1988, 1990). Sample extracts derived from 0.2 to 1 g (wet weight) of packed cells were twice treated with charcoal as described by Mayr (1988) in order to remove interfering nucleotides. As controls for losses of inositol phosphates by charcoal treatment, samples were also analysed without this treatment. The inositol phosphates were separated employing two different elution systems on Mono-Q columns (250 mm × 5 mm). One is the strongly acidic HCl elution system for further separation of more highly phosphorylated inositols and the other is a KCl eluant adjusted to pH 8.5 for further separation of the InsP_2 and InsP_3 isomers (Freund *et al.*, 1992). Standardization of InsP_7 and InsP_8 was as described by Mayr *et al.* (1992) and by Stephens *et al.* (1993).

Cell labelling with [^{3}H]inositol and analysis of metabolites

Cells that had been starved for 5 h were harvested in PB and electroporated in the presence of 60 μCi *myo*-[^{3}H]inositol (10^8 cells in 1 ml) as described (Van Haastert *et al.*, 1989). After electroporation, cells were washed and incubated in PB. Samples were drawn 75 min after electroporation with [^{3}H]inositol. Reactions were stopped by addition of an equal volume of TEI buffer [18% TCA (w/v), 10 mM EDTA and 100 μg/ml InsP_6 hydrolysate] and centrifuged for 2 min at 15 000 *g*. The supernatant containing polar inositol phosphates was extracted three times with 3 vols of water-saturated ether. The pellet containing inositol phospholipids was extracted with chloroform/methanol/HCl (20:40:1, by volume). Samples were analysed by HPLC or TLC.

For mass determination of PtdIns(4,5)P_2, total lipid was extracted from cells with chloroform/methanol/HCl (as above) and cleaved by hydrolysis with 1 M KOH (Bominaar *et al.*, 1993). The Ins(1,4,5)P_3 levels were determined by isotope dilution assay and taken as a measure for PtdIns(4,5)P_2 mass. [^{3}H]PtdIns(4,5)P_2 was added during the isolation as an internal standard for determination of recovery and formation of Ins(1,4,5)P_3.

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