

Invited Mini Review

Phosphoinositide turnover in Toll-like receptor signaling and trafficking

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Lipid components in biological membranes are essential for maintaining cellular function. Phosphoinositides, the phosphorylated derivatives of phosphatidylinositol (PI), regulate many critical cell processes involving membrane signaling, trafficking, and reorganization. Multiple metabolic pathways including phosphoinositide kinases and phosphatases and phospholipases tightly control spatio-temporal concentration of membrane phosphoinositides. Metabolizing enzymes responsible for PI 4,5-bisphosphate (PI(4,5)P₂) production or degradation play a regulatory role in Toll-like receptor (TLR) signaling and trafficking. These enzymes include PI 4-phosphate 5-kinase, phosphatase and tensin homolog, PI 3-kinase, and phospholipase C. PI(4,5)P₂ mediates the interaction with target cytosolic proteins to induce their membrane translocation, regulate vesicular trafficking, and serve as a precursor for other signaling lipids. TLR activation is important for the innate immune response and is implicated in diverse pathophysiological disorders. TLR signaling is controlled by specific interactions with distinct signaling and sorting adaptors. Importantly, TLR signaling machinery is differentially formed depending on a specific membrane compartment during signaling cascades. Although detailed mechanisms remain to be fully clarified, phosphoinositide metabolism is promising for a better understanding of such spatio-temporal regulation of TLR signaling and trafficking. [BMB Reports 2014; 47(7): 361-368]

PHOSPHOINOSITIDE METABOLISM

Inositol-containing phospholipids (collectively called phosphoinositides) are phosphorylated derivatives of phosphatidylinositol (PI). These membrane lipids are distinct from other phospholipids as they undergo phosphorylation and dephosphorylation.

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phorylation of the three hydroxyl groups present in the inositol ring head (Fig. 1) (1, 2). Numerous studies have demonstrated that each phosphoinositide is distributed to subcellular membranes with different concentrations and has a distinct function in specific membrane compartments (Fig. 2). For example, PI 4,5-bisphosphate (PI(4,5)P₂) and PI 3,4,5-trisphosphate (PI(3,4,5)P₃) are present mainly in the plasma membrane, whereas PI 3-phosphate and PI 4-phosphate (PI(4)P) are relatively more abundant in the early endosome and Golgi, respectively (3). Such differential distribution of the phosphoinositides underlies vesicle trafficking of cargo proteins.

A wide range of polyphosphoinositide kinases and phosphatases are responsible for enzymatic reactions to produce various phosphoinositides with different numbers of phosphate groups at different positions (Fig. 1) (4, 5). Polyphosphoinositide kinases and phosphatases are conserved from yeast to humans, and many different isoforms are expressed in mammalian cells (5, 6). The lipid metabolizing enzymes specifically mediate turnover of their target substrates, thereby playing a crucial role in many important membrane events related to membrane signaling, trafficking, actin cytoskeletal dynamics, and organelle membranous integrity (5, 7, 8). Human diseases, including cancer, bipolar disorder, metabolic syndrome, Charcot-Marie-Tooth disorder, and Lowe syndrome are caused by genetic mutations and defects in polyphosphoinositide kinases and phosphatases, indicating that phosphoinositide metabolism is a matter of vital importance (9, 10).

PHYSIOLOGICAL ROLES OF PI(4,5)P₂

PI(4,5)P₂ is a critical membrane lipid that regulates cell physiological events at the cell surface (11,12). PI(4,5)P₂ is produced from PI(4)P by type I PI(4)P 5-kinase (PIP5K) and also from PI 5-phosphate (PI(5)P) by type II PI(5)P 4-kinase (13). Due to the relative abundance of PI(4)P over PI(5)P, the type I PIP5K family members comprising three isoforms (PIP5K α , PIP5K β , and PIP5K γ) are the main route for PI(4,5)P₂ synthesis (14-16). The three PIP5Ks are thought to exert distinct roles, although their overlapping functions have also been reported. PI(4,5)P₂ level in the plasma membrane is maintained at a low level but rapidly increases with extracellular stimuli (2). In response to soluble ligands that activate their specific cell sur-

