Interplay between Rab5 and PtdIns(4,5)P$_2$ controls early endocytosis in the Drosophila germline

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Summary

Phosphoinositides have emerged as key regulators of membrane traffic through their control of the localization and activity of several effector proteins. Both Rab5 and phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P$_2$] are involved in the early steps of the clathrin-dependent endocytic pathway, but little is known about how their functions are coordinated. We have studied the role of PtdIns(4,5)P$_2$ and Rab5 in the Drosophila germline during oogenesis. We found that Rab5 is required for the maturation of early endocytic vesicles. We show that PtdIns(4,5)P$_2$ is required for endocytic-vesicle formation, for Rab5 recruitment to endosomes and, consistently, for endocytosis. Furthermore, we reveal a previously undescribed role of Rab5 in releasing PtdIns(4,5)P$_2$, PtdIns(4,5)P$_2$-binding budding factors and F-actin from early endocytic vesicles. Finally, we show that overexpressing the PtdIns(4,5)P$_2$-synthesizing enzyme Skittles leads to an endocytic defect that is similar to that seen in rab5 loss-of-function mutants. Hence, our results argue strongly in favor of the hypothesis that the Rab5-dependent release of PtdIns(4,5)P$_2$ from endosomes that we discovered in this study is crucial for endocytosis to proceed.

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Key words: Rab5, Endocytosis, Phosphatidylinositol (4,5)-bisphosphate, Drosophila, Oogenesis

Introduction

Regulation of the level of phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P$_2$] within a cell and recruitment of the small GTPase Rab5 to clathrin-coated vesicles (CCVs) are required to promote the formation and maturation of these vesicles. Although these two mechanisms appear to participate in the same step of clathrin-dependent endocytosis, the potential interdependency between them is not known. The existence in Drosophila of a well-characterized endocytic route in the oocyte makes its oogenesis a very attractive model in which to address this issue in a physiological context. The ovarian follicle is composed of a 16-cell germline cyst – one cell of which is determined as the oocyte – that is surrounded by follicle cells (Fig. 1A) (for a review, see Spradling, 1993). The most-studied endocytic process in the oocyte is the uptake of yolk proteins (Yp1-Yp3) via clathrin-mediated endocytosis (DiMario and Mahowald, 1987). From stages 8 to 11, the oocyte intakes, via endocytosis, a large quantity of vitellogenins, which are yolk-protein precursors that are synthesized in the fat body and the follicle cells. After their entry into the oocyte, yolk proteins are stored inside large late endocytic compartments, yolk granules, which will later provide the reserves necessary for embryonic development (Spradling, 1993). This specific accumulation of embryonic reserves inside the oocyte is achieved by the localization of the vitellogenin receptor Yolkless (Yl) at the plasma membrane (PM) of the oocyte during stages 8 to 11 (Schonbaum et al., 2000). The morphology of the endocytic intermediates in insect oocytes is well described at the ultrastructural level. For instance, it is inside the ovary of Aedes aegypti that coated vesicles were observed for the first time (Roth and Porter, 1964). Furthermore, the succession of these intermediates has also been well described by means of a thermosensitive dominant negative allele of shibire, the Drosophila homolog of dynamin (Tsuruhara et al., 1990). The vitellogenins are first found in clathrin-coated pits (CCPs) at the oocyte PM; after fission, these CCPs form CCVs. These vesicles then lose their coat and form tubular intermediates (Fig. 2A, ‘T’) that fuse with the forming yolk-protein storage granules, thus filling the lumen of these granules with yolk proteins to form mature storage granules (Fig. 2A) (Tsuruhara et al., 1990).

The small GTPase Rab5 is a well-known regulator of early endocytosis in mammals (for a review, see Zerial and McBride, 2001). Rab5 is involved in the budding of CCVs in vitro (McLauchlan et al., 1998) and also regulates their subsequent maturation by promoting the fusion of early endocytic vesicles (EEVs) with sorting endosomes (Bucci et al., 1992). This contribution to endocytic-vesicle maturation relies on the recruitment of several effector proteins that trigger a local enrichment in the endosomal membrane of phosphatidylinositol 3-phosphate [PtdIns(3)P] (Christoforidis et al., 1999; Erdmann et al., 2007; Hyvola et al., 2006; Shin et al., 2005). The Rab5-dependent formation of the PtdIns(3)P-positive endosomal domain on early endosomes participates in the recruitment of endosomal factors regulating various aspects of early-endosome function, such as tethering, fusion and mobility (Schnatwinkel et al., 2004; Zerial and McBride, 2001). Another aspect of endocytosis-related phosphatidylinositol (PtdIns) regulation is the control of PtdIns(4,5)P$_2$ levels during early endocytic steps (for reviews, see Di Paolo and De Camilli, 2006; Wenk and De Camilli, 2004). PtdIns(4,5)P$_2$ plays a crucial role in the selective recruitment of endocytic proteins to the PM for CCV formation. It binds to endocytic clathrin adaptor complexes such as AP2 to initiate the assembly of the coat and also to dynamin, which controls the fission reaction (Di Paolo and De Camilli, 2006). Consistent with this, lower
levels of PtdIns(4,5)P$_2$ impair endocytosis (Abe et al., 2008; Di Paolo et al., 2004; Varnai et al., 2006; Zoncu et al., 2007). There is yet another requirement in the regulation of PtdIns(4,5)P$_2$ after CCV formation. The PtdIns(4,5)P$_2$ 5-phosphatase activity of Synaptojanin (Synj) is necessary for the hydrolysis of PtdIns(4,5)P$_2$ from EEVs, thereby triggering coat-component shedding (Cremona et al., 1999) and downregulating actin polymerization (Sun et al., 2007).

