In vivo analysis of 3-phosphoinositide dynamics during Dictyostelium phagocytosis and chemotaxis

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Summary

Phagocytosis and chemotaxis are receptor-mediated processes that require extensive rearrangements of the actin cytoskeleton, and are controlled by lipid second messengers such as phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3] and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2]. We used a panel of pleckstrin homology (PH) domains with distinct binding specificities for PtdIns(3,4,5)P3 and PtdIns(3,4)P2 to study the spatiotemporal dynamics of these phosphoinositides in vivo. During phagocytosis and macropinocytosis PtdIns(3,4,5)P3 levels transiently increased at sites of engulfment, followed by a rapid PtdIns(3,4)P2 production round the phagosome/macropinosome upon its internalisation, suggesting that PtdIns(3,4,5)P3 is degraded to PtdIns(3,4)P2. PTEN null mutants, which are defective in phagocytosis, showed normal rates of PtdIns(3,4,5)P3 degradation, but unexpectedly an accelerated PtdIns(3,4)P2 degradation. During chemotaxis to cAMP only PtdIns(3,4,5)P3 was formed in the plasma membrane, and no PtdIns(3,4)P2 was detectable, showing that all PtdIns(3,4,5)P3 was degraded by PTEN to PtdIns(4,5)P2. Furthermore, we showed that different PtdIns(3,4,5)P3 binding PH domains gave distinct spatial and temporal readouts of the same underlying PtdIns(3,4,5)P3 signal, enabling distinct biological responses to one signal.

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Key words: Phagocytosis, Chemotaxis, Phosphoinositide, PH domain, Imaging

Introduction

In recent years phosphorylated inositol lipids (phosphoinositides) have emerged as important second messenger molecules. In particular, the 3-phosphoinositides are involved in a variety of distinct cellular functions ranging from the control of cell growth, survival, proliferation, endocytosis, membrane traffic and cell movement to their involvement in human disease and cancer (Stephens et al., 2002; Vanhaesebroeck et al., 2001).

3-Phosphoinositide levels are controlled by several enzymes. Multiple isoforms of phosphoinositide 3-kinases (PI3-kinases) phosphorylate phosphatidylinositol (PtdIns), phosphatidylinositol (4)-phosphate [PtdIns(4)P] and phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P2] at the 3-OH position of the inositol ring, generating PtdIns(3)P, PtdIns(3,4)P2 and PtdIns(3,4,5)P3, respectively (Vanhaesebroeck et al., 2001). These 3-phosphoinositides, in turn, are recognised by a variety of lipid binding domains including PH, PX and FYFE domains (Lemmon, 2003), enabling the recruitment of specific proteins to the membrane sites where these lipids were generated and regulating downstream signalling events. Phosphatases terminate the responses by removing phosphate groups from the inositol ring. In the case of PtdIns(3,4,5)P3, the major product of agonist-stimulated PI3-kinase activity, there appear to be two main routes of degradation. One involves the removal of the 3-phosphate by the 3-phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10), which was originally identified as a tumour suppressor gene, generating PtdIns(4,5)P2 (Maehama et al., 2001). The other route is via 5-phosphatases like SHIP (Src homology 2 domain-containing inositol 5-phosphatase), which can remove the phosphate group from the 5-position of the inositol ring to form PtdIns(3,4)P2 (Rohrschneider et al., 2000).

In Dictyostelium amoebae and leukocytes 3-phosphoinositides have been implicated in the control of chemotactic cell movement. When these cells are exposed to shallow gradients of their respective chemoattractants (cAMP in Dictyostelium, fMLP in neutrophils), with a concentration difference over the length of the cell of as little as ~5%, the cells are able to detect the small differences in receptor occupancy and respond with a rapid localised accumulation of the PI3-kinase products PtdIns(3,4,5)P3/PtdIns(3,4)P2 at the side of the plasma membrane facing the higher concentration of chemoattractant. This has been successfully visualised in vivo using several GFP-tagged PH-domain-containing proteins like the Dictyostelium CRAC (cytosolic regulator of adenylyl cyclase) (Parent et al., 1998) and Akt/PKB (Meili et al., 1999; Servant et al., 2000), which bind to PtdIns(3,4,5)P3 and PtdIns(3,4)P2 and therefore localise to the leading edge of chemotactic cells. The internal PtdIns(3,4,5)P3 gradient appeared much steeper than the extracellular chemoattractant.
gradient suggesting the involvement of an amplification step in gradient sensing and cell polarisation (Iijima et al., 2002; Merlot and Firtel, 2003). In Dictyostelium the localised PtdIns(3,4,5)P₃ generation at the leading edge requires the interplay between the PI3-kinases and PTEN. The PI3-kinase homologues DdPIK1 and DdPIK2 translocate rapidly to the front of the cell where they generate PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ (Funamoto et al., 2002), while PTEN dissociates from the leading edge and binds the lateral and posterior plasma membrane where it presumably degrades PtdIns(3,4,5)P₃ to PtdIns(4,5)P₂, restricting PtdIns(3,4,5)P₃ distribution to the front of the cell (Funamoto et al., 2002; Iijima and Devreotes, 2002).

Another important role for PtdIns(3,4,5)P₃ is in the regulation of phagocytosis, which shows some similarities to chemotaxis as the cells have to extend protrusions to engulf particles like bacteria or apoptotic cells, involving major rearrangements of the actin cytoskeleton (Stephens et al., 2002). Here, too, PtdIns(3,4,5)P₃ is locally generated at phagocytic cups, the membrane protrusions where the particles are being engulfed (Marshall et al., 2001). The PtdIns(3,4,5)P₃ signal is very transient and rapidly disappears once the membrane-enclosed phagosome has been formed.

