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Phosphoinositides as membrane compartmental identifiers

Phosphoinositides regulate membrane traffic, define organelle identity and mediate signal transduction. Different phosphoinositides map to the membranes of different organelles[1-4], in relative amounts that reflect the activities of organelle-associated phosphoinositide synthases and specific inositol kinases and phosphatases. In endocytic traffic, PtdIns(4,5)P_2 marks the plasma membrane, and PtdIns3P and PtdIns4P mark distinct endosomal compartments[3,4]. Early genetic studies in yeast showed that generation of PtdIns3P by an endosomal phosphatidylinositol 3-kinase, Vps34, is a determinant of compartmental identity in membrane traffic[5,6], while leaving open the question of where and with what timing phosphoinositide conversion occurs.

Endocytic proteins modulate coat assembly and cargo selection, membrane deformation and scission, vesicle translocation and fusion. That many of these proteins interact *in vitro* with PtdIns(4,5)P_2, PtdIns3P, PtdIns4P and PtdIns(3,4)P_2 suggests interconversion of phosphoinositides along the routes of endocytic traffic. A connection between PtdIns(4,5)P_2 and an endocytic protein was first shown for AP2, the endocytic clathrin adaptor, which binds PtdIns(4,5)P_2 selectively[7]. Acute depletion of PtdIns(4,5)P_2 blocks association of cytosolic AP2 with the plasma membrane, prevents coated pit initiation and stalls partially formed coated pits[8,9]. Other endocytic proteins such as dynamin, epsin, eps15, and SNX9 bind phosphoinositides *in vitro*, but their low affinity and relative promiscuity have made the specificity of their lipid interactions difficult to establish[10-14].

A particularly important characteristic of a phosphoinositide compartmental identifier in vesicular membrane traffic is that the onset of any modification that can be detected experimentally or functionally marks the separation of the vesicle from the partner membrane. The modified lipid can no longer diffuse from the budded carrier, nor can the lipid identifier of the parent membrane replace it, and components such as auxilin can then sense vesicle closure, even tens of nanometers from the point of scission.

Clathrin-coat specific phosphoinositide sensors

Auxilin1 (Aux1) has a phosphoinositide binding N-terminal PTEN-like domain, a central clathrin-binding region, and a C-terminal, Hsc70 binding J-domain (Fig. 1a)[15,16]. A burst of auxilin1 and auxilin2/GAK recruitment immediately after closure of an endocytic clathrin-coated vesicle at the plasma membrane determines the onset of uncoating[15,16] (Extended Data Fig. 1a, Supplementary Video 1). Partial or complete removal of the PTEN-like domain[15-17] (Extended Data Fig. 1a,

Supplementary Video 1) or mutation of its lipid binding surface[17] eliminates recruitment, but the J-domain is dispensable for this initial auxilin association with the clathrin lattice[15,16] (Extended Data Fig. 1a). A number of other proteins with functions completely unrelated to auxilin or to clathrin-coat dynamics bind specific phosphoinositides through interactions mediated by lipid-binding domains of similar size but of structure unrelated to that of the Aux1 PTEN-like domain. We created live-cell imaging sensors by replacing the PTEN-like domain of EGFP-Aux1 with other domains of defined phosphoinositide specificity (Fig. 1a, Extended Data Fig. 1j), causing recruitment of the modified Aux1 chimeras to coated pits or coated vesicles depending on the phosphoinositide composition of the underlying membrane.

We verified that all the Aux1-based sensors of defined lipid binding specificity interacted with the correct phosphoinositides in membrane strips (Extended Data Fig. 3a). We also verified that expression of the Aux1-based sensors at the relatively low levels used in the experiments had no detectable effect on clathrin-coated pit dynamics (Extended Data Fig. 3b) and clathrin-mediated transferrin uptake (Extended Data Fig. 3c). Moreover, the recruitment properties of the Aux1-based sensors were the same in cells stably (Extended Data Fig. 4b) or transiently expressing them (Fig. 2) and also in other cell types such as monkey COS-7 and human dermal fibroblasts[18,19] (Extended Data Fig. 4c, d). Chimeric proteins with the clathrin-binding domain of epsin1 fused to various lipid-binding domains in place of the epsin1 ENTH domain confirmed the coincidence detection principle embodied in our Aux1-based constructs (Extended Data Fig. 4e).

We showed that recruitment of the PtdIns(4,5)P_2 sensor did not require a proposed dynamin-binding region between the PTEN-like domain and the clathrin-binding domain, but only a linker from Dishevelled2[20] long enough to bridge the ~100 Å gap between the clathrin lattice and the lipid headgroup layer (Extended Data Fig. 1c-i), so that the sensor can make both lipid and clathrin contacts at the same time. Finally, we saw unaltered recruitment of the PtdIns(4,5)P_2 sensor to coated pits when we used a mutant of the phosphatase module of OCRL unable to hydrolyze PtdIns(4,5)P_2 (Extended Data Fig. 2c).

