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Phosphoinositide-specific Phospholipase C in Dictyostelium discoideum, A molecular genetic approach to study its function and biochemical properties

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SUMMARY

Phosphoinositide-specific phospholipase C (PLC) is generally considered to be one of the central effector enzymes in transmembrane signalling, generating the second messengers $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol. PLC activity in the cellular slime mould *Dictyostelium discoideum* was shown to be regulated via cAMP receptors and G-proteins (1). Previous studies have suggested $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol play a role in chemotaxis and development. In this thesis the role of the enzyme PLC in *Dictyostelium discoideum* growth, chemotaxis and development has been investigated by generating a cell line which no longer contains PLC activity.

Comparison of the processes involved in signal transduction between different organisms shows that many pathways and proteins have been evolutionary conserved (chapter 7). The fact that many components are genetically conserved in higher and lower eukaryotes was used to search for a PLC gene in *Dictyostelium* (chapter 2). Primers encoding highly conserved amino acid regions in mammalian and *Drosophila* PLC isoforms were used in the polymerase chain reaction to identify PLC in *Dictyostelium*. One PLC-like sequence was found; the deduced amino acid sequence of the full protein resembles mammalian $\text{PLC}\delta$. A mutant with a disrupted PLC gene (plc^-) was generated and analyzed for development and cAMP-mediated responses (chapter 3). It was demonstrated that PLC activity was absent in plc^- cells, and unexpectedly, no defect in growth, chemotaxis or development was observed. Although no PLC activity could be detected in the mutant, the level of $\text{Ins}(1,4,5)\text{P}_3$ was found to be nearly normal. This suggests there are alternative pathways

to generate this second messenger besides PLC-mediated hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$.

The deduced amino acid sequence of *Dictyostelium* PLC does not predict any membrane-spanning regions, yet the substrate and proteins interacting with PLC such as cAMP receptors and activated G-proteins are membrane-associated. In chapter 4 localisation of PLC was investigated, as the cellular distribution of the enzyme may provide a way to regulate its activity. Eighty percent of the PLC protein was localized to the soluble fraction of both resting and cAMP-stimulated cells, whereas the highest amount of PLC activity was measured in the particulate fraction. Particulate PLC was found to be regulated by a heat-stable component from the soluble fraction, which was required to express full enzyme activity. These studies suggest only a small part of the available PLC in a cell is in a suitable location to hydrolyse $\text{PtdIns}(4,5)\text{P}_2$ in the particulate fraction, whereby an additional, unidentified, factor in the soluble fraction is required to activate particulate PLC.

In chapter 5 an account is given of the various procedures employed to produce recombinant PLC and subsequently to measure activity in the recombinant enzyme. Expression of *Dictyostelium* PLC in *Escherichia coli* was successful in producing sufficient amount of the protein to raise antibodies. Initially most of the recombinant PLC was retrieved as an insoluble component in inclusion bodies in *E.coli*, which did not show enzyme activity. Changing culture conditions and expression vectors lead to the production of soluble, recombinant PLC. Unfortunately however, it proved not possible to obtain active recombinant *Dictyostelium* PLC, although

second messenger besides hydrolysis of PtdIns(4,5)P₂. The amino acid sequence of PLC does not predict any conserved regions, yet the proteins interacting with PLC are receptors and activated G-proteins membrane-associated. In *Dictyostelium*, the localisation of PLC was studied to provide a way to regulate its activity. About 50 percent of the PLC protein is found in the soluble fraction of both unstimulated and GMP-stimulated cells, whereas the remaining amount of PLC activity was found in the particulate fraction. It was found to be regulated by a component from the soluble fraction. It was required to express full length PLC. These studies suggest only a small amount of the available PLC in a cell is involved in the hydrolysis of PtdIns(4,5)P₂ to generate the particulate fraction, whereby an unidentified factor in the soluble fraction is required to activate particulate PLC.

An account is given of the various procedures employed to produce recombinant PLC and subsequently to purify it in the recombinant enzyme. The cloning of *Dictyostelium* PLC in *Escherichia coli* was successful in producing a large amount of the protein to raise antibodies against most of the recombinant protein. It was retrieved as an insoluble protein in inclusion bodies in *E. coli*, but did not show enzyme activity. Under various conditions and expression systems, the production of soluble, active PLC. Unfortunately however, it was not possible to obtain active recombinant *Dictyostelium* PLC, although

various substrates and reaction conditions were tested.

