Regulation of early-endosome dynamics by phosphatidylinositol 3-phosphate binding proteins

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Introduction
The endocytic pathway plays an important role in regulating the activities of signalling receptors. Experiments that block the clathrin-mediated internalization of epidermal growth factor receptors have shown that the relative strengths of various signalling pathways associated with the activated receptor are significantly altered as a consequence [1]. Recycling through the early endosome and exposure to the acidic lumen can be used for re-activation of desensitized G-protein-coupled receptors [2], while sorting to later endosomal compartments can target receptors for degradation. One might therefore expect a dialectical relationship between receptor-mediated signal transduction and receptor trafficking.

Joly et al. [3] have shown that platelet-derived growth factor receptor down-regulation is inhibited by the phosphoinositide 3-kinase (PI 3-kinase) inhibitor wortmannin and by mutation of the site for recruitment of the p85/p110 PI 3-kinase complex. This is not due to a block of initial uptake, but rather to a block in the transfer of the receptor from the sorting endosome to lysosomally directed elements [4]. With this in mind, we set out to explore the requirement for PI 3-kinase activity of several aspects of endosomal dynamics using the inhibitors wortmannin and LY294002.

Endosome fusion
Early endosomes or sorting endosomes undergo constant remodelling from a plethora of vesicle fusion, fission and vesicle budding events [5]. We measure the so-called homotypic fusion of early endosomes in a cell-free assay. Fusion is defined as homotypic because fusion partners are operationally defined by identical protocols of internalization of a fluid-phase marker prior to cell homogenization. Following incubation of membrane fractions with cytosol plus the requisite salts and ATP-regenerating system, complex-formation of the two internalized markers, which can only result from membrane fusion, is recorded. This fusion event is known to require the ubiquitous fusion factors NSF (soluble N-ethylmaleimide-sensitive factor) and α-SNAP (soluble NSF attachment protein) [6,7], as well as an endosome-specific fusion factor, the small GTPase Rab5 [8]. Our earliest results showed a dose-dependent inhibition of endosome fusion by wortmannin or LY294002 at concentrations entirely consistent with inhibition of a PI 3-kinase enzyme (IC₅₀ for wortmannin ≈ 15 nM) [9].

Li et al. [10] published similar results, together with a further interesting observation. They observed that when a constitutively active mutant of Rab5 (Q79L, which is defective in GTP hydrolysis) was included in the assay, it was rendered insensitive to wortmannin [10]. The simplest explanation for this result would be that PI 3-kinase activity is required upstream of Rab5 activation on the fusion pathway. Initially we were unable to reconcile their data with our own results, which showed that under the particular configuration of our assay, which can proceed following pre-incubation with guanosine 5'-[γ-thiotriphosphate, thereby locking endogenous Rab5 into the active state, the assay remained sensitive to wortmannin [11]. Titration of the levels of the Rab5 Q79L mutant in the assay provided an explanation. We found that we could render endosome fusion refractory to wortmannin inhibition if we included the mutant at levels far in excess of endogenous levels, i.e. in excess of the amount of Rab5 that could possibly have been inactivated in the first instance. We proposed that PtdIns3Ps were required at the same step of the fusion pathway as Rab5, perhaps to recruit a further fusion factor, and that excess Rab5 could overcome the requirement for these phosphatidylinositols by a mass-action effect [11]. One should note that this interpretation does not rule out a role for PtdIns3Ps in Rab5 activation as originally proposed and for which further evidence has been put...
forward [12], but highlights a late requirement for PtdIns3Ps beyond endogenous Rab5 activation.

**Involvement of early endosomal antigen I (EEA1) in endosome fusion**

Patki et al. [13] conducted a search for proteins that are redistributed to the cytosol from membrane fractions in response to wortmannin. They identified a protein with these properties as EEA1, a well-defined early-endosome marker [14]. EEA1 is predicted to form an extended coiled-coil structure over most of its length, flanked by two zinc-finger domains. The C-terminal domain belongs to the FYVE class of zinc fingers (named from the first letters of four proteins containing it), which has now been shown to represent a specific binding domain for PtdIns3P [15-17].

We tested the requirement of early endosome fusion for EEA1 [18]. We first confirmed that EEA1 dissociates from early endosomes of BHK cells following wortmannin treatment, and then explored the possibility that this redistribution can account for the wortmannin sensitivity of endosome fusion. Three lines of evidence have highlighted the involvement of EEA1 in an in vitro assay of homotypic endosome fusion [18]: (i) polyclonal antibodies raised against the C-terminal domain of EEA1 inhibit endosome fusion; (ii) incorporation of the C-terminal domain of EEA1 into the fusion assay displaces endogenous EEA1 from membranes and inhibits endosome fusion with a similar dose-dependence; and (iii) depletion of EEA1 from the system (by salt treatment of membranes and/or immunoabsorption of cytosol) results in a diminution of fusion.