In Drosophila, Rab5 has been found to localize on PtdIns(3)P-containing early endosomes at the neuromuscular junction, where it is required for synaptic-vesicle recycling (Wucherpfennig et al., 2003). It has also been demonstrated that Rab5 function is required for the formation of PtdIns(3)P-containing early endosomes (Wucherpfennig et al., 2003). Thus, Rab5 is, in Drosophila, a fundamental regulator of the early endocytic pathway, similar to its mammalian homologs.

In this study, we used complete loss of rab5 function in the germline cyst to study the consequences on the endocytic pathway. We found that Rab5 is required for maturation of the EEV and yolk-protein endocytosis in the oocyte. Using loss of function of skittles (sktl), coding for a type I phosphatidylinositol 4-phosphate 5-kinase, we show that PtdIns(4,5)P$_2$ is required for endocytic-vesicle formation, for Rab5 recruitment and accordingly for yolk-protein endocytosis. Furthermore, we reveal a previously undescribed role for Rab5 in controlling the release of PtdIns(4,5)P$_2$-binding budding factors and F-actin from EEVs. Finally, we show that PtdIns(4,5)P$_2$-binding coat components and F-actin leads to the formation of an abnormal early endocytic compartment containing Rab5, PtdIns(4,5)P$_2$-binding coat component and F-actin, and, second, affects yolk-protein endocytosis. Hence, our results argue strongly in favor of the hypothesis that Rab5-dependent release of PtdIns(4,5)P$_2$ from EEVs is crucial for endocytosis to proceed.

**Results**

Rab5 is required for endocytosis in the oocyte

In order to investigate whether Rab5 function is required for endocytosis in the oocyte, we first analyzed the intake of yolk proteins in rab5 mutant oocytes. Since embryos homozygous for rab5 mutation die at the end of embryogenesis (Wucherpfennig et al., 2003), we used the FLP-FRT system (Chou and Perrimon, 1996; Xu and Rubin, 1993) to generate germline clones (GLCs) that were homozygous for a null rab5 allele, rab5$^{2}$ (Wucherpfennig et al., 2003) (hereafter abbreviated as rab5$^{2}$ GLCs). The internalized yolk proteins, stored in autofluorescent granules, were not visible in the cytoplasm of rab5$^{2}$ mutant oocytes (compare Fig. 1D and Fig. 1E; see also Fig. 1F,G). This observation shows that yolk-protein storage in the oocyte requires Rab5 function. Consistently, eggs laid by females in which the germline is devoid of any Rab5 activity are flaccid and they collapse (100%, n=60) (data not shown). The process of yolk-protein intake in the oocyte relies on both the correct transport of the vitellogenin receptor Y1 along the secretory pathway to the oocyte PM and on the clathrin-dependent endocytic pathway (Culi and Mann, 2003; DiMario and Mahowald, 1987). In rab5$^{2}$ GLCs, most Y1 protein was, as in controls, detected at and close to PM of the oocyte during vitellogenic stages (Fig. 1H, I). A subset of the Y1 was also mislocalized inside the oocyte cytoplasm (Fig. 1I, arrows), suggesting either that not all of the protein reached the PM or that some Y1 was trapped inside the cytoplasm after its internalization. Nevertheless, the presence of Y1 at the oocyte PM suggests that the secretory pathway is not significantly affected in rab5 mutant oocytes. Thus, the defect in yolk-protein uptake strongly suggests a conserved role of Rab5 in clathrin-dependent endocytosis in the oocyte. The involvement of Rab5 in endocytosis was further confirmed by the absence of FM4-64 internalization in rab5$^{2}$ GLCs (data not shown).

Ultrastructure of the endocytic compartment in rab5$^{2}$ oocytes

To gain insight into the nature of the endocytic defects that we observed in rab5$^{2}$ GLCs, we analyzed the organization of the endocytic compartments by electron microscopy (EM) during stages 9 and 10. We took advantage of the previous characterization of the morphology of endocytic intermediates in the Drosophila oocyte (Tsuruhara et al., 1990) (the outcome of this study is summarized in Fig. 2A). In wild type (wt), during stages 9 and 10, all the endocytic intermediates were found in a narrow region of 3–4 μm beneath the PM (n=6) (Fig. 2C). CCPs (Fig. 2C, double arrowheads) were observed at the PM. The next endocytic intermediate corresponded to small circular CCVs with yolk-proteins apposed to the luminal side of their membrane. The CCVs then lose their coat and form naked EEVs (NEEVs). Although no clearly identifiable coat was visible, we could not conclude that there were no coat components in these vesicles. Thus, CCVs and NEEVs were considered together to be EEVs (Fig. 2C, double arrowheads). EEVs then formed tubular intermediates (T) (Fig. 2C, arrow) that fuse with forming yolk granules (Y1 to Y2) (Fig. 2C). The only endocytic intermediates observed deeper within the oocyte were condensed yolk granules that accumulated progressively inside the oocyte cytoplasm during vitellogenic stages (Y3) (Fig. 2B). It is important to note that the electron-dense material found between the oocyte and the follicle cells does not correspond to yolk protein prior to its uptake by the oocyte but rather to vitelline bodies (v), which remain in the perivitelline space and will coalesce at the end of stage 10 to form the vitelline membrane around the oocyte (D’Alterio et al., 2005; Schlichting et al., 2006).