Crucial to our understanding of 3-phosphoinositide signalling in these processes is a detailed knowledge of which species of phosphoinositide is generated, as well as when and where inside the cell. However, many studies have relied on PH domains that are not specific for just one phosphoinositide, e.g. PtdIns(3,4,5)P₃, but that bind both PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ in vitro. The PH domains of Akt/PKB and CRAC are two examples (Freh et al., 1997; Huang et al., 2003). Although it might, in many cases, be reasonable to equate membrane binding of these probes to elevated PtdIns(3,4,5)P₃ levels, there could be instances where only PtdIns(3,4)P₂ is generated, or even both PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂, especially as there is growing evidence that PtdIns(3,4)P₂ can be generated independently from PtdIns(3,4,5)P₃ and might act as a second messenger in its own right (Banfic et al., 1998; Van der Kaay et al., 1999). Dual specificity PH domains might therefore not always be suited to examining 3-phosphoinositide distribution.

We decided to overcome these potential problems by using a combination of mono- and dual-specific PH domains to visualise and dissect the 3-phosphoinositide signalling pathways in the genetically tractable Dictyostelium system. We generated GFP fusion proteins with the PH domains of the proteins listed below, and expressed them in Dictyostelium cells. The PH domain of GRP1 (general receptor of phosholiposides) binds specifically with high affinity to PtdIns(3,4,5)P₃ (Klarlund et al., 1997); the PH domain of DAPP1 (dual adaptor for phosphotyrosine and 3-phosphoinositides) has been shown to bind both PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ (Dowler et al., 1999) and so does the PH domain of CRAC (Huang et al., 2003). The PtdIns(3,4)P₂-specific probes include the mutated DAPP1G176A PH domain, carrying a single amino acid substitution that removes the PtdIns(3,4,5)P₃ binding activity (Thomas et al., 2001), as well as the C-terminal PH domain of TAPP1 (tandem PH-domain containing protein 1) (Dowler et al., 2000). In addition, we used the PtdIns(4)P-specific PH domain of FAPP1 (phosphatidylinositol-4-phosphate adaptor protein-1) to detect possible phosphoinositide intermediates in the generation and/or degradation especially of PtdIns(3,4)P₂ (Dowler et al., 2000).

We studied both macrophagocytosis, which has previously been shown to require PI3-kinase activity, and phagocytosis during the vegetative stage of the Dictyostelium life cycle when cells are growing and dividing, as well as cells during the different developmental stages when they become responsive to the chemotactant cAMP and aggregate to form multicellular structures (Weijer, 1999). In vegetative cells we found that the PtdIns(3,4,5)P₃-specific GFP probes accumulate at the plasma membrane at the sites of fluid/particle uptake, which is followed by a rapid decrease following internalisation of membrane enclosed vesicles. This has been shown previously, but we show for the first time that PtdIns(3,4)P₂-specific probes transiently bind to these internalised membranes suggesting a role for PtdIns(3,4)P₂ in this process. In aggregation stage cells we find that cAMP stimulation only induces the production of PtdIns(3,4,5)P₃, but no PtdIns(3,4)P₂ is formed, showing a different route of PtdIns(3,4,5)P₃ degradation. In chemotactic cells the PtdIns(3,4,5)P₃-binding proteins GRP1 and CRAC show distinct spatial and temporal patterns of membrane localisation, indicating that the readout of the internal PtdIns(3,4,5)P₃ gradient depends on the binding properties of the PH-domain. This could be used to target effector molecules very specifically to sites like the leading edge or to wider areas of the plasma membrane.

Materials and Methods

Constructs, strains and cell culture

The cell line expressing the CRAC-PH-GFP fusion protein was the same as described previously (Dormann et al., 2002b). The other PH-domain constructs were obtained by digesting the mammalian GST PH domain expression vectors for DAPP1, FAPP1, TAPP1 and GRP1 with BamHI (Dowler et al., 2000). The 800-1300 bp fragments were then inserted in a BamHI-digested and -dephosphorylated pB15 expression vector in frame with S65T GFP (cloned as a BamHI-XhoI fragment) and which gives constitutive expression of the PH-domain-GFP fusion protein under the control of the actin15 promoter. Site-directed mutagenesis of the DAPP1 vector was carried out using a Quickchange Kit. AX2 cells were electroporated with the vector constructs and stable transfectants selected in 10 µg/ml G418. All constructs were tested for their appropriate lipid-binding specificity by a modification of the protein lipid overlay technique (Dowler et al., 2000). Briefly, 2 µl of the various phospholipids (L-α-D-myo-phosphatidylinositol 3-monophosphate, diC16, L-α-D-myo-phosphatidylinositol 4-monophosphate, diC16, L-α-D-myo-phosphatidylinositol 5-monophosphate, diC16, L-α-D-myo-phosphatidylinositol 3,4-bisphosphate, diC16, L-α-D-myo-phosphatidylinositol 3,5-bisphosphate, diC16, L-α-D-myo-phosphatidylinositol 3,4,5-trisphosphate, diC16) (CellSignals, Lexington, KY) dissolved in a 1:2 mixture of chloroform and methanol were spotted on Hybond C Extra (BDH) nitrocellulose membrane. After drying for 1 hour at RT, membranes were blocked for 1 hour using 3% (w/v) fatty acid free BSA (Sigma) in TBST (20 mM Tris/HCl, 136 mM NaCl, 0.05% (v/v) Tween-20). Membranes were washed repeatedly with TBST and then incubated with cytosolic cell extracts. To obtain these extracts 2-4×10⁶ cells were washed once in ice-cold KK2 buffer (20 mM KH₂PO₄/K₂HPO₄, pH 6.8), resuspended in ice-cold phosphate buffer (150 mM potassium phosphate, pH 7.5; 4 mM EDTA) and lysed by passing through 5.0 µm Isopore membrane filters (Millipore). Cell lysates were centrifuged for 2 minutes at 20,800 g (4 °C). The supernatant and an
equal volume of ice-cold TBST containing 5 mM EDTA and 5 mM EGTA were added to the membrane, which was incubated on a platform shaker at 4°C overnight. Finally, the membranes were washed with TBST, mounted between two glass plates and photographed using a Fuji LAS-1000 Imaging system. Dictyostelium development was induced as described previously (Dormann and Weijer, 2001).