In the main text of this paper, we describe sensors for PtdIns3P, PtdIns4P, PtdIns(4,5)P_2, and PtdIns(3,4)P_2. We could not detect association of PtdIns5P or PtdIns(3,4,5)P_3 sensors with clathrin-coated structures, but we cannot rule out that lack of association was due to the low affinity of the relevant sensor for these lipids[21,22], rather than to their absence from the coated structures. We did not test for PtdIns(3,5)P_2, as there is no identified lipid binding domain specific for this headgroup[23].

Roles of phosphatidylinositol 4-kinase PI4KIIIα and type I phosphatidylinositol 4-phosphate 5-kinase PIPKIγ and inositol 5-phosphatases synaptojanin1 and OCRL

The plasma membrane phosphatidylinositol 4-kinase, PI4KIIIα, converts phosphatidylinositol to PtdIns4P[24]. We treated cells for 10 min with the selective PI4KIIIα inhibitor A1 (100 nM)[25], and confirmed an increase in the number of coated pits with longer lifetimes (Extended Data Fig. 6a), as first shown in mouse embryonic cells constitutively lacking PI4KIIIα[24]. We detected a substantial reduction in the steady PtdIns4P sensor accumulation during pit formation but a somewhat increased subsequent burst (Fig. 3a) for which we presently do not have an explanation, and confirmed the reversibility of A1 inhibition (Extended Data Fig. 6b). Previous reports have shown that acute inhibition of PtdIns4P synthesis has only a minor effect on the amount of PtdIns(4,5)P_2 associated with the plasma membrane[24-26]; consistent with these observations, the PtdIns(4,5)P_2 sensor was still recruited in the presence of the A1 inhibitor (Extended Data Fig. 6c). We conclude that the PI4KIIIα catalyzed reaction is a source of PtdIns4P in a nascent coated pit but not a principal generator of the PtdIns4P burst that follows budding of a coated vesicle. PIPKIγ is known to convert PtdIns4P into PtdIns(4,5)P_2[27] and its depletion by siRNA led to a ~30% reduction in the recruitment of the PtdIns(4,5)P_2 sensor to forming coated pits (Extended Data Fig. 6d); this observation suggests that PIPKIγ might be partially responsible for the presence of PtdIns(4,5)P_2 in coated pits located at the plasma membrane.

Another potential route to PtdIns4P is the reaction catalyzed by 5-phosphatases such as synaptojanin1 (Synj1), which can interact with clathrin coat components[28-30] and associates with clathrin-derived structures at the plasma membrane[31,32], or OCRL, which also associates with clathrin-derived structures at the plasma membrane and peripheral early endosomes when ectopically expressed as an EGFP fusion[18,33,34]. EGFP-Synj1 expressed in gene-edited EGFP-Synj1$^{+/+}$ SUM159 cells (Extended Data Fig. 6e) was also recruited to clathrin-coated pits and vesicles (Extended Data Fig. 6f). We found, however, that single elimination of Synj1 by CRISPR/Cas9 mediated knockout in SUM159 cells (Extended Data Fig. 6g) or its partial knockdown mediated by lentivirus transduction using shRNA in either SUM159 or COS-7 cells (Extended Data Fig. 6h, i) affected neither the steady, low-level recruitment of the PtdIns4P sensor to assembling coated pits nor the final burst of recruitment to coated vesicles seen in control cells. The same was true for single knockout or knockdown of OCRL in SUM159 cells (Extended Data Fig. 7e, f) and knockdown in COS-7 cells (Extended Data Fig. 7g). Transient depletion of OCRL in SUM159 cells was accompanied by an increased level of Synj1 mRNA (Extended Data Fig. 7j). We further found that EGFP-OCRL expressed at physiological levels in gene-edited SUM159 EGFP-OCRL$^{+/+}$ cells was not present in clathrin-coated pits whereas 2–8 OCRL molecules were typically recruited to coated vesicles after

vesicle scission (Extended Data Fig. 7a–d). Single elimination of Synj1 minimally increased the recruitment of the PtdIns(4,5)P_2 sensor, while elimination of OCRL had no effect (Extended Data Fig. 7h).

Elimination of Synj1 together with ~80% depletion of OCRL (Synj1-KO + OCRL-KD) led to an increased amount of PtdIns(4,5)P_2 sensor during the late stages of pit formation and in the coated vesicles, together with a delayed release of the sensor during the uncoating process (Fig. 3b and Extended Data Fig. 7h). Consistent with the impaired dephosphorylation of PtdIns(4,5)P_2 observed with the combined removal of Synj1 and OCRL, we found a decrease in the burst of the PtdIns4P sensor at the time of uncoating (Fig. 3c). Thus, upon depletion of ~80% of OCRL, we expect an average recruitment of ~ one OCRL molecule / per clathrin-coated vesicle, still sufficient to generate a PtdIns4P burst during the uncoating stage. Assuming that the catalytic activity of OCRL is about one dephosphorylation event / 20 ms, and that a coated vesicles contains ~100–200 PtdIns(4,5)P_2 molecules, then PtdIns(4,5)P_2 would still be consumed, but at a slower rate, e.g. in ~3 sec. We note that the small amount of PtdIns4P detected during the coated pit phase remained unchanged (Fig. 3c); this result is consistent with the observation that most of PtdIns4P associated with coated pits comes from the plasma membrane pool generated by the kinase activity of PI4KIIIα, as shown in Fig. 3a.