In rab5$^{2}$ GLCs (n=5), no condensed yolk granules (Y3) in the oocyte cytoplasm were observed (Fig. 2D). CCPs were still present at the oocyte PM (compare Fig. 2C and Fig. 2E, arrowheads). Below the PM, the density of EEVs appeared largely increased when compared with controls (compare Fig. 2C and Fig. 2E, double arrowheads). The tubular intermediates and yolk granules were rare (compare Fig. 2C and Fig. 2E). When present, yolk granules were always immature (Y1-Y2) (compare Fig. 2C and Fig. 2E). Interestingly, in rab5$^{2}$ mutant oocytes, we also observed abnormal structures deeper in the cytoplasm, which were never seen in the control (Fig. 2D, red circles; the red arrowhead indicates the structure shown at higher magnification in G). These correspond to aggregates of clearly recognizable intermediates in the endocytic pathway. Numerous structures corresponding to EEVs were found (Fig. 2G, double arrowheads). Tubular intermediates and immature yolk granules were also found (Fig. 2G).

To gain insight into the overall effect of rab5 loss of function in the oocyte, we quantified the density of endocytic intermediates in one rab5$^{2}$ oocyte and one wt control oocyte, both at the end of stage 9 (Fig. 2F). The densities calculated above can be extrapolated to a typical egg chamber, in which the region 0 to 3.5 μm below the perivitelline space (PVS) corresponds to 18% of the overall surface of a section in the oocyte (Fig. 2F). Quantification showed a fourfold increase in the density of EEVs in the rab5$^{2}$ oocyte. In this context, the later endocytic intermediates (tubular intermediates to Y3 considered together) were fewer (75%) decrease). These observations suggest a requirement for Rab5 in the transition from
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Rab5 and PtdIns(4,5)P$_2$ localization during oogenesis

Because we had show that Rab5 is required during early endocytic steps, we next investigated whether Rab5 distribution in the oocyte was compatible with this requirement. During early oogenesis, a large pool of Rab5 was homogeneously distributed in the cytoplasm of germ line cells (Fig. 3A). Then, concomitantly with the onset of yolk-protein uptake in the oocyte, Rab5 became enriched in the vicinity of the PM (Fig. 3B,C). This enrichment was more pronounced in the oocyte (Fig. 3B,C). The localization of Rab5 that was observed during the vitellogenic stages is thus consistent with the observed role in early endocytic traffic close to the PM. Similar to the activity of Rab5, the regulation of the level of PtdIns(4,5)P$_2$ is necessary during early endocytic stages in mammals (Di Paolo and De Camilli, 2006), so we therefore analyzed the distribution of Rab5 with regards to that of PtdIns(4,5)P$_2$. In egg chambers, PtdIns(4,5)P$_2$, visualized with a construct in which the Pleckstrin homology domain of the phospholipase-C$_\delta$ is fused to GFP [PH(PLC$_\delta$)-GFP] (Downes et al., 2005), was found at the PM of all cells composing the egg chamber (Fig. 3D,E,G). Rab5 staining partially overlapped with the PH(PLC$_\delta$)-GFP signal and was also seen just below the PM (Fig. 3D,F,G, arrowheads). This localization in the close proximity of the PtdIns(4,5)P$_2$-containing PM is consistent with a role of Rab5 and PtdIns(4,5)P$_2$ in the early endocytic step in the oocyte.

PtdIns(4,5)P$_2$ is required for Rab5 localization and yolk-protein endocytosis

We examined the potential interconnections between the regulatory roles of PtdIns(4,5)P$_2$ and Rab5 in endocytosis. We first analyzed the localization of Rab5 when the PtdIns(4,5)P$_2$ level was lowered. To do so, we used loss of function of sktl, coding for a type 1 phosphatidylinositol 4-phosphate 5-kinase (Hassan et al., 1998). As null alleles of sktl led to an arrest early in oogenesis, we used a viable combination of hypomorphic alleles (Gervais et al., 2008). When compared with the control, PH(PLC$_\delta$)-GFP signal intensity at the oocyte PM, stained with Lycopersicon esculentum lectin, appeared reduced in sktl$^{2.3}$/Cyo$^2$ (100%, n=18) (see Materials and Methods for details on the quantification procedure). This indicates that the PtdIns(4,5)P$_2$ level at the PM of the oocyte is lower in sktl$^{2.3}$/Cyo$^2$ homozygous mutants (n=20) when compared with the sktl$^{2.3}$/Cyo heterozygous control (n=18) (Fig. 3H-R, and compare arrows in Fig. 3I-L and Fig. 3O-R). Quantification showed a decrease of 29±5% (± s.e.m.) in PH(PLC$_\delta$)-GFP fluorescence intensity at the oocyte anterior margin in sktl$^{2.3}$/sktl$^{5}$ mutants (Fig. 3H,R, and compare arrows in Fig. 3I-L and Fig. 3O-R). Because the presence of the PH(PLC$_\delta$)-GFP transgene could affect the levels of available PtdIns(4,5)P$_2$ at the PM, we confirmed that the loss of Rab5 cortical recruitment in sktl$^{2.3}$/sktl$^{5}$ oocytes was also observed independently of the PH(PLC$_\delta$)-GFP transgene (100%, n=30, data not shown). These observations are indicative of a requirement of PtdIns(4,5)P$_2$ for Rab5 subcortical recruitment during vitellogenic stages.