**Phagocytosis assay**

Phagocytosis of TRITC-labelled yeast cells by Dictyostelium cells was carried out as described (Maniak et al., 1995). Bacterial uptake was assessed as described (Zhou et al., 1998). Live Klebsiella aerogenes were washed twice in KK2 buffer and added at a final density of OD$_{600nm}$=0.6 to 1×10$^6$ cells/ml Dictyostelium cells that had also been washed twice in KK2 buffer. The cell suspension was incubated at 22°C on a shaker at 150 rpm, and at 1 hour intervals duplicate samples were taken and the OD$_{600nm}$ was determined. Phagocytosis of 1 µm beads (yellow-green fluorescent Fluospheres polystyrene microspheres, Molecular Probes) was measured in 15 minutes and at 50-fold excess of beads had been added to a suspension of Dictyostelium cells (Temesvari et al., 1996). For experiments with the PI3-kinase inhibitor LY294002 (Sigma), cells were pre-incubated for 15 minutes with the inhibitor or its solvent DMSO alone (0.25% final concentration) before the addition of particles.

**Live cell microscopy**

Samples for the microscopic in vivo analysis of the developmental stages were prepared and recorded as described previously (Dormann et al., 2002b). For phagocytosis experiments vegetative cells were washed once in KK2 buffer (20 mM KH$_2$PO$_4$/K$_2$HPO$_4$, pH 6.8), resuspended and placed on 1% KK2 agar plates. When cells had settled on the agar surface the supernatant was removed and the remaining fluid was allowed to evaporate. After heat-killed yeast (Saccharomyces cerevisiae) or 1 µm latex beads had been added, the piece of agar was cut out and put upside down on a glass coverslip mounted in an Attofluor chamber (Molecular Probes). To prevent desiccation the agar was covered with silicone oil (Dow Corning 200/20cs) and confocal imaging started immediately using a Leica TCS SP2 system with a 100×/1.4NA PL APO objective. cAMP stimulation was performed as described previously (Dormann et al., 2002b). For chemotaxis experiments aggregation-competent cells were allowed to settle on a glass coverslip submerged in KK2 buffer, and a micropipette containing 10$^{-4}$ M cAMP was used to generate a gradient.

**Image processing and analysis**

All image analysis was performed using custom written macros for the Optimas (version 6.1, MediaCybernetics) image processing software. We measured the changes in membrane fluorescence during phagocytosis at the plasma membrane at the proximal site of the phagocytic cup and later in the phagosomal membrane in a 5×5 pixel window and in the adjacent cytosol (see inset Fig. 1C). To correct for the cytosolic fluorescence (c) this value was subtracted from the plasma membrane intensity (p) and the result divided by the cytosolic fluorescence intensity (p-c)/c. Because of large differences in the amplitudes between cells and the various strains due to differences in expression levels, the data for individual cells were subsequently rescaled between 0 and 1. For the graphical representation the time point at which the phagosome detached from the cell cortex was defined as t=0 seconds. To detect changes in membrane translocation following cAMP stimulation and during development we measured plasma membrane and cytosolic fluorescence and calculated the ratio (p/c). The periodic membrane translocation of PH-domain probes in aggregation streams was visualised by measuring the average intensity in a 200×200 pixel window, for CRAC-PH cells following background subtraction (Dormann et al., 2002b). Active contours were calculated as described (Dormann et al., 2002a). The results were plotted as polar plots using custom written macros for the Open Source IBM Visualisation DataExplorer (4.1.3).

**Results**

 Dictyostelium cells can phagocytose different particles, such as bacteria, latex beads and yeast cells. Previous studies in Dictyostelium suggested that PI3-kinase signalling is only required for macropinocytosis, not phagocytosis (Cardelli, 2001). However, in our hands phagocytosis assays using yeast as substrate in the presence or absence of the PI3-kinase inhibitor LY294002 showed a dose-dependent effect of the inhibitor, with 50 µM LY294002 causing a considerable reduction in yeast uptake, suggesting that phagocytosis of large particles like yeast is also dependent on PI3-kinase activity.

![Fig. 1.](image-url) The PI3-kinase inhibitor LY294002 affects phagocytosis. (A) Wild-type AX3 cells were incubated with TRITC-labelled yeast and at the indicated time points the fluorescence of internalised particles was measured. Average and standard deviation of three independent experiments are shown. (B) Uptake of fluorescent 1 µm beads by AX3 cells. Average and standard deviation of three independent experiments are shown. (C) Effect of LY294002 treatment on phagocytosis of bacteria by AX3 cells. The internalisation of bacteria was measured as a reduction of the optical density at 600 nm, here plotted as a percentage of the OD$_{600}$ at t=0 hours. The curve ‘bacteria’ refers to a control with bacteria but without Dictyostelium cells. The average and standard deviation of three independent experiments are shown.
As this requirement for PI3-kinase activity might depend on the particle size (Vieira et al., 2001) we measured the uptake of smaller fluorescent beads of 1 µm diameter (Fig. 1B) and observed again a strong inhibitory effect of LY294002 on the phagocytosis of these beads, indicating that the uptake of 1 µm particles is PI3-kinase dependent. Similarly, when we assessed the phagocytosis of bacteria, measured as a decrease in the optical density of a suspension of bacteria with *Dictyostelium* cells, we found that LY294002 treatment reduced the uptake of bacteria. As these results suggested the involvement of PI3-kinases in particle uptake we measured phosphoinositide dynamics with high spatial and temporal resolution during phagocytosis of yeast cells and beads using a variety of GFP-tagged PH domains. First, we confirmed, by using a modified protein lipid overlay assay in which lipid binding was detected directly using the GFP fluorescence, that these PH domain GFP fusion proteins showed the expected phosphoinositide binding specificity. Typical blots are shown in Fig. 2. CRAC binds both PtdIns(3,4)P3 and PtdIns(3,4,5)P3, whereas GRP1 binds to PtdIns(3,4,5)P3, TAPP1 to PtdIns(3,4)P3 (Fig. 2A, B). DAPP1 has a similar binding specificity to CRAC – it binds PtdIns(3,4,5)P3 as well as PtdIns(3,4)P2 (Fig. 2C).

