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Phosphoinositides, kinases and adaptors coordinating endocytosis in *Trypanosoma brucei*

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In the kinetoplastid parasite *Trypanosoma brucei* clathrin-mediated endocytosis is essential for survival and aids immune evasion in the mammalian host. The formation of endocytic clathrin coated vesicles in *T. brucei* is *via* a unique mechanism owing to an evolutionarily recent loss of the adaptor protein (AP)2 complex, a central hub in endocytic vesicle assembly. Despite this loss, recent studies examining endocytic clathrin coat assembly have highlighted a high degree of conservation between trypanosomes and their mammalian hosts. In particular phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) and its putative effectors, TbCALM and TbEpsinR, are central to clathrin-mediated endocytosis in the trypanosome, just as they are in animal cells. In addition to providing insights into the cell biology of *T. brucei*, these studies also suggest an ancient, possibly *pan*-eukaryotic connection between PtdIns(4,5)P_2 and endocytosis.

Phosphoinositides are derivatives of phosphatidylinositol (PtdIns), reversibly phosphorylated at the 3, 4 or 5 positions of the inositol ring, giving 7 potential differentially phosphorylated species. These lipid species are produced and eliminated through the concerted actions of specific phosphoinositide kinases, phosphatases and phospholipases. Together with small GTPases of the ARF and Rab families, phosphoinositides constitute an organelle identity code on the cytosol-facing membrane leaflet of endomembrane compartments. Local phosphoinositide levels and active small GTPases are sensed by effector proteins that carry phosphoinositide-specific binding domains. In many cases these also function as GTPase coincidence detectors, whereby recruitment to the membrane depends upon both local phosphoinositide levels and the presence of a particular active GTPase.[1] It is suggested that this co-incidence detection mechanism of adaptor recruitment allows greater precision in adaptor protein specificity than either system functioning in isolation.[1] Thus, spatially and temporally regulated phosphoinositide gradients are central to maintenance of intracellular membrane traffic and hence proper cellular compartmentalization. Well-characterized examples of phosphoinositide regulation of membrane trafficking are endosomal phosphatidylinositol 3-phosphate (PI3P) controlling endosome fusion and maturation.[2,3,4] and phosphatidylinositol 4-phosphate (PI4P) controlling peripheral membrane protein recruitment to the *trans*-Golgi network.[1,5,6] The ability of kinases and phosphatases to rapidly modulate the levels of specific phosphoinositides also facilitates swift changes to compartmental address codes and integration with cellular signal transduction pathways important for responses coordinating other biological processes.

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is highly enriched at the plasma membrane of mammalian and yeast cells where, among other roles, it aids the driving of endocytic clathrin coated pit formation through PI(4,5)P_2-binding clathrin adaptors, including the central AP2 hub and peripheral adaptors such as CALM/AP180.[7,8,9] The kinetoplastid parasite *Trypanosoma brucei,* and close relatives within the African trypanosome lineage, are unique in having dispensed with the AP2 complex while at the same time relying on extensive clathrin mediated endocytosis to aid in host immune evasion as well as nutrient uptake.[10–14] Two recent studies have revealed roles for a phosphoinositide kinase and its product PtdIns(4,5)P_2, as well as likely effector molecules bearing phosphoinositide binding domains in clathrin mediated endocytosis in this organism.[15,16]

All endocytic and exocytic events in trypanosomatids occur in a specialized plasma membrane domain termed the flagellar pocket. This structure is a membrane invagination segregated from the bulk plasma membrane by a tight junction-like assemblage, the flagellar pocket collar that acts as a diffusion barrier for plasma membrane resident proteins and lipids. The recent characterization of TbPIPKA, localized predominantly at the flagellar pocket neck, suggests a mechanism for directing this highly polarized interaction between the endomembrane system and the plasma membrane.[15] The authors showed that TbPIPKA acts as a PI4P-5-kinase that is essential in both the insect stage of the parasite life cycle as well as the much more endocytically active mammalian infective stage. Additionally, depletion of this kinase caused severe endocytic defects, similar to those seen following depletion of clathrin heavy chain.[10] Furthermore, the same study

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demonstrated enrichment of $PI(4,5)P_2$, derived from TbPIPKA activity, at the cytosolic face of the flagellar pocket in the insect stage, although attempts to localize these lipid species in mammalian infective forms were surprisingly unsuccessful, given the higher rate of endocytosis in this life stage.

The strong phenotype observed following TbPIPKA depletion suggests that there is little or no redundancy in the generation of flagellar pocket $PtdIns(4,5)P_2$. However the trypanosome genome encodes a total of 4 putative phosphatidylinositol phosphate kinase (PIPK) genes, which suggests that $PI(4,5)P_2$ can be formed by other routes.[11,15] While the roles of these other gene products are currently unknown, phylogenetic reconstruction (**Fig. 1**) demonstrates that one gene likely encodes a PIP5K3 homolog, with the other proteins falling into a poorly resolved clade containing both the PIP5K1 and PIP4K2 metazoan gene families, together with the characterized MSS4 PI4P-5-kinase from *Saccharomyces cerevisiae*. In agreement with previous studies,[17] the only strong support in this clade is within eukaryotic super-groups, suggesting multiple independent PIPK gene family expansions following divergence of the eukaryotic supergroups some one billion years ago. Clearly functional analysis is required to understand the significance of these gene family expansions in *T. brucei* as simple inference from animals and fungi is unlikely to be insightful.