As Rab5 activity is required for the formation of yolk granules, we investigated the potential role of Sktl in yolk-protein endocytosis. In total, 60% of the eggs laid by sktl$^{2.3}$/sktl$^{5}$ females were flaccid, whereas, in this context, Y1 was properly localized at the oocyte PM (Fig. 3T,U) (100%, n=28). To exclude a potential contribution...
Fig. 2. Ultrastructure of the endocytic compartment in rab52 mutant oocytes. (A) Schematic representation of the morphology of intermediates in the clathrin-dependent vitellogenins endocytic pathway. FCCP, forming clathrin-coated pit; CCP, clathrin-coated pit (arrowheads in C,E,G); EEV, early endocytic vesicles (double arrowheads in C,E,G); CCV, clathrin-coated vesicles; NEEV, naked early endocytic vesicles; T, tubular intermediates (arrows in C,E,G); Y1 and Y2, forming yolk granules; Y3, condensed yolk granules. (B,C) Electron micrographs of late stage 9 wt egg chambers showing the oocyte (B) and a representative region of the lateral cortex (C). Note the large quantity of dark yolk granules in the oocyte cytoplasm in B and that all intermediates in the clathrin-dependent vitellogenin endocytic pathway are found in C. (D,E) Electron micrographs of rab52 GLC late stage 9 egg chambers showing the oocyte (D) and a representative region of the lateral cortex (E). Note the absence of dark yolk granules and the presence of endocytic intermediate aggregates (red circles) in the oocyte cytoplasm (D). Green arrowhead indicates the aggregate shown in supplementary material Fig. S1G,H. (E) Note that EEV density is increased, and that tubules and forming yolk granules are very rare in this region. (F) Top: the density of endocytic intermediates ~3.5 μm below the oocyte PM per μm² of oocyte cytoplasm is shown. The numbers in brackets in the first column represent the surface of oocyte cytoplasm considered for each genotype. The numbers in brackets in the remaining columns represent the number of structures counted. Middle: the density of endocytic intermediates found deeper than 3.5 μm below the oocyte PM per μm² of oocyte cytoplasm is shown. The numbers in brackets in the first column represent the surface of oocyte cytoplasm considered for each genotype. The numbers in brackets in the remaining columns represent the number of structures counted. Schematic representation of the oocyte showing, in orange, the subcortical region ~3.5 μm below the oocyte PM and, in pink, the remaining oocyte cytoplasm. Bottom: the density of endocytic intermediates per μm² of oocyte cytoplasm calculated with the densities determined in the tables above, and considering that the region 3.5 μm below the PVS represents 18% of the oocyte cytoplasm. CCP, clathrin-coated pit; EEV, early endocytic vesicles; Y1-Y2, forming yolk granules; Y3, condensed yolk granules. (G) High magnification of the early endocytic intermediate aggregate indicated by the red arrowhead in B. oo, oocyte; fc, follicle cells; nc, nurse cells; v, vitelline bodies. The larger size of vitelline bodies and longer follicle-cell microvilli in E compared with C correspond to the fact that this egg chamber, although in stage 9 (similar to the control), is slightly older than that shown in C. Scale bars: (B,D) 2.5 μm; (C,E,G) 500 nm.
Fig. 3. PtdIns(4,5)P2 is required for Rab5 localization and yolk endocytosis. (A-C) Rab5 localization during oogenesis in wt egg chambers. (A) From germarium (upper left) to stage 6 (bottom right); (B) stage 8; (C) stage 10. Note the recruitment of Rab5 in the oocyte cortical region during the vitellogenic stages (B,C). (D-G) Rab5 and PH(PLCδ)-GFP localization in wt stage 10 egg chambers. (F; red in D,G) Rab5; (E; green in D,G) PH(PLCδ)-GFP. (E-G) Correspond to the region inside the boxed area in D. Rab5 is visible at and in a narrow region below the oocyte PM. Green, PH(PLCδ).[...]

of the loss of Sktl activity in the somatic cell in this defect, we generated sktl2.3 GLCs, in which Sktl activity is only impaired in the germline. In such cases, the number of yolk granules in the oocyte cytoplasm was largely reduced (Fig. 3V,W).

In order to explore the nature of the endocytic defects that we observed in sktl2.3 GLCs, we analyzed the organization of the endocytic compartments in this context by EM during stage 9. In sktl2.3 GLCs, a small number of condensed yolk granules were observed in the oocyte cytoplasm and all early endocytic intermediates were limited to the region 3.5 μm below the PM. In this region, two different profiles were found, which were randomly distributed within all oocytes that were considered (n = 4): either numerous CCPs were observed on abnormally long PM invaginations (Fig. 4B, red arrowhead), or large accumulations of yolk proteins were visible at the oocyte PM (Fig. 4C, green arrowhead). A possible explanation for this phenotypic variability is that the sktl mutant context used is hypomorphic and that the remaining PtdIns(4,5)P2-synthesizing activity might not be homogeneously distributed. In all cases, the density of yolk-protein-containing profiles apparently not connected with the PM was lower than in the wt control and, when present, they were often abnormal (Fig. 4B, red arrows). Together, these observations indicate that: (1) in the oocyte, Sktl-dependent PtdIns(4,5)P2 synthesis is required for the formation of CCVs and (2) Sktl-dependent PtdIns(4,5)P2 synthesis acts prior to, or concomitantly with, Rab5 during endocytosis.