**PtdIns(3,4,5)P3 dynamics during phagocytosis**

We examined PtdIns(3,4,5)P3 distribution in vegetative *Dictyostelium* cells by monitoring the binding of the PtdIns(3,4,5)P3-specific GRP1-PH-GFP and noted some basal plasma membrane binding at the leading edge of moving cells (Fig. 3A). To study the distribution of GRP1-PH during phagocytosis, heat-killed yeast cells were added. At timepoint 0 seconds there was already an extended region with strong GRP1-PH-GFP membrane localisation at the leading edge, which was followed by a further rise in fluorescence at the phagocytic cup as the cell started to extend the plasma membrane to engulf the yeast cell (30 seconds) (Fig. 3A, Fig. 4A). The fluorescence intensity peaked around the time of phagosome closure (60 seconds), when the yeast was completely surrounded by a distinct phagosomal membrane, which had lost contact with the plasma membrane. Subsequently, GRP1-PH-GFP binding to the phagosomal membrane decreased rapidly and was undetectable at 150 seconds. Our data suggest that the disappearance of PtdIns(3,4,5)P3 from the phagosomal membrane starts directly after phagosome closure (Fig. 3A, Fig. 4A).

**PtdIns(3,4)P2 levels increase transiently during phagocytosis**

The rapid breakdown of PtdIns(3,4,5)P3 on phagosome formation could be due to a dephosphorylation by PTEN to form PtdIns(4,5)P2 or by SHIP to form PtdIns(3,4)P2. We examined the cellular distribution of PtdIns(3,4)P2 by following the localisation of the PtdIns(3,4)P2-specific TAPP1-PH-GFP. PtdIns(3,4)P2 was never detected at the plasmamembrane of motile vegetative cells; however, brightly labelled intracellular vesicles were visible (Fig. 3A, 0 seconds); these were probably macropinosomes, which are used for liquid uptake (see below). During phagocytosis TAPP1-PH-GFP binding increased slowly at the forming phagocytic cup (Fig. 3A) (TAPP1, 30-60 seconds). The fluorescence continued to increase until a peak was reached around the time when the phagosome detached from the cell cortex (90 seconds); this was followed by a slow dissociation from the phagosomal membrane (Fig. 3A, 210 seconds, Fig. 4A). In a few cases this was followed by a second but much weaker – and again, transient – peak of TAPP1-PH-GFP binding (data not shown).

The processes described above are not specific to the
phagocytosis of large yeast cells, since during the uptake of small 1 µm latex beads a rapid binding and dissociation of GRP1-PH and TAPP1-PH to and from the phagosome was observed (Fig. 3B). This shows that the transient generation of PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ is common to all the phagocytic processes studied here.

**Binding of PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ dual specificity PH domains**

We next examined the binding of the dual specificity PH-domains CRAC and DAPP1, which bind both PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$, and found that their membrane association followed both the changes in PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ (Fig. 3A, Fig. 4B); however, CRAC-PH and DAPP1-PH show lower basal membrane binding than GRP1-PH. The DAPP1-PH domain showed less strong accumulation at the plasma membrane during the early phases of the particle uptake than CRAC-PH (Fig. 3A, 0-15 seconds, Fig. 4B), and basically followed the kinetics observed with TAPP1-PH, although in vitro data clearly show that DAPP1-PH has the ability to bind PtdIns(3,4,5)P$_3$ as well (Fig. 2C) (Dowler et al., 1999). The PtdIns(3,4)P$_2$-specific DAPP1-PH$^{G176A}$ mutants showed no discernible plasma membrane localisation at membrane protrusions at all, but once the phagosome was formed, its distribution was comparable to TAPP1-PH (Fig. 3A). The similarity between DAPP1-PH and DAPP1-PH$^{G176A}$ binding suggests that DAPP1 binds to PtdIns(3,4,5)P$_3$ with lower affinity in this assay than CRAC and GRP1 (Fig. 4B), which is supported by in vitro PtdIns(3,4,5)P$_3$ binding studies comparing DAPP1 and GRP1 (Fig. 2C). DAPP1 only bound to the membrane at high concentrations of PtdIns(3,4,5)P$_3$, whereas GRP1 displayed considerable binding at levels ten times lower (Fig. 2C).