The requirement of endosome fusion for EEA1, which associates with endosomes in a PI 3-kinase-dependent manner, is sufficient to account for the inhibition of fusion by wortmannin. PtdIns3Ps are not the sole determinant of EEA1 membrane localization. Simonsen et al. [19], as well as presenting data very similar to those described above, provided evidence that Rab5 cooperates with lipid in binding EEA1 to endosomes. Co-operativity between two relatively low-affinity reactions can result in high-affinity binding. This may explain the ability of excess amounts of activated Rab5 to overcome wortmannin inhibition, i.e. by mass action. On the basis of similarities with Usol, a yeast protein involved in transport from the endoplasmic reticulum to the Golgi, we suggested that the role of EEA1 in endosome fusion may be to act as a tethering molecule that mediates the initial attachment between fusion partners, prior to the formation of a trans-complex between cognate SNARE (SNAP receptor) molecules [5]. This has recently been shown to be the case using an assay that separates endosome attachment from endosome fusion [20,21].

**FYVE proteins**

The identification of the FYVE domain as a PtdIns3P binding motif was a major advance [13, 15-17]. There are five proteins with this domain in budding yeast, of which three (Vac1, Vps27 and Fab1) have been implicated in the regulation of membrane traffic. Vac1p is involved in the fusion of Golgi-derived vesicles with endosomes, and binds both PtdIns3P and the Rab5 homologue Vps21p [22]. On this basis it is proposed that Vac1p fulfils the function of EEA1 in this system. Vac1p also binds to the t-SNARE Pep12p, and to Vps45p [22], a protein related to the n-sec1 family of proteins that have been proposed to be negative regulators of SNARE-complex assembly through a tubular SNARE (t-SNARE) interaction [23,24]. This suggests an additional function for Vac1p beyond initial attachment of the vesicle, perhaps regulation of the availability of Pep12p for participation in SNARE-complex assembly. Vps27, a class E Vps mutant, has been implicated in transport between endosomes and the vacuole [25], while Fab1 is a PtdIns3P 5-kinase that may regulate the formation of intraluminal vesicles in multivesicular bodies (MVBs) of the endocytic pathway (see below) [26,27].

One important issue is whether the endosomal pathway represents the sole site of membrane localization of FYVE-domain-containing proteins, perhaps because it is the major site of PtdIns3P generation. If so, what is the degree of overlap between different FYVE proteins? In mammalian cells Smad anchor for receptor activation (SARA) localizes to internal vesicles reminiscent of endosomes and is involved in the recruitment of Smad2 to the transforming growth factor-β receptor [28]. In our laboratory we are undertaking localization studies on two further FYVE-domain-containing proteins, the growth factor substrate protein Hrs [29] and KIAA0371 [30], a protein of unknown function. Hrs has been shown to localize to early endosomes [31] on the basis of co-localization with internalized transferrin. In our hands we observe only a partial overlap between endogenous EEA1 and Hrs, epitope-tagged with haemagglutinin at the C-terminus, expressed at low levels. The degree of
Wortmannin inhibits transfer of horseradish peroxidase from early endosomes to a lighter fraction

(A) Schematic diagram of experimental protocol. BHK cells are labelled with the fluid-phase marker horseradish peroxidase (HRP) by a 5 min pulse at 37 °C. The cells are then incubated at 4 °C for 10 min in the presence or absence of 100 nM wortmannin and then chased at 37 °C for 30 min in the presence or absence of 100 nM wortmannin. The cells are then homogenized and loaded on to a velocity flotation gradient with sucrose concentrations as indicated. Fractions accumulated at the 35/25% and 25/8% (w/v) sucrose interfaces are collected and assayed for total HRP activity. EE, early endosomes; LE, late endosomes.

(B) The fraction of HRP accumulated at the 35/25% (w/v) sucrose interface that enriches early endosomes is expressed as a percentage of the sum of HRP in this fraction plus the HRP at the density of ECVs and late endosomes.

PI 3-kinase requirement for MVB formation

Although internalized fluid-phase markers can clearly reach compartments containing late-endosomal markers in the presence of wortmannin [32], the efficiency of this process has not been accurately quantified. We have used an assay that measures the formation of endosomal carrier vesicles (ECVs) or MVBs from early endosomes of BHK cells by their separation on a velocity flotation gradient [33,34]. Following a pulse-chase protocol, accumulation of fluid-phase marker at a low-density interface, compared with early-endosome labelling at a higher-density interface, is measured as an index of ECV formation [33]. In the presence of wortmannin, the marker can no longer reach the lower-density compartment, indicating a block to ECV/MVB formation, at least as defined by their centrifugation profile (Figure 1). Our interpretation is supported by recent studies of human melanoma cells which showed that wortmannin inhibits the pinching-off of intraluminal vesicles, which is presumably required for bona fide ECV/MVB formation [35]. This can explain the wortmannin block of platelet-derived growth factor receptor trafficking to the late endosomes described by Joly et al. [4], because tyrosine kinase growth factor receptors destined for down-regulation probably require sorting into...
these intraluminal vesicles, as exemplified by the epidermal growth factor receptor [36]. Our results also agree with data from studies on budding yeast indicating that the PtdIns3P 5-kinase activity of Fab1 is required for MVB formation [27] and that Vps27 is required for endosome-to-vacuole transport [25]. We propose that wortmannin blocks ECV/MVB formation in BHK cells by denying substrate to a mammalian Fab1 homologue and/or by influencing Vps27 function.