Rab5 controls the release of PtdIns(4,5)P2 from the membrane of early endocytic intermediates

Maturation of EEVs requires both Rab5 recruitment (Wucherpfennig et al., 2003) (and this study) and the removal of PtdIns(4,5)P2 (Cremona et al., 1999). Considering this, we wondered whether the recruitment of Rab5 could influence the removal of PtdIns(4,5)P2 from EEVs. We thus analyzed the distribution of PtdIns(4,5)P2 in rab52 GLCs. In this context, PtdIns(4,5)P2 was no longer restricted to the PM (compare Fig. 5A and Fig. 5B). Large PH(PLCδ)-GFP-positive compartments were observed within the cytoplasm of the oocyte (100% of stage 9-10 oocytes, n = 50) (Fig. 5B,C). Some were in close proximity to the oocyte PM, but others did not appear to be connected with it (Fig. 5B,C; Fig. 5D, cross-section). We next investigated whether these structures might correspond to the ectopic yolk-protein-containing endocytic aggregates that were observed by EM (Fig. 2D,G). To do so, we stained rab52 GLCs with an antibody directed against one of the yolk proteins, YP1 (Butterworth et al., 1999). In wt controls, YP1 was found inside the PH(PLCδ)-GFP-positive compartments (Fig. 5E, inset). In rab52 GLCs, YP1 was found within the cytoplasm and all early endocytic intermediates were limited to the region 3.5 μm below the PM. In this region, two different profiles were found, which were randomly distributed within all oocytes that were considered (n = 4): either numerous CCPs were observed on abnormally long PM invaginations (Fig. 4B, red arrowhead), or large accumulations of yolk proteins were visible at the oocyte PM (Fig. 4C, green arrowhead). A possible explanation for this phenotypic variability is that the sktl mutant context used is hypomorphic and that the remaining PtdIns(4,5)P2-synthesizing activity might not be homogeneously distributed. In all cases, the density of yolk-protein-containing profiles apparently not connected with the PM was lower than in the wt control and, when present, they were often abnormal (Fig. 4B, red arrows). Together, these observations indicate that: (1) in the oocyte, Sktl-dependent PtdIns(4,5)P2 synthesis is required for the formation of CCVs and (2) Sktl-dependent PtdIns(4,5)P2 synthesis acts prior to, or concomitantly with, Rab5 during endocytosis.
the case, the components of the early endocytic machinery, such as
the coat component α-adaptin (Gonzalez-Gaitan and Jackle, 1997),
the recruitment of which relies on their binding to PtdIns(4,5)P$_2$,
could be retained on these ectopic PH(PLCδ)-GFP-positive
structures. To test this hypothesis, we characterized the distribution
of PH(PLCδ)-GFP and α-adaptin during vitellogenic stages in
rab$_{52}$ GLCs. In controls, α-adaptin was found at, and in a narrow
region below, the oocyte PM (Fig. 5I). In rab$_{52}$ GLCs,
α-adaptin was always found in the ectopic PH(PLCδ)-GFP-positive
cytoplasmic compartments (100%, n=10) (Fig. 5J-L). The localization of the
sub-cortical α-adaptin seemed unaltered. We made the same
observation with dynamin, another early endocytic factor whose
recruitment relies on the presence of PtdIns(4,5)P$_2$ (data not shown).
In summary, we confirm that Rab5 loss of function leads to the
formation of ectopic early endocytic intermediate aggregates in the
cytoplasm. Furthermore, we show that Rab5 controls the release of
PtdIns(4,5)P$_2$ from these structures. This defect is associated with
the impairment of localization of PtdIns(4,5)P$_2$-binding budding factors.

Rab5 regulates F-actin distribution on early endocytic
intermediate membrane
PtdIns(4,5)P$_2$ is known to recruit actin-polymerization factors
(Niggli, 2005). Moreover, the disappearance of PtdIns(4,5)P$_2$ has
been proposed to be required for the downregulation of actin
polymerization, which is necessary for endocytosis to proceed
(Cremona and De Camilli, 2001; Sun et al., 2007). Considering the
effect of Rab5 on the distribution of PtdIns(4,5)P$_2$, we decided to
further investigate the potential role of Rab5 in the organization of
the actin cytoskeleton. In wt oocytes, a dense actin network was
found below the PM (Fig. 5M,Q). In rab$_{52}$ GLCs, we observed the
presence of actin fiber in all large PH(PLCδ)-GFP-positive
structures seen in the cytoplasm and below the cortex of the oocyte
(Fig. 5N-P,R-T) (100% n=30). These observations therefore suggest
that Rab5 is required to repress actin polymerization around EEVs.
Furthermore, in line with our finding that the PtdIns(4,5)P$_2$-
containing ectopic endocytic-compartment aggregates contain F-
actin, we observed on the electron micrograph fibrous structures
within these aggregates (supplementary material Fig. S1F, triple-
Fig. 5. Rab5 controls the removal of PtdIns(4,5)P2 from early endocytic intermediate membrane. (A–D) PH(PLCδ)-GFP localization in wt (A) and rab52-GLCs (B–D) stage 9 egg chambers. (C) Inset on the anterior margin. (D) Cross-section through the broken line in B. Note the presence of large PLC(PLCδ)-GFP-positive structures in the oocyte cytoplasm when Rab5 is absent which are never seen in control (A) (projection of optical sections). Scale bars: 50 µm, except in C, 25 µm. (E–H) YP1 and PH(PLCδ)-GFP localization in wt (E) and rab52-GLC (F–H) stage 10 egg chambers. (G; red in E,H) YP1; (F; green in E,H) PH(PLCδ)-GFP; (blue in E,H) DNA. (Inset, E) In controls, YP1 is absent which are never seen in control (A) (projection of optical sections). Scale bars: 50 µm, except in C, 25 µm. (I–L) YP1 and PH(PLCδ)-GFP localization in wt (I) and rab52-GLCs (J–L) stage 9 egg chambers. (K; red in I,L) YP1; (J; green in I,L) PH(PLCδ)-GFP. Note that, when Rab5 is absent, large PH(PLCδ)-GFP-positive structures in the oocyte cytoplasm are associated with α-adaptin. Scale bars: 30 µm. (M–T) F-actin and PH(PLCδ)-GFP localization in wt (M,Q) and rab52-GLC (N–P,R,T) stage 9 egg chambers. (O,S; red in M,Q,T) F-actin; (N,R; green in M,Q,T) PH(PLCδ)-GFP. Note that the large PH(PLCδ)-GFP-positive structures in the oocyte cytoplasm observed when Rab5 is absent contain F-actin (projection of optical sections).