**Correlation of 3-phosphoinositides with actin dynamics**

To correlate the timing of the observed changes in phosphoinositide levels during phagocytosis with previously described changes in the actin cytoskeleton, we analysed the accumulation of f-actin at the forming phagocytic cup. We used the GFP-tagged actin associated protein coronin (Maniak et al., 1995) as well as the f-actin specific ABD-GFP, which contains the N-terminal actin binding domain of the actin cross-linking protein ABP120 (Pang et al., 1998). Both probes briefly labelled a ring-like f-actin-containing structure surrounding the phagosome (Fig. 5A, 20 seconds; Fig. 5B, 30 seconds) around...
the time when PtdIns(3,4,5)$P_3$ levels reach a maximum. The f-actin ring usually disappeared from the side facing the cell interior (Fig. 5A, 30 seconds; Fig. 5B, 50 seconds), while the fluorescence increased at the interface between phagosome and plasma membrane (30-35 seconds), as seen most prominently with coronin-GFP. The apparent restructuring of the phagosomal actin coat was usually accompanied by a rapid inward movement of the phagosome (Fig. 5C). The timing of this phagosome detachment from the cell cortex coincided with the peak of PtdIns(3,4)$P_2$ production (Fig. 5C).

PTEN null mutants are defective in phagocytosis

It is possible that some of the PtdIns(3,4,5)$P_3$ generated during phagocytosis is dephosphorylated to PtdIns(4,5)$P_2$ by PTEN. We found that phagocytosis of yeast cells was significantly impaired in PTEN null cells (Fig. 6A), which could be rescued by addition of moderate concentrations (20-50 µM) of LY294002 (Fig. 6A). However, this effect was abolished at high concentrations of LY294002 (100 µM). These experiments suggest that levels of 3-phosphoinositides need to be tightly regulated to ensure efficient phagocytosis. There was no significant difference between PTEN null cells and wild-type cells in the uptake of bacteria (data not shown).

Observation of the localisation of the PTEN-GFP fusion protein during phagocytosis showed that PTEN, which is normally associated with the plasma membrane, disappears from the parts of the plasma membrane in direct contact with the yeast particle (Fig. 6B). The PTEN-GFP distribution is thus complementary to the distribution of the PtdIns(3,4,5)$P_3$-specific PH domains; this is similar to what has been described for chemotactically moving cells, where PTEN-GFP is also excluded from the leading edge (Iijima and Devreotes, 2002) and thus contributes to the accumulation of PtdIns(3,4,5)$P_3$ at the leading edge.

We therefore investigated PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$ dynamics in a Dictyostelium PTEN knockout strain. GRP1-PH dissociated from the phagosomal membranes with the same rapid kinetics as in wild-type cells (Fig. 6C), indicating that PTEN is not involved in the degradation of PtdIns(3,4,5)$P_3$ on phagosomal membranes, as expected from its localisation (Fig. 6B). However, to our surprise we observed that PtdIns(3,4)$P_2$ as measured by TAPP1-PH membrane association was degraded more rapidly than

![Fig. 4.](image)

**Fig. 4.** Quantitative analysis of membrane localisation during phagocytosis. Fluorescence was measured at the plasma membrane (p) at the phagocytic cup/phagosome as well as in the adjacent cytoplasm (c), as indicated in inset in (A), and the ratio calculated as described in experimental procedures. Curves were aligned using ‘phagosome closure’, the formation of a membrane enclosed phagosome as $t=0$ seconds. Data were averaged from 10 (GRP1), 13 (TAPP1), 7 (CRAC-PH), 9 (DAPP1-PH) and 12 (DAPP1G176A) cells.

![Fig. 5.](image)

**Fig. 5.** Localisation of f-actin specific probes during phagocytosis. (A) Coronin-GFP localises to the site of particle engulfment, followed by a complex redistribution process (25-45 seconds) until the phagosome is propelled into the cytosol. Yeast marked by asterisks. Bar, 5 µm. See also supplementary material Fig. S2. (B) The f-actin specific probe ABD-GFP shows a similar localisation pattern during the uptake of a yeast cell (marked by asterisks). Bar, 5 µm. (C) Correlation of phagosome movement with changes in GRP1/TAPP1 binding to phagosomal membranes. The averaged fluorescence intensity data for GRP1 and TAPP1 are taken from Fig. 4. The movement of yeast particles during engulfment was tracked and the average velocity from five phagocytosis events from different cells plotted. Phagosome closure occurs at $t=0$ seconds.
Visualisation of phosphoinositide dynamics

Visualisation of phosphoinositide dynamics in wild-type, showing that a PTEN-dependent process normally slows down the dephosphorylation of PtdIns(3,4)P₂ (Fig. 6C).

PtdIns(3,4)P₂ on phagosomal membranes is not made from PtdIns(4)P

To understand whether PtdIns(3,4)P₂ is degraded further to PtdIns(4)P or whether PtdIns(3,4)P₂ could possibly be made de novo from PtdIns(4)P by PI3-kinases on the phagosomal membrane, we examined the distribution of PtdIns(4)P using the PH-domain of FAPP1 (Dowler et al., 2000). We did not observe any binding of FAPP1-PH to plasma or phagosomal membranes (18 phagocytic events) but instead it was found to be associated with highly dynamic vesicular and tubular structures, possibly the Golgi, suggesting that PtdIns(4)P is not an intermediate in the generation/degradation of PtdIns(3,4)P₂ in this process (Fig. 7).

Phagocytosis and macropinocytosis show similar changes in phosphoinositide dynamics

During macropinocytosis we observed the same characteristic changes in PH domain binding as during phagocytosis (Fig. 8). Binding of PH domain-containing proteins like CRAC or Akt/PKB to macroinosomes had been described previously; however, the exact nature of the phosphoinositides in the membranes of these vesicles was unknown (Parent et al., 1998; Rupper et al., 2001a). Only the PtdIns(3,4,5)P₃-specific PH-domains localised to the plasma membrane of the endocytic cups where small fluid drops were taken up. Subsequently, a strong accumulation of PtdIns(3,4)P₂ occurred on the membranes of macroinosomes accompanied by the disappearance of PtdIns(3,4,5)P₃ (see GRP1-PH).

