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Phosphatidylinositol 3-kinase regulation of fluid phase endocytosis

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Abstract Endocytosis of the fluid phase marker, horse radish peroxidase, into baby hamster kidney cells is inhibited by treatment of cells with the fungal metabolite wortmannin. The IC_{50} of approximately 5 nM is consistent with the well-described action of wortmannin upon phosphatidylinositol (PI) 3-kinase. Analysis of the kinetics of uptake indicates a >50% decrease in the initial rate of marker internalisation, a concomitant decrease in the volume of the early endosome and an increased efficiency of recycling of that marker which is internalised. As PI 3-kinase binds to activated growth factor receptors our data suggest that receptor activation can be coupled to receptor internalisation (down regulation) by localising PI 3-kinase stimulation of endocytosis.

Key words: PI 3-kinase; Endocytosis; Wortmannin; Receptor down-regulation; Dynamin

1. Introduction

Phosphatidylinositol (PI) 3-kinase is a signalling protein which is activated upon stimulation by a number of growth factor receptors (reviewed in [1]). It is a stable heterodimer comprising a 110 kDa catalytic sub-unit and an 85 kDa regulatory sub-unit [2] which binds, via SH_2 domains, to phosphotyrosine containing sequences of activated receptors. The 110 kDa catalytic sub-unit has been shown to be related to the yeast *S. cerevisiae* Vps34 protein which is involved in sorting of proteins to the vacuole [3]. Due to the conservation of mechanism between yeast and mammalian cells and between various intracellular membrane trafficking events [4,5] a more widespread function in membrane trafficking for PI 3-kinase has therefore been proposed.

The fungal metabolite wortmannin has become an important tool for the investigation of mammalian PI 3-kinase function. It was originally identified as an inhibitor of myosin light chain kinase [6], but recent work has shown that it will specifically inhibit PI 3-kinase at 100-fold lower concentrations [6,7,8,9]. Wortmannin covalently modifies the catalytic sub-unit of PI-3 kinase [8]. A study of neutrophils determined that the only discernible protein labelled by a radioactive derivative of wortmannin over the relevant concentration range was indeed the 110 kDa sub-unit. Use of this drug has revealed a requirement for PI 3-kinase kinase activity in several examples of regulated exocytosis [8,10] and our laboratory has recently obtained evidence for involvement of a wortmannin sensitive factor in an in vitro assay of early endosome fusion (Jones and Clague, manuscript submitted). In this paper we show that wortmannin exerts a profound inhibition upon the fluid phase endocytic uptake of horseradish peroxidase (HRP) into baby hamster kidney (BHK) cells. These data support the idea that interaction with PI 3-kinase is an important factor in the down regulation of activated receptor tyrosine kinases [11].

2. Materials and methods

Cell culture reagents were from Gibco, HRP was from Biozyme, wortmannin was from Sigma.

Confluent monolayers of BHK cells were split 1:4 into 35 mm diameter dishes the day before an experiment. 30 min before commencing the experiment the indicated concentration of wortmannin was added. Immediately prior to the experiment, cells were washed with Dulbecco's phosphate buffered saline (DPBS) at room temperature. At time zero, dishes were transferred to a metal plate held at 37°C, whilst simultaneously exchanging the DPBS for prewarmed (37°C) Dulbeccos Modified Eagles Medium (DMEM) supplemented with 20 mM HEPES, 5 mM D-glucose (pH 6.85) and the indicated concentrations of HRP.

After the indicated incubation time, the cells were washed extensively, on a rocking metal plate held at 4°C, by 10 × 5 min incubations with DPBS supplemented with 1% bovine serum albumin followed by 2 rinses with cold DPBS. Liquid in the dishes was aspirated off and then 0.25 ml of 1% Triton X-100 was added to each dish and incubated on a rocker at room temperature for 5 min. This liquid was then transferred to a microfuge tube and spun at 5,000 × *g* for one minute. The HRP activity of an aliquot of each supernatant was then determined by a colourimetric assay at 455 nm using *o*-dianisodine and H_2O_2 as substrates.