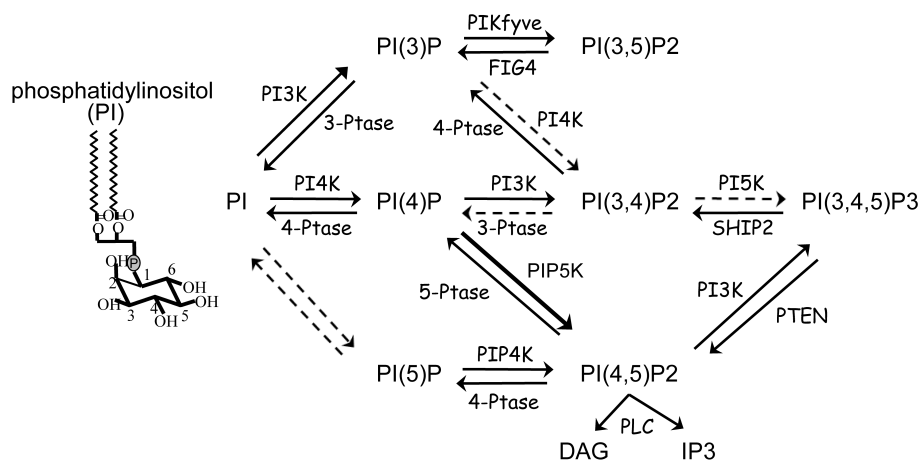


Fig. 1. Phosphoinositide metabolism. The three hydroxyl groups of the inositol ring are phosphorylated by phosphoinositide kinases or dephosphorylated by phosphoinositide phosphatases, generating seven different phosphorylated derivatives of PI called phosphoinositides. Each phosphoinositide differs in number and position of phosphate. PI(4,5)P₂ is also degraded into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) by phospholipase C (PLC). The metabolic pathways shown in dotted lines indicate the uncertainty of physiological relevance *in vivo*.

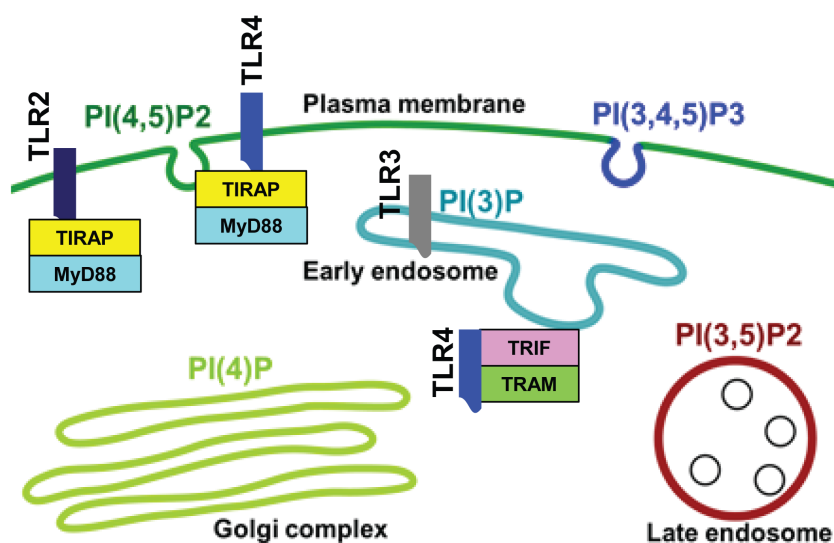


Fig. 2. Differential distribution of phosphoinositides and Toll-like receptors (TLRs). Phosphoinositides are present on the plasma and organelle membrane compartments in a non-uniform fashion. Each phosphoinositide has a distinct enrichment in the indicated individual membranes. There is specificity of the subcellular localization of TLRs; TLR2 and TLR4 are localized to the plasma membrane while TLR3 is a cytoplasmic TLR that localizes to intracellular endosomes.

face receptors, local PI(4,5)P₂ synthesis by PIP5Ks is enhanced (17). PI(4,5)P₂ is further metabolized to PI(3,4,5)P₃ by class I PI 3-kinase or to PI(4)P by polyphosphoinositide 5-phosphatases such as synaptojanin and OCRL (oculocerebrorenal syndrome of Lowe). The PI(3,4,5)P₃ formation induces recruitment of Akt and phosphoinositide-dependent kinase 1 (PDK1) to plasma membrane and the Akt phosphorylation by PDK1, a

critical step in Akt activation. In addition, phospholipase C (PLC) degrades PI(4,5)P₂ to generate diacylglycerol and inositol 1,4,5-trisphosphate (IP₃), the two important second messengers for protein kinase C activation and intracellular calcium release from endoplasmic reticulum, respectively (1,12). PLC-mediated PI(4,5)P₂ hydrolysis is an important downstream event of growth factor receptor signaling and G pro-

tein-coupled receptor signaling (18). Through the multiple PI(4,5)P₂ metabolic pathways, this phosphoinositide concentration is tightly regulated and the lipid metabolites are closely related to cell survival, proliferation, and differentiation (11, 12, 19).

