Research article

**Phospholipase Cδ regulates germination of Dictyostelium spores**

Peter Van Dijken and Peter JM Van Haastert*

Address: Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

E-mail: Peter Van Dijken - P.vanDijken@id.dlo.nl; Peter JM Van Haastert* - Haastert@chem.rug.nl

*Corresponding author

**Abstract**

**Background:** Many eukaryotes, including plants and fungi make spores that resist severe environmental stress. The micro-organism *Dictyostelium* contains a single phospholipase C gene (PLC); deletion of the gene has no effect on growth, cell movement and differentiation. In this report we show that PLC is essential to sense the environment of food-activated spores.

**Results:** Plc-null spores germinate at alkaline pH, reduced temperature or increased osmolarity, conditions at which the emerging amoebae can not grow. In contrast, food-activated wild-type spores return to dormancy till conditions in the environment allow growth. The analysis of inositol 1,4,5-trisphosphate (IP$_3$) levels and the effect of added IP$_3$ uncover an unexpected mechanism how PLC regulates spore germination: i) deletion of PLC induces the enhanced activity of an IP$_3$ phosphatase leading to high IP$_3$ levels in plc-null cells; ii) in wild-type spores unfavourable conditions inhibit PLC leading to a reduction of IP$_3$ levels; addition of exogenous IP$_3$ to wild-type spores induces germination at unfavourable conditions; iii) in plc-null spores IP$_3$ levels remain high, also at unfavourable environmental conditions.

**Conclusions:** The results imply that environmental conditions regulate PLC activity and that IP$_3$ induces spore germination; the uncontrolled germination of plc-null spores is not due to a lack of PLC activity but to the constitutive activation of an alternative IP$_3$-forming pathway.

**Background**

Many extracellular signals activate inositol-specific phospholipase C (PLC) thereby producing the second messengers Ins(1,4,5)P$_3$ and diacylglycerol [1]. Three types of PLC are known, PLC-β, PLC-γ and PLC-δ which are regulated by G-proteins, receptor tyrosine kinases, and Ca$^{2+}$, respectively [2]. Animals such as human and rat, but also *C. elegans*, *Artemia*, *Loligo forbesi* and *Drosophila* possess all three PLC isoforms [3–6]. However, in non-animals exclusively PLC-δ has been identified, e.g. in soybean [7] and catfish [8], and the lower eukaryotes *Dictyostelium discoideum*[9], *Saccharomyces cerevisiae*[10–12] and *Schizosaccharomyces pombe*[13]. This phylogenetic distribution of PLC isozymes is in accordance with the deduced three dimensional structure, suggesting that PLC-δ is the ancient isofom to which specific domains were added in PLC-β and PLC-γ[14,15].

*Dictyostelium* cells live in the soil where they feed on bacteria. When the bacteria become scarce, starvation induces the expression of a cAMP sensory system that mediates cell aggregation. A fruiting body is formed consisting of spores embedded in a slime droplet on top of a stalk. When nutrients are available, spores germinate...
and amoebae search for bacteria by chemotaxis. Spores are relatively safe to environmental stress; no intake of food is required. Furthermore, spores resist extreme temperatures, humidity and pH, and they can pass the digestive track of birds and nematodes [16].

Previously, a Dictyostelium mutant was constructed in which the single PLC-δ gene was disrupted resulting in cells without detectable PLC activity [17]. Besides the unexpected finding that these cells contained near-normal IP_3 levels due to an alternative route of IP_3 synthesis [18], they also showed no abnormal phenotype. At optimal laboratory conditions neither growth nor development were affected [17]. However, optimal conditions are not likely to occur for a very long period in the habitat of Dictyostelium, which is the upper layer of the soil. Therefore we analyzed the survival of the species at suboptimal conditions.

Results

Normal stress resistance in plc-null cells

A Dictyostelium cell lacking the single PLCδ gene shows no aberrant phenotype at laboratory conditions. Therefore we measured the kinetics and dose-dependencies for survival of the amoebae at various stress conditions, including temperature, pH, osmolarity, and removal of extracellular Ca^{2+}. We never observed a significant differences between plc-null and control cells (data not shown), suggesting that Dd-PLCδ is not essential to protect the amoebae. This is in strong contrast to another second messenger enzyme, guanylyl cyclase, which protects Dictyostelium cells against osmotic stress [19].

