SUMMARY

Phosphoinositide-specific phospholipase C (PLC) is generally considered to be one of the central effector enzymes in transmembrane signalling, generating the second messengers Ins(1,4,5)P3 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 Ins(1,4,5)P3 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 evolutionarily 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 PLC6. 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 Ins(1,4,5)P3 was found to be nearly normal. This suggests there are alternative pathways to generate this second messenger besides PLC-mediated hydrolysis of PtdIns(4,5)P2.

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 PtdIns(4,5)P2 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...
8. Summary

Second messenger besides hydrolysis of PtdIns(4,5)P₂.

The amino acid sequence of PLC does not predict any binding regions, yet the proteins interacting with PLC receptors and activated G-proteins are membrane-associated. In localisation of PLC was used to determine the cellular distribution of the PLC protein in the soluble fraction of both MP-stimulated cells, whereas the particulate fraction was found to be regulated by components from the soluble fraction. These studies suggest only the available PLC in a cell is in the particulate fraction, whereby an identified factor in the soluble fraction is required to activate particulate PLC.

It was found to be regulated by Ca²⁺ in the recombinant enzyme. Dicyostelium PLC in Dictyostelium was also found to be regulated by Ca²⁺. 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 single 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 that it 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)P₆ 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*?

The results obtained in the described studies clearly demonstrate PLC is not essential for normal growth, chemotaxis and development in *Dictyostelium discoideum*. Then why has PLC been evolutionary conserved in *Dictyostelium*? It is possible that the plc- cells are unique in that they have been carefully cultured to survive, and employ salvage pathways to adapt to the new situation in the absence of PLC. On the other hand, there could be an advantage for cells to have PLC in their original surroundings, which is not necessary in laboratory conditions. Culturing of cells in less optimal and more stressful surroundings and comparing survival rates of plc- and unmutated strains might give an answer, but 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 diacetyl glycerol 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 Ga2 protein, and Frigid C, with an unidentified defect, have demonstrated the Frigid C gene product and Ga2 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 Ga2. 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 Ca**2⁺** homeostasis, leading to inhibition of chemotaxis. In Ga1-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).
Summary

It has been established that adenylyl and guanylyl cyclases play an essential role in signal transduction pathways involving cAMP and cGMP. These enzymes are involved in the regulation of various cellular processes, including chemotaxis, development, and cell signaling. Therefore, impaired development in Frigid A can be attributed to defective phosphodiesterase and adenylyl cyclase.

Chemotaxis is absent under normal conditions, but cells are capable of responding to chemotactic stimulants. This could be explained by the reduced number of cAMP receptors present in Frigid C. The plc^− mutant has been found to lack PLC activity. The absence of PLC is not consistent with the chemotactic movement and elevated cAMP levels observed in Frigid C mutants. It is plausible that the Frigid C mutation affects another, unidentified effector that stabilizes PLC.

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 3NH-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)P^3^ and diacylglycerol, which are proposed to be synthesized by alternative routes (double hatched arrows). Cytosolic Ca^2+^ can be supplied by release from Ins(1,4,5)P^3^-sensitive and Ins(1,4,5)P^3^-insensitive stores (open box), or by receptor-mediated Ca^2+^ influx across the membrane (open arrow). Mutants showing aberrant chemotaxis and isolated genes are indicated by italics. Mutant FgdC 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; InsP^3^, inositol 1,4,5-trisphosphate; InsP^4^, other phosphorylated myo-inositol compound; DG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.
In this cell line 3'NH-cAMP is not an antagonist of chemotaxis, supporting the hypothesis that inhibition of PLC activity 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.

REFERENCES