PI(4,5)P₂ critically regulates vesicle trafficking of cargo proteins such as membrane receptors. PI(4,5)P₂ is necessary for synaptic vesicle exocytosis, and its production by PIP5K γ and hydrolysis by synaptojanin also participate in clathrin-mediated endocytosis (20-22). Various synaptic proteins are recruited to the presynaptic membranes through specific interactions with PI(4,5)P₂. Genetic ablation of both enzymes results in significant defects in synaptic vesicle recycling (20, 21). Accumulating evidence indicates that PI(4,5)P₂ produced by PIP5Ks is involved in growth factor receptor trafficking and modulation of intracellular signaling cascades. Besides this endocytic membrane remodeling, PI(4,5)P₂ production is required for phagocytosis, which mediates clearance of invading microorganisms, contributing to innate immunity (23, 24). PI(4,5)P₂ is a key factor for actin cytoskeletal reorganization, which is engaged in diverse cell physiology including endocytic and phagocytic membrane remodeling, and cell adhesion and migration (2, 11, 19, 25). Various actin binding proteins directly interact with PI(4,5)P₂ and this lipid-protein interaction changes their conformation and activity.

Various phosphoinositides are crucial for maintaining membrane integrity and are recognized as important regulators of vesicle trafficking (2, 8, 26). Soluble proteins can translocate to membrane compartments with corresponding phosphoinositides. Such roles for phosphoinositides as membrane docking sites for proteins are due to their specific interaction with cytosolic proteins. A wide range of cytosolic proteins related to actin cytoskeletal membranes, lipid-mediated cell signaling, and protein trafficking harbor phosphoinositide-binding domains such as pleckstrin homology (PH), phox homology, and FERM (band4.1, ezrin, radixin, moesin) domains (27, 28). The lipid-protein interaction also underlies recruitment of cytosolic proteins to phosphoinositide-specific membranes.

TOLL-LIKE RECEPTOR SIGNALING

Toll-like receptors (TLRs) are critical players responsible for innate immunity (29, 30). TLRs recognize molecular patterns of a wide range of invading bacteria and viruses and transduce immune responses against them (31, 32). These pattern recognition receptors contain leucine-rich repeat motifs in extracellular membrane and the cytoplasmic Toll/IL-1 receptor (TIR) domain. TLR family members are expressed in various types of cells including monocytes and macrophages and are engaged in the inflammatory response, phagocytosis, adaptive immunity, and other signaling pathways. In brain tissue, microglia and astrocytes express most TLRs and function as immune effector cells (33, 34). The TLRs in microglia and astrocytes become active in response to endogenously generated

soluble factors such as aggregated proteins, which mediate neuroprotective function (35-39). However, aberrant TLR signaling is associated with neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, leading to neurocytotoxicity, as well as inflammatory diseases (40-44).

TLR signaling is transmitted via association with intracellular adaptor proteins containing the TIR domain. Upon ligand binding to TLRs, the cytoplasmic TIR domain physically interacts with the TIR domains of the adaptor proteins (45). These adaptor proteins are myeloid differentiation factor 88 (MyD88), TIR domain-containing adaptor protein (TIRAP, also called MyD88 adaptor-like), TIR domain-containing adaptor inducing IFN- β (TRIF), and TRIF-related adaptor molecule (TRAM). Distinct recruitment of adaptor proteins to TLRs determines specificity of TLR signaling. MyD88 is necessary for activation of all TLRs except TLR3, which is dependent solely on TRIF. TIRAP is another necessary adaptor for TLR2 and TLR4 activation. Among TLR family members, TLR4 signaling pathways have been widely studied using lipopolysaccharide (LPS), a ligand for TLR4. All four TIR domain adaptor proteins are utilized for activation of TLR4 signaling pathways through MyD88-dependent and independent pathways (32, 45, 46).