Normal differentiation in plc-null cells

A common theme in species that have only the PLCδ isoform is the formation of spores or seeds, which can survive extreme environmental conditions. Sporulation to survive stress comes to an evolutionary advantage when germination of spores occurs only at conditions that allow growth of the emerging organism. We investigated the role of PLC in sporulation and germination. During Dictyostelium development about two-thirds of the cells differentiates into viable spores, whereas one-third develops into dead stalk cells. We observed that the proportion of stalk and spore cells is not different in plc-null fruiting bodies (data not shown). Next, plc-null cells with a Neo marker were mixed 1:1 with wild-type cells containing a Bsr marker conferring resistance to the antibiotics G418 and blasticidin, respectively. Spores were isolated from the resulting fruiting bodies, and inoculated in microtiter plates at low density such that about 40% of the cultures showed growth. Subsequently cultures were transferred to media with G418 or blasticidin, allowing growth of plc-null and wild-type cells, respectively. In three independent experiments we observed that from a total of 101 cultures, 29 did grow in G418, 31 grew in blasticidin, whereas 41 showed growth in both G418 and blasticidin. This experiment demonstrates that the spores have essentially the same plc-null/wild-type ratio as the 1:1 mixture of cells from which they were derived, suggesting that Dd-PLCδ does not play a role in the formation of spores.

Aberrant spore germination in plc-null cells

Germination of spores is strictly controlled by environmental factors such as pH, osmolarity and temperature [21]. Figure 1 shows that the germination rate is nearly identical for wild-type and plc-null spores at 22°C (half times 4.0 ± 0.2 h and 4.2 ± 0.2 h, respectively). Germination at 16°C is reduced considerably for wild-type spores as compared to 22°C (half-time 17.8 ± 2.6 h). In contrast, plc-null spores germinate with almost the same rate at 16°C as at 22°C (half-time 6.6 ± 0.5 h). Similar data were obtained at other temperatures (Table 1). Thus, whereas at 22°C both strains germinate at the same rate, at 13°C plc-null spores germinate 15-fold faster than wild-type spores.

Osmotic pressure and pH of the medium are other environmental factors known to affect spore germination. Germination of wild-type spores is more strongly inhibited by sucrose than germination of plc-null spores (Figure 1B). Addition of 0.3 M sucrose inhibits wild-type germination 7-fold while germination of plc-null spores is retarded only 2-fold (Table 1). At acidic pH, spore germination is inhibited about 25-fold at pH 4.7, 5-fold at pH 5.2, and about 2-fold at the mildly acidic pH of 5.5 (Table 1); no statistical significant differences can be observed between germination of wild-type and plc-null spores. At a slightly alkaline pH up to pH 7.8 spore germination is not strongly affected if compared to the optimal pH, but at more alkaline pH germination is inhibited. As with temperature and osmolarity, germination is significantly more affected for wild-type than for plc-null spores.

To evaluate the effect of PLCδ deletion on survival of the Dictyostelium species, we tested the growth rate of amoebae at stress conditions that influence spore germination. Amoebae grown under optimal conditions were incubated in growth medium at reduced temperature, alkaline or acid pH, or increased osmolarity. The results (Figure 2) show that growth of the amoebae was hardly affected at pH 5.2. In contrast, cells grew about 5-fold slower at 15°C compared to 22°C, while cells did not grow at pH 8.7 or in 0.2 M sucrose. The data reveal no differences in growth rates between wild-type and plc-null cells. A role of Dd-PLCδ in spore germination is consistent with the expression of the Dd-PLC gene, which is ab-
sent in the multicellular slug, but reappears to high levels during culmination and formation of spores [9].

**IP<sub>3</sub> levels during spore germination**

A simple model can explain the effect of PLC disruption on spore germination: unfavourable conditions activate PLC resulting in a return to dormancy of food-activated spores; in *plec*-null cells PLC can not be activated, so activated spores can not return to dormancy. However, we have observed that amoebae lacking PLC activity have near-normal IP<sub>3</sub> levels due to an alternative route of IP<sub>3</sub> formation, obtained from the degradation of IP<sub>5</sub>[18].

Therefore, we have measured IP<sub>3</sub> levels in wild-type and *plec*-null spores germinating at 22°C and 16°C.

The IP<sub>3</sub> content of *plec*-null spores is significantly 50% higher than the IP<sub>3</sub> content of wild-type spores (figure 3). The identity of IP<sub>3</sub> as the Ins(1,4,5)P<sub>3</sub> isomer was confirmed using its sensitivity to degradation by specific enzymes (see [18]; data not shown). The IP<sub>3</sub> concentration of wild-type spores does not change much during germination at 22°C. However, at 16°C the IP<sub>3</sub> levels decrease 53% at three hours after incubating the spores in growth medium. Subsequently, IP<sub>3</sub> levels return to basal levels, which coincides with the return of activated spores to dormancy. In *plec*-null spores no significant alteration of the IP<sub>3</sub> concentration occurs during incubation of spores at 22°C or 16°C. These results suggest that unfavourable conditions in wild-type cells induces the inhibition of PLC activity, and that high IP<sub>3</sub> levels are essential to complete germination.