The combined removal of Synj1 and the substantial depletion of OCRL had no detectable effects on the dynamics of coated vesicle formation as assessed by the distribution of lifetimes (Extended Data Fig. 7h). This is the expected behavior since the observed reduced PtdIns4P burst was accompanied by a normal PtdIns3P burst (Extended Data Fig. 7i) (these are the lipids that cause the recruitment of auxilin1 and auxilin2/GAK in a burst that accompanies uncoating)[15,16].

Roles of the class II phosphatidylinositol 3-kinase PI3K-C2α and class III phosphatidylinositol 3-kinase Vps34 and 4-phosphatase INPP4A in generating PtdIns3P in clathrin-coated vesicles

The class II phosphatidylinositol 3-kinase PI3K-C2α, known to associate with clathrin-coated structures at the plasma membrane[35-37], can generate PtdIns3P and PtdIns(3,4)P_2[36,38]. We studied recruitment of this enzyme to clathrin-coated pits and vesicles in EGFP-PI3K-C2α$^{+/-}$ SUM159 cells gene-edited in a single allele to express EGFP-PI3K-C2α (Extended Data Fig. 8a). As observed before when using ectopic expression[35], the continuous recruitment of EGFP-PI3K-C2α was congruent with the accumulation of clathrin throughout the clathrin cycle (Extended Data Fig. 8b, d). Subsequent EGFP-PI3K-C2α loss paralleled the abrupt release of the clathrin coat during the uncoating step (Extended Data Fig. 8b, d). It is therefore unlikely that PI3K-C2α generates the

PtdIns(3,4)P_2 that appears late in these vesicular carriers (Fig. 2d). EGFP-PI3K-C2α was present during the appearance of the PtdIns3P burst, however, suggesting that it may have generated PtdIns3P from phosphatidylinositol (Extended Data Fig. 8c). Support for this hypothesis comes from the observation that reducing the availability of PI3K-C2α by siRNA knockdown greatly reduced the amplitude of the PtdIns3P burst detected with the PtdIns3P sensor in SUM159 cells (Fig. 3d, Extended Data Fig. 8e, f) or in COS-7 cells (Extended Data Fig. 8i), while recruitment of the PtdIns(3,4)P_2 and PtdIns4P sensors was largely unaffected (Extended Data Fig. 8g-i).

The class III phosphatidylinositol 3-kinase Vps34 known to regulate steps associated with intracellular vesicular trafficking including autophagosome formation[39] uses phosphatidylinositol as a substrate to generate PtdIns3P[40]. Since the selective inhibitors PIK-III (5 μM for 1 hour; Fig. 3e) or VPS34-IN1 (5 μM for 1 hour; Extended Data Fig. 8j)[41,42] had minimal effects in the burst recruitment of the PtdIns3P sensor to endocytic coated vesicles, we infer that this kinase does not generate the PtdIns3P detected in the early steps of endocytosis.

Since INPP4A generates PtdIns3P from PtdIns(3,4)P_2[43] and coated vesicles contain PtdIns(3,4)P_2, it is possible that the burst of PtdIns3P we observe after scission is in part due to INPP4A activity. Indeed, siRNA depletion of INPP4A led to significant reduction of in the amount of PtdIns3P in coated vesicles (Extended Data Fig. 8k). INPP4A depletion also led to stalling of a fraction of coated pits (Extended Data Fig. 8k) and to enlargement of the endosomes as shown before[43].

Rab5 recruitment

Because Rab5 is required for fusion with early endosomes, it was proposed that coated vesicles either carry Rab5 captured from the plasma membrane or acquire it en route to endosomes[44-46]. More recent work led to the proposal that hRME-6, a Rab5 guanine exchange factor (GEF), recruits Rab5 to clathrin-coated vesicles, and that Rab5 is required for uncoating[47]. But recruitment of Rab5 to coated pits or coated vesicles is at odds with the absence of all three Rab5 isoforms in coated vesicles analyzed by mass spectrometry[48], with the incomplete effect on transferrin or EGF uptake following joint depletion of all three Rab5 isoforms[49], with the absence of Rab5 in coated pits or coated vesicles (Fig. 4a, Extended Data Fig. 9) and with the absence of any effect on coated pit and coated vesicle assembly and disassembly dynamics (Extended Data Fig. 10c). We find instead that Rab5 appears in almost all endocytic carriers soon after they lose their clathrin coat and that its recruitment coincides with an abrupt increase in carrier motion. We further show that Rabex5 has a role in Rab5 recruitment to the endocytic carriers similar to that of hRME-6 and that Rab5 recruitment

becomes negligible if both are depleted (Extended Data Fig. 10c). Rab5 might itself recruit a motor, or a third component, linked to the presence of PtdIns(3,4)P_2, might directly or indirectly recruit both. Either alternative is consistent with our experimental observation, that endocytic carriers recruit Rab5 before they fuse with early endosomes.

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