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function would lead to an endocytic defect similar to those observed in rab5 loss-of-function mutants. We thus generated GLCs containing synj1/D, a strong loss-of-function allele of synj (Verstreken et al., 2003). In this context, neither yolk-protein endocytosis (compare supplementary material Fig. S2B and Fig. S2E) nor actin restriction at the cortex (compare supplementary material Fig. S2C and Fig. S2F) were impaired in the oocyte. This result suggests that either Synj is not alone responsible for PtdIns(4,5)P2 removal from endocytic vesicles or that PtdIns(4,5)P2 removal from endocytic vesicles is not required in the oocyte.

To distinguish between these two hypotheses, we overexpressed Sktl in the germline by means of the UAS/GAL4 system. In 45% of UAS-RFP-Sktl-expressing oocytes, PtdIns(4,5)P2, besides its distribution to the PM, was also found in large PH(PLCδ)-GFP-positive cytoplasmic compartments (compare Fig. 6A,C,E with Fig. 6B,D,F). Moreover, these structures were always associated with RFP-Sktl (Fig. 6B”-B”, D”-D”, F”-F”). We next investigated whether those aggregates were associated with early endosomal markers. We observed that α-adaptin (Fig. 6A-B”), Rab5 (Fig. 6C-D”) and Yl (Fig. 6E-F”) were found on these structures. It is noteworthy that these markers were also found at the level of PM as in wt, indicating that Yl secretion (Fig. 6E-F”), and α-adaptin (Fig. 6A-B”) and

Skittles overexpression impairs endocytosis

In vivo, the removal of PtdIns(4,5)P2 from EEVs has so far been attributed to Synj (Cremona et al., 1999; Sun et al., 2007). We thus wondered whether the role of Rab5 in the spatial restriction of PtdIns(4,5)P2 that we found in the oocyte could rely on the regulation of Synj activity. Under this hypothesis, loss of Synj activity in vivo, the removal of PtdIns(4,5)P2 from EEVs has so far been attributed to Synj (Cremona et al., 1999; Sun et al., 2007). We thus wondered whether the role of Rab5 in the spatial restriction of PtdIns(4,5)P2 that we found in the oocyte could rely on the regulation of Synj activity. Under this hypothesis, loss of Synj activity.
Fig. 6. See next page for legend.
Rab5 (Fig. 6C-D) recruitment, at the level of the PM were not compromised. These observations suggest that an excess of PtdIns(4,5)P_2-synthesizing enzyme leads to the formation of abnormal early endocytic compartments. To determine whether the PH(PLCδ)-GFP/RFP-Sktl-positive structures were indeed endosomal structures, we stained RFP-Sktl-expressing oocytes with YP1 antibody. YP1 was found inside yolk granules in mutant oocytes. In wt, Sktl was enriched at or close to the PM (Fig. 6O). In rabs^{2} GLCs, Sktl was also found in the PH(PLCδ)-GFP-positive abnormal endosomal compartment (Fig. 6O-P). This observation supports the hypothesis that Rab5 could be also required to repress Sktl localization and/or activation on early endosomes in order to control PtdIns(4,5)P_2 spatial restriction.

In summary, an excess of the PtdIns(4,5)P_2-producing enzyme Sktl led to the formation of abnormal early endocytic compartments containing PtdIns(4,5)P_2, α-adaptin, Rab5, Y1, YP1 and actin. This defect is associated with a reduction of yolk-protein endocytosis. This strongly suggests that removing PtdIns(4,5)P_2 from the endosomal membrane is necessary for endocytosis to proceed.

Discussion

In this study, we used complete loss of Rab5 function in the germline cyst to study its consequences on the endocytic pathway. We found that Rab5 is required for EEV maturation and yolk-protein endocytosis in the oocyte. Using loss of function of sktl, coding for a type I phosphatidylidyinositol 4-phosphate 5-kinase, we show that PtdIns(4,5)P_2 is required for endocytic-vesicle formation, for Rab5 recruitment on early endosomes and accordingly for yolk-protein endocytosis. Furthermore, we unveil a previously undescribed role of Rab5 in controlling the removal of PtdIns(4,5)P_2, PtdIns(4,5)P_2-binding coat components and F-actin from EEVs. Finally, we show that overexpressing the PtdIns(4,5)P_2-synthesizing enzyme Sktl leads to the formation of abnormal early endocytic compartments containing Rab5, PtdIns(4,5)P_2-binding coat component and actin, and thus it affects yolk endocytosis. Hence, our results strongly suggest that Rab5-dependent PtdIns(4,5)P_2 restriction from endosomes is crucial for endocytosis to proceed.