**Concluding remarks**

The relevant lipid in both aspects of endosome dynamics we have discussed is PtdIns3P, which acts to facilitate binding of EEA1, to serve as a substrate for Fab1 and perhaps to influence Vps27 function. How is this lipid generated within cells? Several PI 3-kinases have now been identified that demonstrate distinct substrate specificities in vitro [37]. However, the hVps34 enzyme is a particularly strong candidate, as it can only use PtdIns as a substrate [38]. Micro-injection of inhibitory antibodies against this enzyme resulted in redistribution of EEA1 to the cytosol, whereas antibodies directed against the p110α catalytic subunit had no effect on this distribution [39]. PtdIns3P is the most abundant 3-phosphoinoside in a resting cell, the levels of which are relatively insensitive to growth factor stimulation [40]. It is perhaps no surprise, then, that such basic aspects of the endocytic pathway are regulated by a constitutively produced lipid.

To return to our starting point, it may be that specific receptor trafficking can be coupled to receptor signalling by PI 3-kinase activation, but the effects of general PI 3-kinase inhibitors cannot be interpreted in these terms. More specific approaches will have to be adopted, including site-directed mutagenesis of receptors and specific inactivation of individual PI 3-kinases.

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Regulation of endocytic membrane traffic by phosphatidylinositol 3-phosphate
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Introduction
Phosphoinositide 3-kinase (PI 3-kinase) phosphorylates phosphatidylinositol (PtdIns) and its derivatives at the 3'-position of the inositol ring. The resulting products, called 3'-phosphoinositides, serve as regulators of vital cellular functions, including signal transduction, membrane trafficking and the organization of the actin cytoskeleton [1]. Here we will discuss some recent data that provide insight into how one of the 3'-phosphoinositides, PtdIns3P, functions in the regulation of endocytic membrane trafficking.

Role of Rab5 and PI 3-kinase in endosome fusion
Endocytosis, the process by which vesicles pinch off from the plasma membrane and fuse with early endosomes, is crucial for the cellular uptake of macromolecules and for the down-regulation of surface receptors [2]. Like other pathways of intracellular trafficking, endocytosis is tightly regulated by a protein machinery. One of the key players in endocytic membrane trafficking is the small GTPase Rab5. This protein is reversibly attached to the cytosolic side of the plasma membrane, endocytic vesicles and early endosomes, and its overexpression leads to an increased rate of endocytosis [3,4]. Rab5 has been reported to regulate the formation of clathrin-coated vesicles from the plasma membrane [5], but its best-documented function is the regulation of fusion between endocytic vesicles and early endosomes, as well as the homo- and heterotypic fusion between early endosomes [6,7]. The latter processes are stimulated by the 'active', GTP-bound, form of Rab5, and the expression of a GTPase-deficient Rab5 mutant leads to the occurrence of giant early endosomes due to increased fusion [8]. The GTP-bound form of Rab5 recruits a cytosolic complex consisting of Rabaptin-5 and Rabex-5, a GDP/GTP exchange factor (GEF) for Rab5. This complex is crucial for endocytic membrane fusion, as its immunodepletion from the cytosol inhibits endocytic-vesicle-endosome and endosome-endosome fusion [7,9]. It is still not clear how the Rabaptin-5–Rabex-5 complex participates in the fusion process, but the association of a GEF with a protein binding to Rab5-GTP suggests a self-amplification mechanism whereby Rabaptin-5–Rabex-5, bound to Rab5-GTP, may convert a neighbouring Rab5-GDP into the GTP-bound form [7]. This could lead to a localized patch of active Rab5 in the membrane.

There is a connection between Rab5 and PI 3-kinase: both of these molecules are required for endocytic membrane fusion [6,10,11], and the presence of excess Rab5-GTP counteracts the inhibitory effect of the PI 3-kinase inhibitor wortmannin on endosome fusion [10]. Initially the latter observation was interpreted as evidence that PI 3-kinase regulates a GEF of Rab5. However, there is no evidence that Rab5 GEFs, such as Rabex-5, are regulated by PI 3-kinase, and the amount of Rab5-GTP required to counteract the wortmannin inhibition is much higher than the amount of endogenous Rab5 present [12]. Therefore alternative explanations have to be sought in order to explain the interplay between Rab5 and PI 3-kinase.

Early-endosomal autoantigen 1 (EEA1), an effector of Rab5 and PtdIns3P in endocytic membrane fusion
EEA1 was originally discovered as an autoantigen in a subset of patients suffering from lupus...