Phosphoinositide dynamics during chemotaxis

In aggregating Dictyostelium cells cAMP induces the transient translocation of the PH-domain-containing proteins like CRAC to the plasma membrane, suggesting a transient rise in PtdIns(3,4,5)P₃ and/or PtdIns(3,4)P₂ (Parent et al., 1998). To determine which of the 3-phosphoinositides are generated following cAMP stimulation, we used our panel of cell lines and found that membrane translocation only occurred in cells expressing PtdIns(3,4,5)P₃ binding PH-domains (GRP1, CRAC-PH, DAPP1-PH) (Fig. 9A). There was no detectable change in the cellular distribution of the PtdIns(3,4)P₂-specific TAPP1-PH and DAPP1-PH<sup>G176A</sup>, suggesting that only PtdIns(3,4,5)P₃ is generated in response to cAMP. As PtdIns(3,4)P₂ does not appear to be a PtdIns(3,4,5)P₃ degradation product in this assay aggregating cells must use
Fig. 8. Changes in 3-phosphoinositide levels during macropinocytosis. Comparison of fluid-phase uptake during macropinocytosis using different PH-GFP fusion proteins. Arrows mark the position of the endocytic cup (0 seconds) and the forming macropinosomes. See also supplementary material Fig. S5.

Fig. 9. Effect of cAMP stimulation on PH-domain localisation. (A) Snapshots of aggregation competent cells just before and after cAMP stimulation (final concentration 5 µM). Only PtdIns(3,4,5)P₃ binding PH domains show plasma membrane translocation. 20 to 40 cells were recorded for each cell line. (B) Kinetics of membrane translocation of the PtdIns(3,4,5)P₃-specific probes. Data averaged from eight (CRAC) to ten cells (DAPP1/GRP1). (C) Membrane localisation of GRP1-PH and CRAC-PH during chemotaxis. Arrows indicate the direction of the cAMP source. (D) Changes in GRP1-PH-GFP localisation following repositioning of a cAMP-filled micropipette (position marked by arrows). See also supplementary material Fig. S6.
another pathway to terminate the \( \text{PtdIns}(3,4,5)P_3 \) signal involving PTEN.

**PtdIns(3,4,5)P\(_3\)** binding PH domains show differences in membrane localisation in chemotactic cells

Although the PH-domains of GRP1, CRAC and DAPP1 bind to the plasma membrane following cAMP stimulation, maximum binding of GRP1-PH was several seconds later than that of the other PH domains and remained at the plasma membrane much longer (Fig. 9A,B). With the exception of a few less motile cells, where GRP1 did return to basal levels within 4-7 minutes in most cases, GRP1 did not completely dissociate from the membrane, as cells started to move again and GRP1 relocated to the newly protruding pseudopods. These differences in the kinetics of membrane association probably reflect differences in the affinity for PtdIns(3,4,5)P\(_3\). CRAC-PH has been shown to associate with the leading edge of cells moving in a cAMP gradient (Parent et al., 1998), and we investigated whether the GRP1 and DAPP1 PH domains showed a similar distribution in chemotactically moving cells. The GRP1-PH domain showed an extended distribution along the plasma membrane, although there was always an anterior-posterior gradient and the strongest binding was found at the leading edge (Fig. 9C,D). DAPP1-PH-GFP membrane localisation was only observed in cells in close proximity to the cAMP-filled micropipette, where the cells experienced the highest cAMP levels (data not shown).

Periodic modulation of PtdIns(3,4,5)P\(_3\) levels in aggregating cells

We have previously characterised the dynamic behaviour of CRAC-GFP membrane binding in cell moving chemotactically in response to naturally occurring cAMP waves during aggregation and mound formation (Fig. 10D-F) (Dormann et al., 2002b). We now investigate the dynamics of GRP1-PH-GFP binding (Fig. 10A-C). In early aggregating cells a strong transient membrane translocation could be observed in response to the cAMP waves; however, once the cells were organised in aggregation streams, a strong residual membrane binding usually with an anterior posterior gradient was observed (Fig. 10A,B). Membrane binding was still periodically modulated following detection of the exogenous cAMP signals as shown for a single cell (Fig. 10B) and as measured over a part of the aggregation stream (Fig. 10C). Cells in mounds also maintained a periodic modulation of GRP1-PH-GFP binding to their leading edge but localisation was less localised than that of CRAC-PH (data not shown).

Although cAMP stimulation only led to the production of PtdIns(3,4,5)P\(_3\), we did observe some membrane localisation in aggregating cells expressing the PtdIns(3,4)P\(_2\)-specific probes TAPP1-PH or Dapp\(_1^{G176A}\)-PH. The cells characteristically showed a V-shaped leading edge and tried to follow or even engulf the cells in front, which were occasionally phagocytosed (Fig. 10G,H). A substantial number of cells showed this behaviour during the course of a recording; however, there was no evidence for spatial coordination of this behaviour.
Polarisation of slug cells

We have previously shown that cells in migrating slugs show constant polarised CRAC-PH binding at their leading edge (Dormann et al., 2002b). To our surprise we observed a uniform membrane binding of GRP1-PH in both prestalk and prespore cells (Fig. 11A,B). Quantification using active contours (Dormann et al., 2002a) showed that there is a nonuniform membrane distribution of PtdIns(3,4,5)P_3 in prestalk cells as measured with CRAC-PH (Fig. 11D) but that this asymmetry cannot be detected by GRP1-PH (Fig. 11C). To show that GRP1-PH bound specifically to PtdIns(3,4,5)P_3, slugs were transferred to agar plates containing 250 µM of the PI3-kinase inhibitor LY294002 leads to rapid loss of GRP1-PH-GFP plasma membrane localisation; however, CRAC-PH-GFP often accumulates in bright cytoplasmic structures (arrows). Bars, 30 µm.