The observation that IP<sub>3</sub> levels decline in wild-type spores at 16°C, but remain constant in *plec*-null cells, predicts that addition of exogenous IP<sub>3</sub> will induce spore germination of wild-type cells at 16°C. Figure 4 describes the effect of IP<sub>3</sub> on germination of wild-type and *plec*-null spores at 22°C and 16°C. At a concentration of 100 µM, IP<sub>3</sub> significantly promotes the germination of wild-type spores at 16°C; it has no effect at 22°C or on the germina-
Table 1: Spore germination at different environmental conditions

| Condition | 50% germination (hours) | Significance |
|-----------|-------------------------|--------------|
|           | wild-type | plc-null |           |
| Temperature (°C) | | | |
| 10        | >250 | 16.9 ± 0.6 | - |
| 13        | 38 ± 7 | 7.1 ± 2.0 | ↔ |
| 16        | 17.4 ± 2.6 | 5.1 ± 0.4 | ↔ |
| 19        | 7.0 ± 0.5 | 4.3 ± 1.1 | * |
| 22 #      | 4.3 ± 0.3 | 4.2 ± 0.2 | NS |
| Osmolarity (M) | | | |
| 0 #       | 4.3 ± 0.3 | 4.2 ± 0.2 | NS |
| 0.10      | 5.5 ± 1.1 | 4.6 ± 0.4 | NS |
| 0.15      | 10.0 ± 0.7 | 5.2 ± 0.9 | ↔ |
| 0.20      | 17.8 ± 1.2 | 6.3 ± 1.4 | ↔ |
| 0.30      | 30.9 ± 3.1 | 9.0 ± 1.0 | ↔ |
| pH | | | |
| 3.0       | >250 | >250 | - |
| 4.0       | >250 | >250 | - |
| 4.7       | 127 ± 21 | 88 ± 18 | NS |
| 5.2       | 19.7 ± 2.6 | 16.8 ± 2.6 | NS |
| 5.5       | 7.6 ± 2.4 | 9.4 ± 3.1 | NS |
| 6.2       | 3.6 ± 1.6 | 4.2 ± 1.7 | NS |
| 7.1 #     | 4.3 ± 0.3 | 4.2 ± 0.2 | NS |
| 7.5       | 5.2 ± 1.5 | 4.6 ± 1.4 | NS |
| 7.8       | 5.6 ± 1.0 | 5.0 ± 1.3 | NS |
| 8.4       | 8.2 ± 0.9 | 5.7 ± 0.8 | * |
| 9.0       | 20.6 ± 3.4 | 9.0 ± 1.1 | ↔ |
| 11        | >250 | >250 | - |

Spore germination was measured at different temperatures, increased osmolality by added glucose or at different pH of the medium; #, normal medium. Data are the means and standard deviations from three independent experiments. NS, the difference between wild-type and plc-null is not significant at P > 0.05; * and **, significant at P < 0.05 and P < 0.01, respectively.

Discussion

The present and previous results suggest that PLCδ has no essential function in cell growth, chemotaxis and differentiation in *Dictyostelium*, but appears to play an essential function during spore germination, which is a complex sequence of events. In the sporehead, germination is inhibited by high osmolality mediated by a specific adenyl cyclase ACG [22]. The activation of spores by nutrients is followed by a lag phase. Subsequently spores swell and amoebae emerge. Once a spore has swollen, germination becomes irreversible, but during the lag phase activated spores can return to dormancy [23]. Several environmental conditions that are harmful to amoebae, but to which spores are resistant, are known to induce dormancy (figure 5). Our observations imply that without environmental stress, plc-null and control spores germinate at the same rate. However, plc-null spore germinate at 16°C, pH 8.7 and 0.2 M sucrose, conditions that inhibit growth of the emerging amoebae. Inhibition of spore germination by high osmolality is probably a dual control by ACG and PLC.

A simple biochemical hypothesis could explain the results: unfavourable conditions activate PLC resulting in IP_3_ formation that inhibits germination; plc-null cells lack the ability to synthesize IP_3_ by which activated spores can not return to dormancy. This hypothesis appears to be incorrect. Firstly, IP_3_ levels of plc-null spores...
are 50% higher than IP₃ levels of wild-type spores. Secondly, in wild-type cells unfavourable conditions inhibit rather than stimulate IP₃ formation. Thirdly, at unfavourable conditions exogenously added IP₃ does not inhibit germination of plc-null spores, but promote germination of wild-type spores. Finally, Lydan and Cotter [24] have demonstrated that in wild-type cells IP₃ acts synergistically with autoactivator to stimulate germination of saponin treated spores, and that EGTA will inhibit swelling of autoactivating spores.