Activated TLR4 undergoes dimerization and forms protein complex with TIRAP and MyD88 at the plasma membrane (47-49). This TIRAP/MyD88-dependent pathway transmits immune signals through signal cascades of IL-1 receptor-associated kinase 1/4, TNF receptor-associated factor 6, and transforming growth factor β -activated kinase 1 that lead to inhibitor of κ B (I κ B) phosphorylation by I κ B kinase, which induces I κ B proteasomal degradation and subsequent activation of nuclear factor- κ B (NF- κ B), a major transcription factor responsible for induction of pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor- α (TNF- α) (32, 46, 50).

PI(4,5)P₂ REGULATION OF TIRAP-DEPENDENT TLR SIGNALING

TIRAP, the essential adaptor for MyD88-mediated TLR2 and TLR4 signaling (45, 48, 49, 51), translocates to the plasma membrane following LPS stimulation through interaction with PI(4,5)P₂ (52). The N-terminal region of TIRAP has broad binding affinity for phosphoinositides, particularly for PI(4,5)P₂ (52). Interestingly, a TIRAP mutant that cannot bind to PI(4,5)P₂ was much less active in inducing cytokine production compared to wild-type TIRAP (52). Upon TLR4 activation, the rapid TLR4 signaling pathway is mediated through formation of the TLR4-TIRAP-MyD88 complex. Therefore, it was proposed that PI(4,5)P₂ recruits TIRAP to the plasma membrane following engagement of TLR4 ligand, thereby triggering the early phase of the MyD88-dependent pathway. In this working model, PI(4,5)P₂-dependent TIRAP serves as a sorting adaptor for connecting TLR4 and MyD88, whereas MyD88 mainly functions as a signaling adaptor for TLR4 activation at the plasma membrane (52, 53). A previous study

demonstrated that *Drosophila* MyD88 harbors a PI(4,5)P2 binding motif at the C-terminus, which enables plasma membrane localization (54). The similarity between *Drosophila* MyD88 and mammalian TIRAP for PI(4,5)P2 binding affinities suggests that the PI(4,5)P2-dependent sorting adaptor function is evolutionarily conserved and important for regulation of Toll signaling.

Because PI(4,5)P2 is necessary for the MyD88-dependent TLR4 pathway, it is likely that plasma membrane PI(4,5)P2 is elevated during activation of TLR4. ADP-ribosylation factor 6 (ARF6) and β 2 integrin may stimulate PIP5K for PI(4,5)P2 production during TLR4 signaling (52). PI(4,5)P2 and PIP5K play an important role coordinating actin-based membrane rearrangement (19). ARF6, a small guanosine triphosphatase (GTPase), also exerts a regulatory effect on various membrane events via upstream action on subcellular localization and catalytic activity of PIP5K (19, 55). TIRAP specifically localizes to the filamentous actin-rich membrane ruffles and ARF6 regulates its plasma membrane localization (52). Expression of the AIP1 protein with GTPase-activating protein activity toward ARF6 reduced PI(4,5)P2 levels inhibits LPS-induced TLR4 signaling events (56). In addition, LPS induces TLR4-dependent membrane ruffle formation. These findings implicate the occurrence of local production of PI(4,5)P2 on membrane ruffles via ARF6 and PIP5K in TIRAP-mediated TLR4 signaling. One can assume spatial location of TLR4 in close proximity to the PI(4,5)P2-enriched membranes such as membrane ruffles.

However, it was not determined which PIP5K isoform was responsible for it. TLR4 activation by LPS leads to upregulation of PIP5K α mRNA and protein expression in primary microglia and BV2 microglial cells (57). Similar results were obtained in primary astrocytes treated with ganglioside, which also has a stimulatory effect on TLR4 activation (58, 59). All type I PIP5K family members are expressed in the glial cells. PIP5K α , a PIP5K isoform, is a major PIP5K expressed in the BV2 microglial cell line (57, 60). It has been further demonstrated from the TLR4-stimulated glial cells that PIP5K α gene knockdown resulting in a decrease in PI(4,5)P2 level significantly attenuates both production of cytokines, such as IL-6 and TNF- α , and phosphorylation of signal proteins such as p38 MAPK, JNK, and NF- κ B (60). Restoration of wild-type PIP5K α , but not of catalytically inactive PIP5K α , increases TLR4 downstream molecular events, indicating that PI(4,5)P2 produced by PIP5K α contributes to TLR4 activation (60). PI(4,5)P2 levels in primary astrocytes and microglia, and BV2 microglia are elevated following stimulation with LPS or ganglioside (57, 58, 60). In addition, there is a temporal correlation between bi-directional TIRAP membrane translocation and PI(4,5)P2 levels upon TLR4 activation, which was abrogated by PIP5K α knockdown (60). These findings implicate a critical role of PIP5K α -derived PI(4,5)P2 in the TIRAP-dependent TLR4 activation. TIRAP plays a role in TLR5 signaling related to the intestinal inflammatory response, where PI(4,5)P2 is also responsible for the TIRAP membrane recruitment (61). In this case, PI(4,5)P2