Ca²⁺ is an important regulator of all PLC isoforms. The amino acid sequence of *Dictyostelium* PLC predicts an EF-hand Ca²⁺-binding motif, a domain found in many Ca²⁺-modulated proteins. In chapter 6 the function of this domain in Ca²⁺-dependent PLC activity was analyzed by introducing point mutations into the EF-hand. *Plc⁻* cells were used in these studies to express the mutated PLC proteins in their native environment. It was demonstrated that this domain is important for enzyme activity, as the maximal enzyme activity decreased with increasing number of point mutations. However, mutation of the EF-hand domain did not affect the affinity of the enzyme for Ca²⁺, demonstrating this is not the site that mediates Ca²⁺-dependence of the PLC enzyme reaction.

PLC-independent Ins(1,4,5)P₃ formation

One of the surprising results in the analysis of the *plc⁻* mutant is that Ins(1,4,5)P₃ levels are only slightly decreased in *plc⁻* cells compared to unmutated cells. It cannot be completely excluded that another, unidentified, PLC isoform is present, however, all genetic and biochemical data suggest this is not the case. PCR reactions performed with various primers encoding highly conserved regions in the A and B domains of PLC did not yield any other PLC-like sequence than the one reported. Reduced stringency-hybridization experiments on Southern blots gave no indications for other PLC sequences. In addition, in *plc⁻* cells no basal or receptor-stimulated PLC activity could be measured, and PLC activity was absent during the complete developmental programme. Together these data indicate the PLC gene which was cloned

accounts for all detectable PLC activity in *Dictyostelium*.

How is Ins(1,4,5)P₃ generated in *plc⁻* cells? Mass determination of inositol phosphate isomers indicated many possible precursors for Ins(1,4,5)P₃ (chapter 3). It is possible that the phosphorylation of lower inositol phosphates or the dephosphorylation of higher inositol polyphosphates could provide Ins(1,4,5)P₃. Recently, a putative route to supply Ins(1,4,5)P₃ has been identified using higher inositol polyphosphates as substrate instead of PtdIns(4,5)P₂. In *Dictyostelium* lysates, Ins(1,3,4,5,6)P₅ was shown to be degraded to Ins(1,4,5)P₃ with both Ins(1,3,4,5)P₄ and Ins(1,4,5,6)P₄ as intermediates (2). Ins(1,3,4,5,6)P₅ can be formed by stepwise phosphorylation of inositol, independently of Ins(1,4,5)P₃ formation (3). In *plc⁻* cells the level of Ins(1,3,4,5,6)P₅ is lower than in control cells (6 compared to 20 pmol/10⁷ cells, see chapter 3), consistent with the hypothesis that Ins(1,3,4,5,6)P₅ could serve as a source for Ins(1,4,5)P₃.

The occurrence of an alternative pathway to generate Ins(1,4,5)P₃ could explain the normal phenotype of the *plc⁻* mutant. The novel phosphatase activities were found in *plc⁻* as well as wild-type cells, indicating it is not a result of disrupting the PLC gene, but is a normally occurring pathway in *Dictyostelium*. The role of this pathway in signal transduction has still to be established. Interestingly, mammalian cells are also capable of synthesising Ins(1,4,5)P₃ by dephosphorylation of higher inositol polyphosphates with the enzyme multiple inositol polyphosphate phosphatase (MIPP) (2,4). This could implicate that PLC-independent formation of Ins(1,4,5)P₃ is a widely occurring event in lower as well as higher organisms.

A role for PLC in *Dictyostelium*?

9 The results obtained in the described
1 studies clearly demonstrate PLC is not
1 essential for normal growth, chemotaxis and
1 development in *Dictyostelium discoideum*.
1 Then why has PLC been evolutionary
1 conserved in *Dictyostelium*? It is possible that
1 the *plc*⁻ cells are unique in that they have
1 been carefully cultured to survive, and
1 employ salvage pathways to adapt to the new
1 situation in the absence of PLC. On the other
1 hand, there could be an advantage for cells
1 to have PLC in their original surroundings,
1 which is not necessary in laboratory
1 conditions. Culturing of cells in less optimal
1 and more stressful surroundings and
1 comparing survival rates of *plc*⁻ and
1 unmutated strains might give an answer, but
1 could require many generations.

In chapter 1 a summary of data from the literature was presented which suggested a role for PLC in *Dictyostelium* chemotaxis and development. Many of the discrepancies can be explained by the fact that *plc*⁻ cells still contain the second messengers diacylglycerol and Ins(1,4,5)P₃. These compounds can still influence chemotaxis and development, but PLC is not essential for producing these messengers.