Discussion

Phosphoinositide dynamics during phagocytosis in Dictyostelium

Using phosphoinositide-specific PH domains tagged with GFP we have shown strong similarities between macropinocytosis and phagocytosis in terms of the spatial distribution and temporal change of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, suggesting that both events might involve similar regulatory mechanisms. We have seen an increase in PtdIns(3,4,5)P_3 during the formation of the phagosome, followed by a rapid decay during internalisation. PtdIns(3,4,5)P_3 production is closely followed by PtdIns(3,4)P_2 formation which peaks shortly after phagosome closure and decays during internalisation. The appearance of PtdIns(3,4)P_2 during endocytosis is intriguing and it is the first time, to our knowledge, that it has been visualised during this process. How is PtdIns(3,4)P_2 generated and what is its function? Most probably, PtdIns(3,4)P_2 is generated from PtdIns(3,4,5)P_3 by removal of the 5-phosphate catalysed by a phosphoinositide 5-phosphatase like SHIP. SHIP phosphatases have been shown to localise to the phagocytic cup in mammalian cell lines (Cox et al., 2001; Marshall et al., 2001) A search in the Dictyostelium genome database for homologs of mammalian
SHIP revealed a sequence with high homology to the inositol phosphate domain of SHIP and this gene was disrupted by homologous recombination. The disruptant did not show altered PtdIns(3,4,5)P$_3$ and or PtdIns(3,4)P$_2$ kinetics or any obvious phenotype (data not shown). Subsequently, it appeared that this gene is identical to DdSP1, which belongs to a recently described five-membered family of 5-phosphatases, of which at least two (DdSP2, DdSP4) are able to degrade PtdIns(3,4,5)P$_3$ in vitro (Loovers et al., 2003). Nothing is as yet known about the cellular localisation of these proteins, but DdSP4 is particularly interesting as gene disruption severely affects growth both in liquid medium as well as on bacterial lawns, suggesting a role in endocytosis. In the view of our findings it would appear that this phosphatase is now a probable candidate to dephosphorylate PtdIns(3,4,5)P$_3$ during macrophagosyosis and phagocytosis. PtdIns(3,4)P$_2$ could be dephosphorylated to PtdIns(4)P. However, the PtdIns(4)P-specific FAPP1 did not localise to the plasma membrane or phagosomes, suggesting that PtdIns(4)P is not a precursor or breakdown product of PtdIns(3,4)P$_2$, making it more likely that PtdIns(3,4)P$_2$ is dephosphorylated to PtdIns(3)P. It has been shown that PtdIns(3)P is transiently generated on phagosomal membranes following phagosome closure in macrophage cell lines (Ellison et al., 2001a; Vieira et al., 2001). Alternatively, the observed PtdIns(3)P could be synthesised directly on phagosomes, given that the class III PI3-kinase Vps34 is recruited to the phagosomal membrane and is required for phagosome maturation (Vieira et al., 2001).

A role for PtdIns(3,4)P$_2$ in phagocytosis?

Interestingly, we observed that in PTEN null mutants the kinetics of PtdIns(3,4,5)P$_3$ disappearance was not altered, showing that PtdIns(3,4,5)P$_3$ is not dephosphorylated by PTEN to PtdIns(4,5)P$_2$ on phagosomal membranes, correlating well with the absence of PTEN-GFP membrane association from the forming phagosome. This is different from the breakdown of PtdIns(3,4,5)P$_3$ during chemotaxis, which is believed to be mainly PTEN mediated. PTEN null mutants are, however, impaired in phagocytosis, and this could be related to the increased rate of PtdIns(3,4)P$_2$ destruction as shown by the TAPP1 binding data (Fig. 6). The PtdIns(3,4)P$_2$ transient during endocytosis could play an important role as a binding site for as yet unknown proteins during phagocytosis and possibly macropinosytosis. PtdIns(3,4)P$_2$ could be targeted by proteins with specific binding motifs. To date, only the tandem-PH-domain-containing-proteins TAPP1, TAPP2 and the PX (phox homology) domain containing p47PHOX have been shown to bind PtdIns(3,4)P$_1$ specifically (Kanai et al., 2001; Karathanassis et al., 2002). Although it remains to be determined whether a wave of PtdIns(3,4)P$_2$ production occurs in other phagocytic cells like neutrophils, it is possible that the binding of p47PHOX to PtdIns(3,4)P$_2$ could play a part in the recruitment of the cytosolic components of the NADPH oxidase complex to the phagosome to form a functional oxidase (Ellison et al., 2001b; Kanai et al., 2001). Interestingly, a mutation in the PX domain of p47PHOX that abolishes PtdIns(3,4)P$_2$ binding is found in patients suffering from chronic granulomatosus disease, which is associated with an inability to generate superoxide and a marked susceptibility to bacterial and fungal infections (Kanai et al., 2001). In Dictyostelium, specific PtdIns(3,4)P$_2$ binding proteins remain to be discovered.

The experiments on PTEN null cells using the PI3-kinase inhibitor LY294002 also indicate that 3-phosphoinositide levels have to be carefully balanced as the reduced yeast uptake by PTEN null cells was rescued to a considerable degree by adding low concentrations of the inhibitor. cAMP-stimulated PTEN null cells show increased and prolonged PtdIns(3,4,5)P$_3$ levels (Iijima and Devreotes, 2002). Although no biochemical data are at present available on PtdIns(3,4,5)P$_3$ levels in vegetative cells they probably exhibit similar defects. A moderate reduction in PtdIns(3,4,5)P$_3$ production by LY294002 could decrease PtdIns(3,4,5)P$_3$ levels sufficiently to enable PTEN null cells to reduce actin polymerisation and to restrict PH domain protein membrane localisation; both of them are processes that are affected in the PTEN null cells (Iijima and Devreotes, 2002) and required for the coordinated formation of cellular extensions like pseudopods or phagocytic cups. If PtdIns(3,4,5)P$_3$ levels are further reduced by high inhibitor concentrations, phagocytosis by PTEN null cells is inhibited as in wild-type cells. The addition of 30 µM LY294002 has also been shown to partially rescue chemotaxis defects of PTEN null cells in a cAMP gradient (Chen et al., 2003).