3. Results and discussion

Wortmannin is a potent inhibitor of fluid phase endocytosis in BHK cells. Fig. 1 shows the dose–response towards wortmannin for a 20 min HRP internalisation. HRP uptake by the cell, is less than 25% the control value at wortmannin concentrations >20 nM. We found some variation between experiments in terms of the absolute level of maximal inhibition at this time point, within the range 50–80% inhibition. Experiments were performed at an external pH of 6.85, which amplified the inhibitory effect about 1.5-fold (data not shown), without significantly affecting HRP internalisation, in accordance with the findings of Davoust et al. [12]. The IC_{50} of approximately 5 nM is entirely consistent with inactivation of PI 3-kinase. Whilst this demonstrates a profound effect on the endocytic pathway, the reduction at this time point could be due to either decreased uptake or increased recycling. We therefore investigated the effects of 25 nM wortmannin on HRP uptake at other time points (Figs. 2 and 3). The measured absorbance is proportional to HRP concentration over the recorded range of Fig. 2; however, in order to obtain a measurable signal after only one minute of internalisation we conducted a separate experiment using a higher concentration of HRP in the internalisation medium (6 mg/ml) (Fig. 3).

Measurement of fluid phase uptake by the cell reveals that

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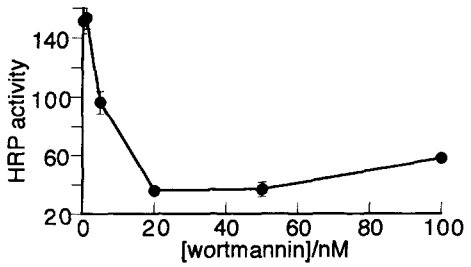


Fig. 1. Dose–response for wortmannin inhibition of fluid phase uptake of HRP. 2 mg/ml HRP was incubated with BHK cells for 20 min at 37°C. Cells were treated with the indicated concentrations of wortmannin for 30 min prior to commencement of the internalisation. *y*-Axis units are arbitrary.

it is biphasic (Fig. 2). This is because in the early stages of the incubation the uptake rate corresponds purely to the rate of internalisation, but as HRP enters recycling compartments the uptake rate at longer time points will correspond to the internalisation rate minus the recycling rate. At steady state this will correspond to the rate of transfer to non-recycling compartments. From Figs. 2 and 3 it is evident that the initial rate of HRP internalisation after wortmannin treatment is about half of the control value. The time point at which the initial slope (Fig. 2) intersects with the final slope gives us an indicator of the time to fill the early endosomal compartment and is close to 10 min in each case. This implies that the volume of the early endosome must decrease in response to wortmannin. As the proportionate volume decrease is essentially the same as the decrease in initial internalisation rate, one can naturally conclude that internalisation rate determines early endosome volume. Reduced volume of the early endosome is consistent with both a maturationist [13] and stable compartmentalist [14] view of the early endosome. An alternative interpretation of this aspect of the data can be fitted to the maturationist framework, this would require that wortmannin increases the rate at which maturing endosomes become refractory to incoming material. Recycling (indirectly measured as initial slope–final slope;

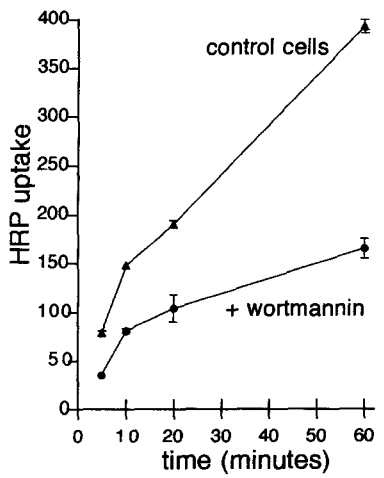


Fig. 2. Time course of HRP internalisation (2 mg/ml HRP) into BHK cells at 37°C. Filled triangles correspond to data points for control cells, filled circles are data points for cells treated with 25 nM wortmannin for 30 min prior to commencement of internalisation. *y*-Axis units are arbitrary.

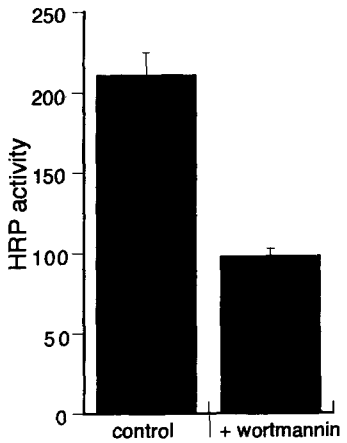


Fig. 3. HRP internalised after 1 min at 37°C by control cells and cells treated with 25 nM wortmannin for 30 min prior to commencement of internalisation. *y*-Axis units are arbitrary.