Analysis of mutants *Frigid A*, with a defect in the α -subunit of the G α 2 protein, and *Frigid C*, with an unidentified defect, have demonstrated the *Frigid C* gene product and G α 2 are essential for chemotaxis and development. It has also been demonstrated that PLC is regulated by these components (1). The fact that chemotaxis and development are normal in *plc*⁻ cells indicates other effector enzymes besides PLC are regulated by *Frigid C* and G α 2. Besides its defect in PLC activation, mutant *Frigid A* is also defective in cAMP-stimulated activation of adenylyl cyclase and guanylyl

cyclase. It has been established that adenylyl cyclase and intracellular cAMP play an essential role in development, while guanylyl cyclase and cGMP are involved in chemotactic movement. Therefore, impaired chemotaxis and development in *Frigid A* can, at least partly, be attributed to defective regulation of guanylyl and adenylyl cyclase. In mutant *Frigid C* chemotaxis is absent under physiological conditions, but cells are not completely defective in chemotactic movement. Approximately 100-fold higher cAMP concentrations are required to induce a chemotactic response. This could be a result of the reduced number of cAMP receptors. The only defect at the second messenger level in *Frigid C* was found to be activation of PLC. The *plc*⁻ mutant demonstrates activation of PLC is not essential for chemotactic movement and development, and it is feasible that the *Frigid C* gene product regulates another, unidentified effector enzyme besides PLC.

It has been argued that a link exists between inhibition of PLC and antagonism of chemotaxis (5). Partial antagonists of cAMP such as 3'NH-cAMP and 8-CPT-cAMP bind to surface cAMP receptors and induce a cGMP response at a concentration proportional to their binding activity. Chemotaxis however, is induced only at very high concentrations. At low concentration these compounds inhibit cAMP-induced chemotaxis. In wild-type cells 3'NH-cAMP and 8-CPT-cAMP decrease Ins(1,4,5)P₃ levels due to inhibition of PLC activity (6). It has been suggested that inhibition of PLC affects chemotaxis. A sudden decrease in Ins(1,4,5)P₃ could affect Ca²⁺ homeostasis, leading to inhibition of chemotaxis. In G α 1-null cells, in which inhibition of PLC does not occur, Ins(1,4,5)P₃ levels remain unchanged after stimulation with 3'NH-cAMP (6).

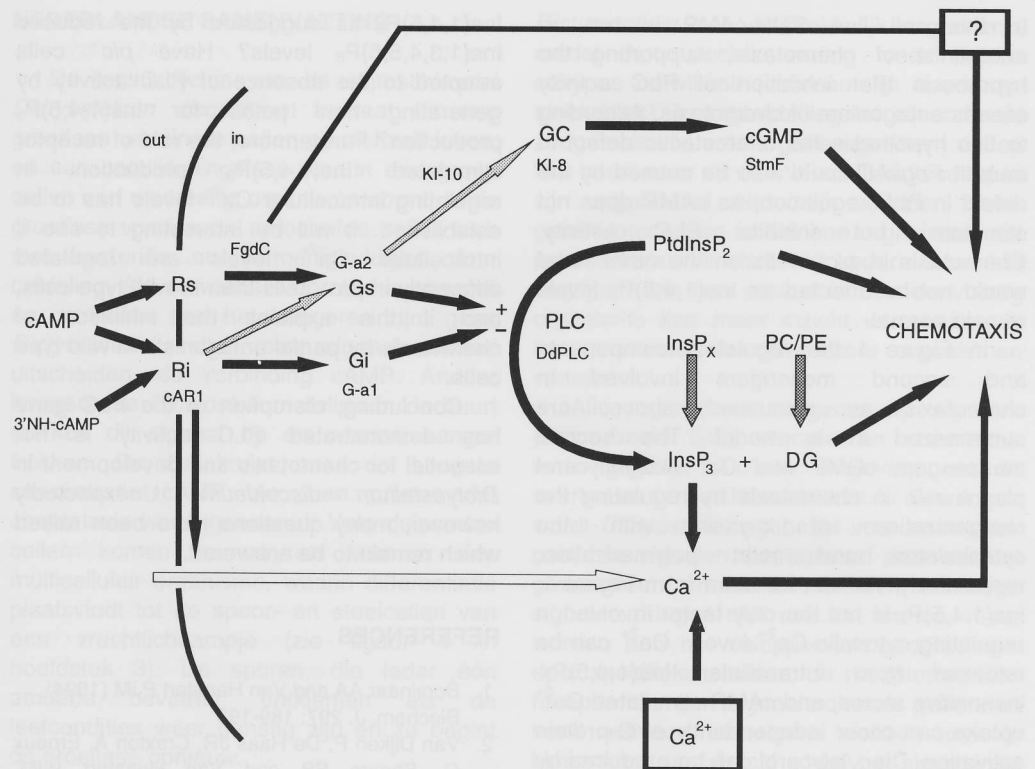


Figure 1: Model for regulation of chemotaxis by second messengers.