is produced by PTEN-mediated PI(3,4,5)P3 hydrolysis, as supported by the evidence that PTEN phosphatase-inactive mutant fails to induce TLR5 activation and TIRAP membrane translocation (61). These observations suggest a functional implication of the PI(4,5)P2-TIRAP interaction as a key step for activating various TLRs such as TLR2, TLR4, and TLR5.

LINK OF PHOSPHOINOSITIDES IN SPATIOTEMPORAL REGULATION OF TLR4

It has been reported that TLRs localize to different subcellular membranes (Fig. 2). TLR1, TLR2, TLR4, TLR5, and TLR6 localize to the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are found mainly in cytoplasmic endosomes (30, 62). Activated TLR4 at the plasma membrane is internalized into cytoplasm by a clathrin- and dynamin-mediated endocytosis. The internalized TLR4 is relocated to early endosomes and associated with TRAM and TRIF, activating the MyD88-independent pathway (63). Endosomal TLR4 then activates TRAF3 and TANK-binding kinase 1, which enhances transcriptional activity of interferon regulatory factor-3 (IRF3), resulting in production of type I interferon- β production as well as late phase NF- κ B transcriptional activity (64-67). These findings indicate that the subcellular locations of TLR4 trigger distinct TLR4 signaling pathways. It was also reported that TLR2 undergoes similar endosomal trafficking in which the TLR2 endocytosis was mediated by clathrin and dynamin, and the internalized TLR2 also induced NF- κ B activation, contributing to the inflammatory response (68).

In the MyD88-independent TLR4 pathway, TRAM acts as a sorting adaptor for endosomal TLR4 and TRIF acts as a signaling adaptor for activation of IRF3 downstream signaling (63). It seems that the binding affinities of TRAM for phosphoinositides are related to its targeting to early endosomes, which facilitate the endosomal TLR4 signaling machinery with TRIF (63). This combined action of TRAM and TRIF at the endosomes resembles that of TIRAP and MyD88 at the plasma membrane, leading to early NF- κ B activation (52, 63). This observation indicates that subcellular location of TLR4 is important for activating distinct signaling pathways. Two distinct TLR4 signaling complexes are formed at different times and in different spaces, which underlies sequential activation of MyD88- and TRIF-dependent signaling. TLR4 relocated into the endosomes via clathrin-mediated endocytosis from cell surface is specific for the latter signaling pathway (Fig. 3). Therefore, it is of great importance to understand the underlying mechanisms of the TLR4 endocytosis.

Notably, PI(4,5)P2 degradation activity of PLC γ 2 has been demonstrated to be critical for LPS-induced TLR4 endocytosis (69, 70). Because defects in the PLC γ 2 activity disrupt TLR4 endocytosis, this result suggests that the receptor trafficking triggered by LPS is accompanied by decreased PI(4,5)P2 levels (69, 70). Given our observation of the up and downregulated PI(4,5)P2 levels during LPS stimulation, it is intriguing to spec-