Similarities between macropinosytosis and phagocytosis

Previous evidence suggested that in Dictyostelium only macropinosytosis requires PI3-kinase activity (Cardelli, 2001). This is partly based on studies using PI3-kinase knockout cell lines in which two of the three identified class I PI3-kinases, DdPIK1 and DdPIK2 (Δdpik1/ddpip2), were inactivated using homologous recombination (Zhou et al., 1995). Double knockouts of DdPIK1 and DdPIK2 and DdPIK2 and DdPIK3 appear to be lethal (Zhou et al., 1995). While fluid phase influx was greatly reduced in Δdpik1/ddpip2 cells, phagocytosis of 1 µm beads was not significantly affected (Buczynski et al., 1997). However, Δdpik1/ddpip2 cells grew slowly on bacterial lawns (Zhou et al., 1995). The PI3-kinase inhibitor LY294002 had neither an effect on the phagocytosis of bacteria (Peracino et al., 1998) nor on the uptake of 1 µm-latex beads (20 µM LY) (Seastone et al., 1998), whereas both LY294002 and wortmannin dramatically reduced fluid uptake (Rupper et al., 2001b). In our hands the inhibitor LY294002 did reduce the phagocytosis of yeast cells, 1 µm beads and bacteria, suggesting the involvement of PI3-kinase activity in the regulation of phagocytosis of all these particles. The discrepancy to previous work can be partly explained by the use of different concentrations of the PI3-kinase inhibitor. At a concentration of 50 µM we see a considerable reduction in particle uptake (Fig. 1A/B), while lower concentrations (20 µM) are less effective (Fig. 1A). These results are in line with observations made on the role of PI3-kinase in phagocytosis in other systems. In macrophages PI3-kinases have been shown to be essential for the cytoskeletal rearrangements during the phagocytic process and have been implicated in the process of phagosome and macropinosome closure (Araki et al., 1995; Stephens et al., 2002). Furthermore, visualisation of PtdIns(3,4,5)P$_3$ signalling during phagocytosis showed a transient PtdIns(3,4,5)P$_3$ accumulation at phagocytic cups (Marshall et al., 2001).
Stimulation with the chemoattractant cAMP results in PtdIns(3,4,5)P$_3$ production, but not PtdIns(3,4)P$_2$

One of the best characterised *Dictyostelium* PH domain-containing proteins, CRAC, necessary for the activation of adenyllycyclase, has been shown to bind both PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ (Huang et al., 2003). However, it was not know whether PtdIns(3,4)P$_2$ is produced in the plasma membrane on cAMP stimulation (Huang et al., 2003). Our data now show that PtdIns(3,4)P$_2$ is not generated in response to cAMP stimulation in the plasma membrane and that all the PtdIns(3,4,5)P$_3$ must be dephosphorylated to PtdIns(4,5)P$_2$ by PTEN (Iijima and Devreotes, 2002).

**Spatial and temporal PH domain localisation reflects binding affinity**

Studies on *Dictyostelium* and leukocytes have shown a strong localisation of GFP-fused PH domain-containing proteins to the leading edge of chemotactically moving cells (Dormann et al., 2002b; Meili et al., 1999; Parent et al., 1998; Servant et al., 2000). The distribution of the PtdIns(3,4,5)P$_3$-specific GRP1-PH domain showed a much more gradual gradient of membrane association. We have previously shown that on cAMP stimulation GRP1 binds to the membrane rapidly with a similar kinetics as CRAC, but dissociated from the membrane more slowly than CRAC, suggesting a high on rate and a lower off rate resulting in high-affinity binding (Dormann et al., 2002a). In aggregation-stage cells PtdIns(3,4,5)P$_3$ is produced very locally and CRAC binds and dissociates fast indicating the true kinetics and extent of PtdIns(3,4,5)P$_3$ production; however, GRP1-PH binding to PtdIns(3,4,5)P$_3$ is so strong that there is a significant diffusion of the GRP1-GFP-PtdIns(3,4,5)P$_3$ complex in the plane of the membrane from the site of its production. This would then result in a shallow gradient in aggregating cells and an equilibration of the complex in slug stage cells where PtdIns(3,4,5)P$_3$ production is permanently polarised.

These observations highlight the interesting point that the binding properties of PH domains influence the temporal and spatial signal readout, possibly just as strong as the binding specificity. GRP1 gives a completely different spatial and temporal signal readout, possibly just as strong as the binding properties of PH domains influence the temporal and spatial signalling kinetics. From these observations it becomes clear that understanding the signalling dynamics of a specific PH domain-containing protein requires the careful study of its behaviour in vivo. We have started to unravel some parts of the complex phosphoinositide signalling pathways in *Dictyostelium* by investigating the spatial-temporal distribution of the 3-phosphoinositides PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ in vivo using phosphoinositide specific PH domains. This approach can be extended to allow simultaneous visualisation of phosphoinositides together with the phosphoinositide kinases and phosphatases that govern phosphoinositide levels. Combined with the analysis of mutants that can readily be generated in *Dictyostelium* and which are already available for several of the key molecules, this should allow us to generate a detailed insight in the in vivo dynamics of phosphoinositide signalling.

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