The first stage of endocytosis, CCV formation, has previously been shown to rely on the presence of PtdIns(4,5)P_2 at the PM for coat-component recruitment and fission in mammals (for review see, Di Paolo and De Camilli, 2006). In Drosophila, the situation was less clear, as early studies attempting to address the requirement of PtdIns(4,5)P_2 for endocytosis were inconclusive (Hassan et al., 1998). Our results in sktl mutant oocytes indicate that, when PtdIns(4,5)P_2 level is lowered, endocytosis is impaired. Moreover, analysis of endocytic-compartment morphology at the ultrastructural level in this context revealed a depletion of all intracytoplasmic endocytic intermediates. This strongly suggests a conserved requirement of PtdIns(4,5)P_2 for the formation of endocytic vesicles in the oocyte. Interestingly, the build-up of coated pits along invaginations of the PM that we observed in sktl mutant oocytes is very similar to what is observed with a dominant-negative allele of dynamin, encoding an essential PtdIns(4,5)P_2-binding regulator of fission (Tsuruhara et al., 1990). Moreover, in sktl mutant oocytes, the subcortical recruitment of Rab5 during vitellogenic stages was affected. Our observations suggest that PtdIns(4,5)P_2-dependent CCV formation is necessary for Rab5 recruitment.

Consistent with a role of Rab5 following EEV formation, our analysis of endocytic-compartment morphology at the ultrastructural level in the absence of Rab5 showed an accumulation of EEVs, which was associated with a depletion of later endocytic structures. This suggests a conserved requirement of Rab5 for EEV maturation (Bucci et al., 1992; Wucherpfennig et al., 2003), but does not exclude that Rab5 might be also required for other steps along the endocytic pathway. Surprisingly, we also observed that loss of function of rabs^{2} impairs the removal of PtdIns(4,5)P_2 from the endosomal membrane.
The importance of PtdIns(4,5)P₂ turnover for endocytic-vesicle maturation was demonstrated by the study of Synj, a PtdIns 5-phosphatase, in synaptic termini. In synj knockout mice or Drosophila mutants, synaptic-vesicle recycling is impaired and CCVs accumulate in the cytoplasm (Cremona and De Camilli, 2001; Verstreken et al., 2003). We observed, in mutants with strong loss of synj function, that neither yolk-protein endocytosis nor actin restriction at the cortex were impaired in the oocyte. Nevertheless, we found that an excess in the PtdIns(4,5)P₂-producing enzyme Sktl led to the formation of an abnormal endocytic compartment containing PtdIns(4,5)P₂ and, accordingly, we also observed a reduction of yolk-protein endocytosis in this context. Our results indicate that, in vivo, besides its known requirement in neuronal synapse maturation, Sktl is essential for endocytosis to proceed in other cell types. Furthermore, we report here a situation different from that in neurons, where removing PtdIns(4,5)P₂ from endosomal membranes is also essential for endocytosis to proceed in other cell types. Alternatively, PtdIns(4,5)P₂ removal from endosomal membranes is also essential for endocytosis to proceed in other cell types. Furthermore, we report here a situation different from that in neurons, where removing PtdIns(4,5)P₂ from endosomal membranes is also essential for endocytosis in S2 cells (Korolchuk et al., 2007). Altogether, these phenotypes suggest that different enzymes could fulfill this function in various cell types. Interestingly, our finding that Rab5 is present on the abnormal endocytic structures induced by Sktl overexpression suggests that, in this context, endocytosis is blocked at the stage when Rab5 is required to proceed further along the endocytic path. Altogether, these observations make our finding of a role of Rab5 in the removal of PtdIns(4,5)P₂ from endosomal membrane all the more relevant.

In the absence of Rab5, PtdIns(4,5)P₂, found in the ectopic endosomal compartments, is associated with the PtdIns(4,5)P₂-binding factors necessary for coat recruitment and fission, and with F-actin aggregates, hence suggesting that the defective PtdIns(4,5)P₂ regulation impairs the dynamics of budding factors and F-actin organization. Although we cannot rule out the involvement of Rab5 in these processes independently of its effect on PtdIns(4,5)P₂ distribution, we favor the interpretation that these phenotypes are a direct consequence of altered PtdIns(4,5)P₂ removal for several reasons. First, recent studies using live-cell imaging have shown that there is an intimate connection between the regulation of PtdIns(4,5)P₂ levels and coat assembly and/or disassembly (Sun et al., 2007; Zoncu et al., 2007). Second, it has been established that the PtdIns(4,5)P₂-dependent shut-down of actin polymerization is required for endocytosis to proceed in yeast (Sun et al., 2007). Third, the phenotypes are reminiscent of those observed when we overexpressed the PtdIns(4,5)P₂-synthesizing enzyme Sktl. These observations raise the issue of a possible link that could exist between Rab5 and the spatial restriction of PtdIns(4,5)P₂. Among the Rab5 effectors known to be involved in PtdIns metabolism, three could directly regulate PtdIns(4,5)P₂ levels: the PtdIns 3-kinase p110 is able to use PtdIns(4,5)P₂ as a substrate to produce PtdIns(3,4,5)P₃, and the PtdIns 5-phosphatases INPP5B and ORCL are able to use both PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ to produce PtdIns(4)P and PtdIns(3,4)P₂, respectively (Hyvola et al., 2006) (Christoforidis et al., 1999; Shin et al., 2005; Erdmann et al., 2007). We found that PtdIns(3,4,5)P₃ accumulates on endosomes along with PtdIns(4,5)P₂ in rab5₂ mutant oocytes (supplementary material Fig. S4). This leads us to favor the assumption that the accumulation of PtdIns(4,5)P₂ that was revealed in this study is more likely to arise from defective PtdIns 5-phosphatase recruitment than defective PtdIns 3-kinase recruitment. Another hypothesis, compatible with our previous assumption, is that Rab5 can also restrict PtdIns(4,5)P₂ synthesis by negatively regulating Sktl activity from the endosomes. Two observations are in line with this scenario: (1) Sktl overexpression led to defects that were similar to those observed in rab5 loss-of-function mutants, and (2) Sktl is found on normally maturing endosomes in rab5₂ mutant oocytes. It may thus prove fruitful in the future to search for a Rab5 effector that is able to restrict Sktl localization from endosomes, and to explore the influence of Rab5-recruited PtdIns 5-phosphatase on the regulation of PtdIns(4,5)P₂ levels along the endocytic pathway.