Fig. 2) is 1.9 times greater in control cells, whereas the rate of entry into non-recycling compartments (final slopes, Fig. 2) is 3.3 times greater, therefore within endosomal compartments wortmannin treatment favours recycling of fluid phase marker, but this may be an indirect effect. This associated phenomenon does not affect the principal finding of this paper, namely that initial fluid phase internalisation steps are PI 3-kinase dependent.

We believe that effects of wortmannin reflect an inhibition of a clathrin coated vesicle-mediated uptake pathway, because this has been shown to account for most of the constitutive fluid phase uptake into BHK cells [12,15]. However, it will be important to confirm our interpretation using an electron microscopic approach. It may be that PI-3 kinase plays a general role in budding of clathrin coated vesicles within the cell including those emanating from the *trans*-Golgi network. The *S. cerevisiae* protein Vps34p, which is homologous to the PI 3-kinase 110 kDa catalytic sub-unit, is involved in sorting of proteins to the vacuole, a process which has also been shown to be clathrin-dependent [16]. It will be interesting to see if effects of wortmannin are observed on trafficking of lysosomal proteins in mammalian cells.

Attention on the role of PI-3 kinase in endocytic events has thus far focussed on internalisation of activated receptors with which it interacts; in particular the platelet derived growth factor (PDGF) receptor. Mutant PDGF receptors lacking a PI 3-kinase binding motif were expressed in human Hep G2 cells. On stimulation, wild type receptors internalised and concentrated in a juxtanuclear region whereas the mutant receptors remained at the cell periphery [11]. One uncertainty with these observations is that the mutated section of the protein also binds the SH2-SH3-containing protein Nck and therefore the requirement for PI 3-kinase binding has not been established unequivocably. We have now shown by the criterion of wortmannin sensitivity that PI 3-kinase activity enhances constitutive endocytosis. Translocation of the enzyme to the plasma membrane following receptor activation might therefore be expected to enhance fluid phase endocytosis. Indeed, treatment of human fibroblasts with EGF has been shown to enhance fluid phase uptake 2.5-fold [17]. This suggests a simple model to couple activation of receptor to its internalisation. PI

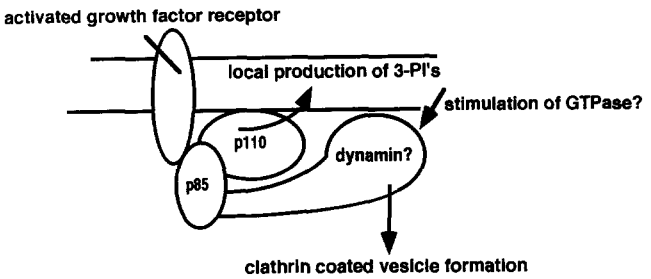


Fig. 4. Diagram indicating proposed model for internalisation of activated growth factor receptor by local generation of 3-PIs and their possible stimulation of dynamin GTPase activity (see text for details).The essential element of the model is the stimulation by 3-PIs of an activity required for endocytosis, which is localised to their point of production by association with p85. This would also apply for target proteins other than dynamin.

3-kinase binds to activated receptor, which would then simply localise endocytosis stimulating activity to the vicinity of the receptor. Further experiments will be required to completely rule out alternative models in which PI 3-kinase stimulates receptor internalisation by enhanced sorting into clathrin coated pits forming independently of receptor activation.

Wortmannin inhibits lipid kinase activity but does not affect growth factor-induced translocation of the p85/p110 complex [18]. This raises the question as to the means by which generation of 3-phosphorylated phosphatidylinositols can lead to enhanced endocytosis. It has recently been shown that dynamin, a protein required for clathrin mediated endocytosis [19,20], can bind to the SH3 domain of p85 [21]. It follows that p85 might act as a three-way adaptor protein, and that this linkage of receptor, PI 3-kinase and dynamin is integral to the receptor internalisation mechanism. As wortmannin seems more likely to affect lipid kinase activity rather than this association, we propose a coupling between phospholipid generation and dynamin activity as a speculative interpretation of our findings (Fig. 4). Acidic phospholipids have been shown to stimulate the GTPase activity of dynamin [22], so it could be that 3-phosphorylated phosphatidylinositols, generated by PI 3-kinase, stimulate the GTPase activity of adjacent dynamin, which has been shown to be required for endocytosis [19].

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