The absence of the AP2 complex suggests that other clathrin adaptors likely control endocytic clathrin coated pit formation in *T. brucei*. To address this question we interrogated the trypanosome genome and identified 2 potential clathrin adaptor proteins orthologous to mammalian CALM/AP180 and EpsinR.[8,16,18-21,] Indeed, these 2 clathrin adaptors were shown to function together in endocytosis from the flagellar pocket of the bloodstream form parasite, with depletion of both proteins leading to a strong inhibition of endocytic activity.[16] Examination of the protein sequence of these 2 adaptors showed that they have highly conserved phosphoinositide-binding domains, suggesting that

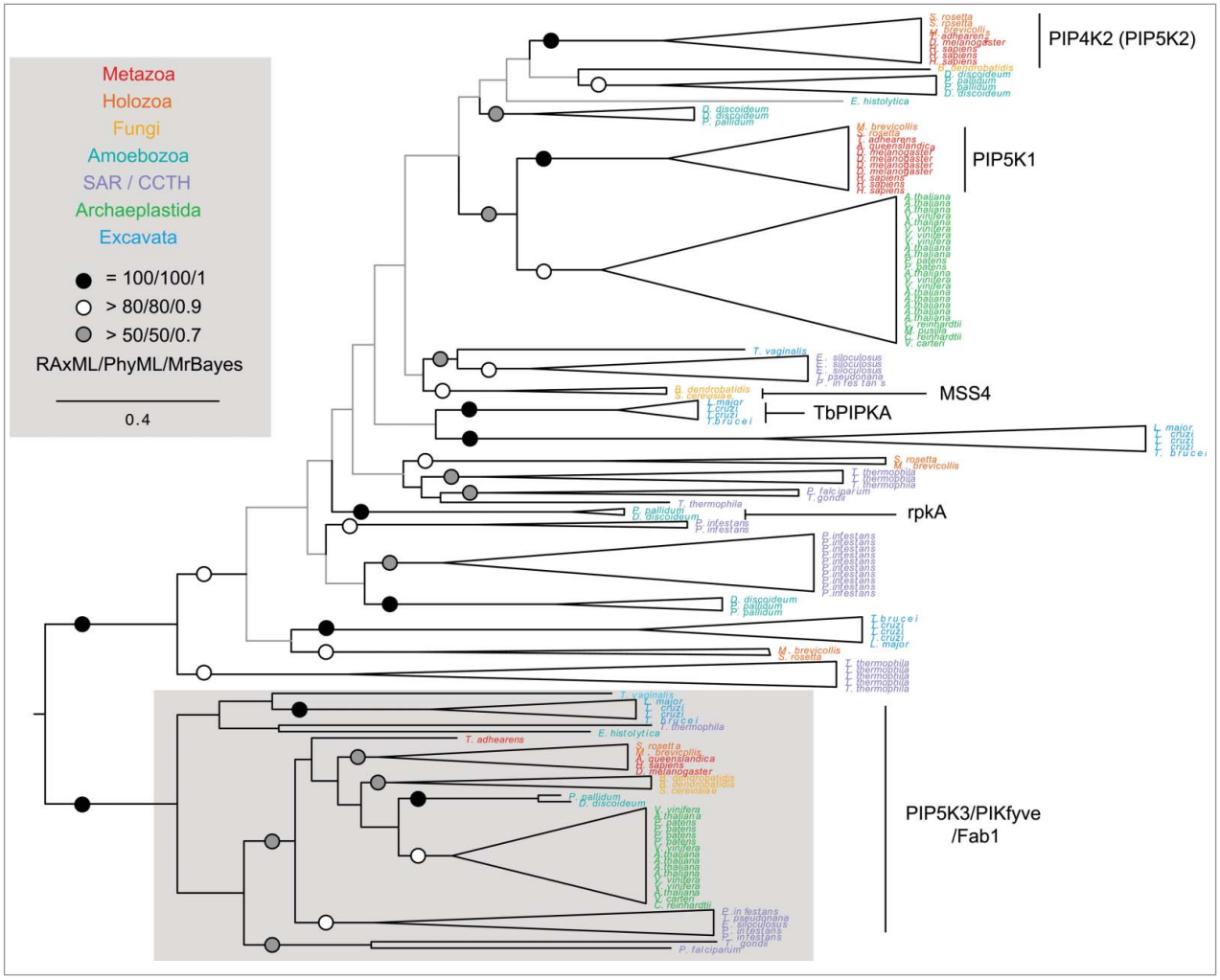

Figure 1. Phylogenetic reconstruction of the PIPK family of phosphoinositide kinases. Taxa are colored by eukaryotic super-group according to the key. Unsupported branches are gray; support levels are indicated with discs according to the key. The topology shown is the best scoring ML topology (RaxML). A well-supported clade containing characterized PIPK3 gene products is found across the eukaryotes. The remaining sequences group according to eukaryotic super-group suggesting multiple, independent instances of gene duplication following divergence of the eukaryotic lineages.