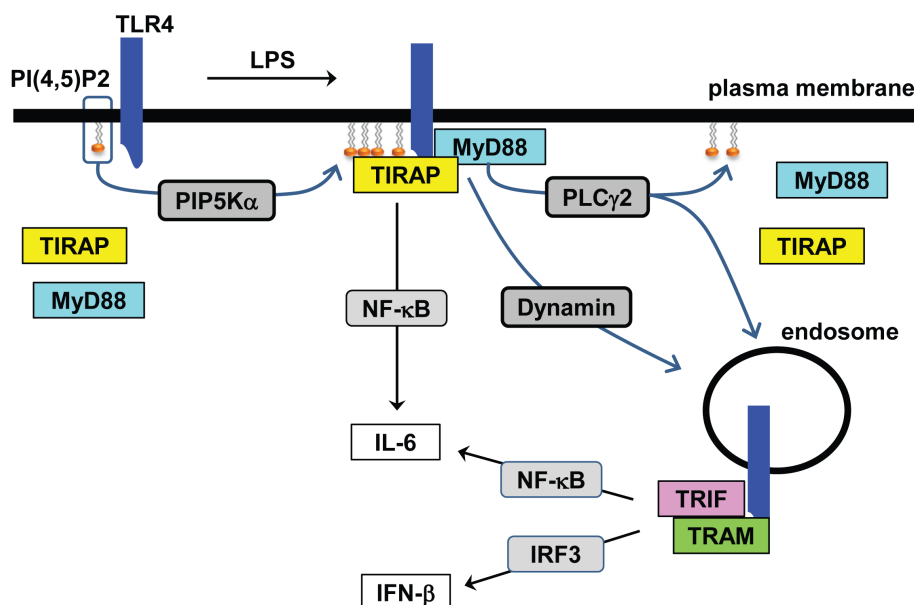


Fig. 3. A model for phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) turnover in Toll-like receptor (TLR)4 signaling and trafficking. Upon TLR4 activation by lipopolysaccharide (LPS), increases in PIP5K α -dependent PI(4,5)P2 induces TIR domain-containing adaptor protein (TIRAP) translocation to the plasma membrane, promoting the TLR4-TIRAP-MyD88 complex formation, which activates nuclear factor kappa beta (NF- κ B) and subsequent pro-inflammatory cytokine such as interleukin-6 (IL-6). TLR4 is then internalized into the endosome through dynamin and clathrin, where interacts with TRIF-related adaptor molecule (TRAM) and TIR domain-containing adaptor inducing IFN- β (TRIF) to induce interferon regulatory factor-3 (IRF3) activation and interferon (IFN)- β production. The endosomal TLR4 is suggested to induce the late phase of NF- κ B activation. Phospholipase- γ 2 (PLC γ 2) participates in TLR4 endocytosis, which implicates that decrease in PI(4,5)P2 level is accompanied with TLR4 internalization and can result in dissociation of MyD88 and TIRAP from TLR4.

ulate that PIP5K α -derived PI(4,5)P2 pool is subsequently degraded by PLC γ 2 (60). PI(4,5)P2 degradation may attenuate MyD88-dependent TLR4 signaling by promoting dissociation of TIRAP from the plasma membrane. In this regard, PI(4,5)P2 degradation may function as a molecular switch for transition from TLR4-TIRAP-MyD88 assembly to TLR4-TRAM-TRIF assembly (Fig. 3). The detailed molecular mechanisms by which PI(4,5)P2 metabolism modulates TLR4 endocytosis and signaling require further investigation.

FUTURE PERSPECTIVE

Recent evidence indicates the specificity of different sub-cellular localizations of TLR family members. This concept is of particular interest, as TLRs signaling cascades are closely related to their locations. Therefore, it is important to understand molecular mechanisms by which TLRs are regulated in a spatiotemporal manner. Considering that receptor signaling and trafficking are tightly coupled, receptor endocytosis is a promising theme in TLR research fields. Notably, phosphoinositides, which are differentially distributed over distinct membrane compartments, have emerged as critical regulators of TLR localization. In particular, PI(4,5)P2 metabolism mediated by PIP5K, PTEN, and PLC plays a role in TLR signaling and/or TIRAP trafficking. In fact, PI(4,5)P2 metabolism is involved in

clathrin- and dynamin-mediated endocytosis and in actin cytoskeleton rearrangement. This raises the question of a functional role of plasma membrane PI(4,5)P2. Considering the pleiotropic roles of PI(4,5)P2 (2), this lipid may play other roles in TLR regulation beyond its engagement in TIRAP membrane translocation. Thus, it will be challenging to precisely delineate changes in PI(4,5)P2 and dissect its significance during the course of TLR signaling and trafficking. In addition, roles of other enzymatic pathways for PI(4,5)P2 turnover are largely unknown. In a similar context, protein trafficking of TLR adaptors and their interaction with phosphoinositides need more detailed clarification. These efforts will provide improved therapeutic opportunities for many TLR-associated diseases including neurodegenerative disorders.

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