PLC activity in *Dictyostelium discoideum* is regulated by a stimulatory pathway and an inhibitory pathway. The guanylyl cyclase (GC) activation pathway (light grey arrows) operates via cAR1 and G- α 2. The chemotactic antagonist 3'NH-cAMP selectively inhibits PLC activity, but activates guanylyl cyclase. In cells with a disrupted *DdPLC* gene, no PLC activity is present, yet cells do contain Ins(1,4,5)P₃ and diacylglycerol which are proposed to be synthesized by alternative routes (double hatched arrows). Cytosolic Ca²⁺ can be supplied by release from Ins(1,4,5)P₃-sensitive and Ins(1,4,5)P₃-insensitive stores (open box), or by receptor-mediated Ca²⁺ influx across the membrane (open arrow). Mutants showing aberrant chemotaxis and isolated genes are indicated by italics. Mutant *KI-10* is defective in receptor-stimulated guanylyl cyclase activity, while *KI-8* has strongly reduced guanylyl cyclase levels. Mutant *StmF* has a defect in cGMP-phosphodiesterase activity resulting in enhanced and prolonged receptor-mediated cGMP elevation, and a prolonged chemotactic response. Mutant *FgdC* is defective in receptor stimulation of PLC activity. The question mark indicates a putative, unidentified effector enzyme regulated by the *FgdC* gene product.

Abbreviations: Rs, stimulatory receptor; Ri, inhibitory receptor; Gs, stimulatory G-protein; Gi, inhibitory G-protein; InsP₃, inositol 1,4,5-trisphosphate; InsP_x, other phosphorylated *myo*-inositol compound; DG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

9. In this cell line 3'NH-cAMP is not an
 10. antagonist of chemotaxis, supporting the
 11. hypothesis that inhibition of PLC activity
 12. causes antagonism of chemotaxis. According
 to this hypothesis the chemotactic defect in
 mutant *Frigid C* could also be caused by the
 defect in PLC regulation, as cAMP does not
 stimulate, but inhibits PLC activity.
 Chemotaxis in *plc*⁻ cells on the other hand
 would not be affected as Ins(1,4,5)P₃ levels
 remain normal.

In Figure 1 the regulatory components
 and second messengers involved in
 chemotaxis as discussed above, are
 summarized in a model. The second
 messengers cGMP and Ca²⁺/diacylglycerol
 play a role in chemotaxis by regulating the
 reorganization of myosin with the
 cytoskeleton and actin polymerization,
 respectively. As can be seen from Figure 1,
 Ins(1,4,5)P₃ is not the only factor involved in
 regulating cytosolic Ca²⁺ levels. Ca²⁺ can be
 released from intracellular Ins(1,4,5)P₃-
 insensitive stores, and cAMP-stimulated Ca²⁺
 uptake can occur independently of G-protein
 activation. Diacylglycerol can be produced by
 other signal activated phospholipases other
 than PLC-induced PtdIns(4,5)P₂ hydrolysis.
 In *plc*⁻ cells normal chemotaxis can be
 attributed to the presence of PLC-
 independent routes to generate these second
 messengers. However, many questions
 concerning PLC activity in wild type cells and
 regulation of Ins(1,4,5)P₃ levels remain
 unanswered. The nearly normal Ins(1,4,5)P₃
 levels in *plc*⁻ cells suggest Ins(1,4,5)P₃ is
 essential for viability. On the other hand, it is
 possible that the contribution of PLC in the
 Ins(1,4,5)P₃ production of wild-type cells is
 much lower than generally expected.
 Concerning PLC-independent Ins(1,4,5)P₃
 formation, do *plc*⁻ cells show a more active
 degradation of Ins(1,3,4,5,6)P₅ to produce

Ins(1,4,5)P₃ as suggested by the reduced
 Ins(1,3,4,5,6)P₅ levels? Have *plc*⁻ cells
 adapted to the absence of PLC activity by
 generating new paths for Ins(1,4,5)P₃
 production? Furthermore, the role of receptor
 stimulated Ins(1,4,5)P₃ production in
 regulating intracellular Ca²⁺ levels has to be
 established. It will be interesting to see if
 intracellular Ca²⁺ levels are regulated
 differently in *plc*⁻ cells than in wild type cells,
 and if this explains the inhibition of
 chemotaxis by partial antagonists in wild type
 cells.

Concluding, disruption of the PLC gene
 has demonstrated PLC activity is not
 essential for chemotaxis and development in
Dictyostelium discoideum. Unexpectedly
 however, many questions have been raised
 which remain to be answered.

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