they may be acting as the link between TbPIPKA derived PtdIns $(4,5)P_2$ at the flagellar pocket membrane and the endocytic clathrin coat. This is supported by the localization of a GFP-fused PI (4,5)P2 binding domain derived from phospholipase C (PLC)δ to the flagellar pocket region of mammalian form parasites (**Fig. 2A**) in line with the previous report for the insect form. We suggest therefore that PtdIns$(4,5)P_2$ is likely a major and critical signal for TbCALM and TbEpsinR recruitment, which in turn generates membrane curvature[22,23] via clathrin recruitment and thereby coated vesicle formation at the flagellar pocket (**Fig. 2B**). While unprecedented in nature, this minimal arrangement of lipid, monomeric clathrin adaptor and clathrin coat has been shown *in vitro* to possess clathrin coated bud forming activity.[8,24]

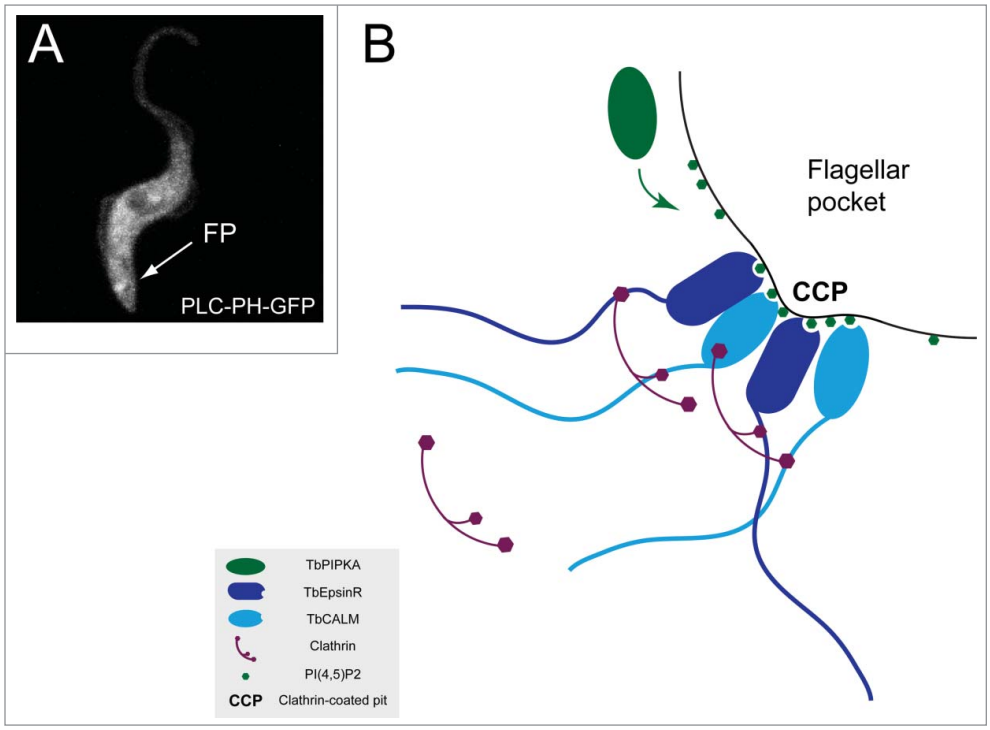

Figure 2. PI(4,5)P2 localization in mammalian life-cycle stage parasites and a model for clathrin coated pit formation. (**A**) GFP-fused PH domain from PLCδ expressed constitutively in mammalian bloodstream form parasites is enriched in the vicinity of the flagellar pocket (FP). (**B**) schema for clathrin coated pit formation at the flagellar pocket of T. brucei. TbPIPKA localized to the pocket neck generates a local increase in PtdIns (4,5)P2 levels. This PI(4,5)P2 is free to diffuse within the cytosolic leaflet of the flagellar pocket membrane but is prevented from traveling further by the flagellar pocket collar. Increased PtdIns(4,5)P2 levels are sensed by TbCALM and TbEpsinR which in turn recruit clathrin and impart membrane curvature, thus driving clathrin coated pit assembly.

In summary, a system that at first appeared paradoxical, i.e. high clathrin-mediated endocytic activity in the absence of the AP2 complex, has actually revealed deep conservation in the actions of phosphoinositide lipids, kinases and effectors in clathrin-mediated endocytosis, which likely extends across the extant eukaryotic lineages. We suggest that this may well provide clues as to the ancient core of endocytic trafficking mechanisms, as the phosphoinositide system may well predate adaptin complex origins.[25]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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