Dictyostelium as well as mammalian cells contain two routes for IP₃ formation, PLC-mediated hydrolysis of the phospholipid PIP₂, and degradation of a specific IP₅-isomer, Ins(1,3,4,5,6)P₅, by the enzyme MIPP. Non-equilibrium labelling experiments with [³H]inositol demonstrate that in wild-type Dictyostelium cells at least 90% of IP₃ is produced by PLC, whereas in plc-null cells all IP₃ is derived from IP₅[18,25]. In addition, our observation of depleted IP₅ levels in plc-null cells, strongly suggesting that the IP₃ pathway from IP₅ is activated in plc-null cells [17].

All experiments are consistent with a more complex hypothesis for the regulation of spore germination in Dictyostelium (Figure 5). Plc-null spores contain an activated route of IP₃ formation from IP₅, leading to enhanced IP₃ levels in comparison to wild-type spores. Activated spores will germinate when IP₃ levels remain high, but return to dormancy at reduced IP₃ levels. Unfavourable conditions inhibit PLC leading to reduced IP₃ levels in food-activated wild-type spores. Apparently IP₃ formation from IP₅ is not inhibited, IP₃ levels do not decrease, and spore germination continues at unfavourable conditions in plc-null spores. Finally, exogenously added IP₃ promotes germination of wild-type spores at conditions that induce a depletion of intracellular IP₃ and a return to dormancy. The depletion of intracellular IP₃ in wild-type cells at reduced temperature is transient. Thus, spore germination is inhibited although IP₃ levels have returned to the high basal levels. Apparently, the transient reduction of IP₃ levels during spore activation has modified the sporulation process.

Many eukaryotes, including plants and fungi make spores that resist severe environmental stress. These spores allow species to preserve their genes even when the individuals that have produced the spores are dead. In contrast, mammals can only transmit their genes while the individual is still living. Gene maintenance through spores requires strict regulation of spore germination, which should only occur when the environment can support growth of the emerging organism. It is clear...
that a return to dormancy of food activated spores at environmental conditions that can not support growth of the emerging organism is of great evolutionary importance. The lack of Dd-PLCδ is a severe disadvantage in the wild, even though under normal laboratory conditions no phenotype of plc-null cells. It is not known whether this function of PLCδ in Dictyostelium is restricted to spore-forming organisms possessing only the δ-isoform of PLC, and that in higher organisms PLCδ fulfils a similar control role, distinct from the functions of the additional PLCβ and PLCγ.

Conclusions

By analyzing spore germination and IP3 levels in wild-type and plc-null strains we conclude that harmful environmental conditions inhibit PLC activity and that the reduced IP3 levels prevent spore germination. In plc-null strains, an alternative pathway for IP3 formation is induced that is not inhibited by harmful environmental conditions. As a consequence, IP3 levels are not inhibited and plc-null spores germinate at environmental conditions where the emerging amoebae can not survive.

Materials and methods

Strains

Two sets of Dictyostelium cells were used. HD10 (plc-null cells) and HD11 (wild-type control for HD10) are G418 resistant clones in an AX3 background [17]. Clone 1.19 (plc-null cells) and o-mut (PLC expressed in 1.19 using an actin 15 promoter) are transformants in a DH1 background [26]. The experiments presented in this report were performed with the combination HD10/HD11; the experiments of figures 1 and 2 were reproduced with the combination 1.19/o-mut.

Spore germination and cell growth

Spores were isolated from 1 to 3 days old fruiting bodies. After treatment with 0.5% NP-40 for 3 minutes to kill remaining amoebae, spores were washed three times with water, and inoculated at a final density of 1.5 × 10⁶ spores per ml in flasks containing 10 ml medium as indicated. The flasks were shaken (150 rpm) at 22°C or 16°C. At various time intervals the spores were observed by phase contrast microscopy and scored for unswollen spores and amoebae. Germination is defined as the fraction of spores that have emerged as amoebae.

Cell growth was measured in medium as indicated in figure 2 using freshly growing cells from a culture at 22°C in HG5 medium at a density of 2 × 10⁶ cells/ml.

Determination of IP3 levels

Two days old spores were incubated at a density of 1.5 × 10⁶/ml in HG5 medium at 22°C or 16°C. At the times indicated in figure 3, 20 μl of the suspension was added to 20 μl ice-cold 3.5% (v/v) perchloric acid. After incubation in a sonication bath for 15 min, the lysates were neutralized with 10 μl KHCO3 (50% saturated at 22°C). IP3 levels were measured in the extracts using an isotope dilution assay [27].

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