Materials and Methods

Flies

Flies were raised under standard laboratory conditions at 25°C. The following stocks were used in this study: w;rab5²/Cyo [provided by Marcos Gonzalez-Gaitan (Wucherpfennig et al., 2003)]; w;rab5²,FRT40A/Cyo; w;FRT40A/synj¹³/Cyo, uhs-GFP-PH(PLCδ); w;uhs-GFP-PH(PLCδ); w;UASP, GFP-Sktl [this construct fully rescues loss of sktl function when expressed at a low level]; w;sktl²/Cyo [provided by Hugo Bellens (Hasan et al., 1998)]; w;FRT42D synj²/Cyo [provided by Hugo Bellens (Verstreken et al., 2003)]; w;FRT2G sktl¹³/Cyo [this sktl allele was isolated in a screen described previously (Januschke et al., 2002)]; w;matutab-GAL4 (Januschke et al., 2002); and OvoD1,FRT40A/dpp+;wg,Mts(2)bw/Cyo; nl52-GLP-FRT40A; nlsGLP-FRT42D syncsFLP; noc/Cyo; w;FRT40A; w;/Bl/Cyo; TM2,TM6; w;noc4X4; w;ub-GFP-PH(GR139); w118 (used as a w control) were provided by the Bloomington Drosophila Stock Center. GLCs were generated as in Januschke et al. (2002).

rab5² RNAi

In vitro transcription was performed on a rab5 cDNA PCR amplification product (oligos: 5'-GTCCTCAGATCTCAGAATCAGCCAGG-3' and 5'-GCAGTAACCTGCCGTTAGAAC-3') with a MEGAscript RNAi kit (Ambion). RNAi was performed as described previously (Échard et al., 2004).

Antibodies, optical imaging and images processing

Staining and imaging were performed as described previously (Januschke et al., 2002). Quantification of autofluorescence (Fig. 1E,G; Fig. 6N) was performed on raw images using the ‘plot profile’ function of ImageJ. Rainbow LUT was applied on raw images using ImageJ in Fig. 6L,‘M’. Quantification of GFP-PH(PLCδ) fluorescence was performed using Leica confocal software (LAS-AF 1.7.0) as follows: raw images of stage 8 or 9 sktl²/sktl² (n=20) and sktl²/Cyo (n=18) egg chambers were acquired in the same condition. Ten measurements were taken at a random location for each sample by drawing ten lines through the anterior margin of the oocyte and taking the maximum fluorescence intensity recorded for each line. The measurements were limited to the anterior margin to avoid the signal from the apical membrane of the adjacent follicle cells. For each individual sample, the ten measurements were averaged to buffer local variations. The estimated variation in GFP-PH(PLCδ) fluorescence level between the two genotypes was obtained by calculating the ratio between the average measurements for sktl²/sktl² egg chambers and the average measurements for sktl²/Cyo egg chambers.

The following antibodies and dyes were used: rat anti-Y1 (1/2000) (Schonbaum et al., 2000), rabbit anti-Sktl (1/2000; provided by Mary Bowes (Butterworth et al., 1999), rabbit anti-Rab5 (1/100), rabbit anti-α-adaptin 1/100 (both provided by Marcos Gonzalez-Gaitan (Gonzalez-Gaitan and Jackle, 1997; Wucherpfennig et al., 2003)); Alexa-Fluor-488-phallolidin and rhodamine-phallolidin (Molecular Probes); and Texas-red Dyocodiscus esculentus lectin (Vector Laboratories). Rab5 anti-Sktl antibody was generated by Eurogentec against both GQDRLKDQPDDENE and QQRSSNSQSNRNIGTEVF peptides. The serum was used at 1:500 for immunofluorescence and 1:25,000 for western blot.

Electron microscopy and quantitation

Ovaries were dissected in PBS and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at room temperature for 2 hours. After washing, samples were post-fixed for 1 hour in 1% OsO₄ in the same buffer, dehydrated in graded alcohols, and embedded in araldite CY212 epoxy resin. Ultrathin sections were cut on a Leica EM UC6 ultramicrotome, stained with uranyl acetate and lead citrate, and then examined with a Philips TEM1212 electron microscope.

For quantitation, four classes of endocytic intermediate were considered using morphological criteria (Tsuruhara et al., 1990): CCVs correspond to budding intermediates containing dense yolk-protein material apposed to the extracellular side of the PM and having no visible connection with the PM; EEVs correspond to small circular vesicles containing electron-dense yolk-protein material apposed to the luminal side and without any visible connection with the PM; tubule corresponds to structures of irregular shape containing electron-dense yolk-protein material apposed to the luminal side and not connected with the PM; Y1-Y3 correspond to large condensing yolk granules in which a condensing yolk sphere was seen inside the...
lumen. Density was calculated taking the ratio between the number of structures observed and surface considered.

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