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Phosphoinositides in FCgamma receptor signaling

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1. ABSTRACT

Fcgamma receptors mediate a variety of immune responses to IgG-containing complexes. Virtually all of these responses appear to be regulated by phosphoinositides, in particular phosphatidylinositol (4,5)P_2 and phosphatidylinositol (3,4,5)P_3. Possible downstream effectors of phosphoinositides involved in multiple FcgammaR-mediated events are discussed, as are elements of the signaling pathways that may couple Fcgamma receptors to the enzymes of phosphoinositide metabolism.

2. INTRODUCTION

2.1. Fcgamma receptors

Fcgamma receptors are widely expressed in immune cells, particularly those of the myeloid lineage (1). They allow cells to respond to multivalent antigen-antibody complexes by recognizing the conserved Fc portion of IgG. Multiple classes of FcgammaR exist. The activating FcgammaR are FcgammaRI, FcgammaRIIA/C (in humans; not present in mice), and FcgammaRIIIA. These each possess an immunoreceptor tyrosine-based activation motif (ITAM), either in the signaling FcR gamma chain that associates noncovalently with the Fc binding polypeptide (for FcgammaRI and FcgammaRIIIA) or within the cytoplasmic domain of the Fc binding polypeptide itself (for FcgammaRIIA/C) (Figure 1). The presence of the ITAM allows these receptors to trigger upon aggregation a variety of responses in different cell types, including phagocytosis by macrophages and neutrophils, antibody-dependent cellular cytotoxicity by natural killer cells, and platelet degranulation. In contrast to the activating FcgammaR, FcgammaRIIB receptors are inhibitory receptors that possessing an immunoreceptor tyrosine-based inhibitory (ITIM) motif. Engagement of these receptors antagonizes cell activation. Finally, FcgammaRIIIB is a glycosyl-phosphatidylinositol-anchored protein expressed in human neutrophils that initiates some signaling through interactions with other membrane receptors (e.g. activating FcgammaR (2)). A large number of downstream effectors have been implicated in signaling from FcgammaR (3). In particular,

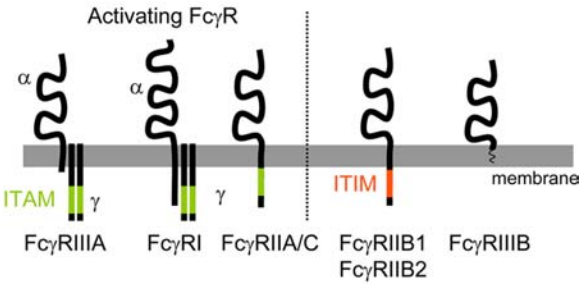

Figure 1. The human Fcgamma receptors. FcgammaRI and FcgammaRIIIA both consist of an Fc binding alpha subunit associated noncovalently with the ITAM-containing common FcR gamma chain. FcgammaRIIA contains an ITAM within its cytoplasmic domain, while the FcgammaRIIB receptors contain an ITIM.

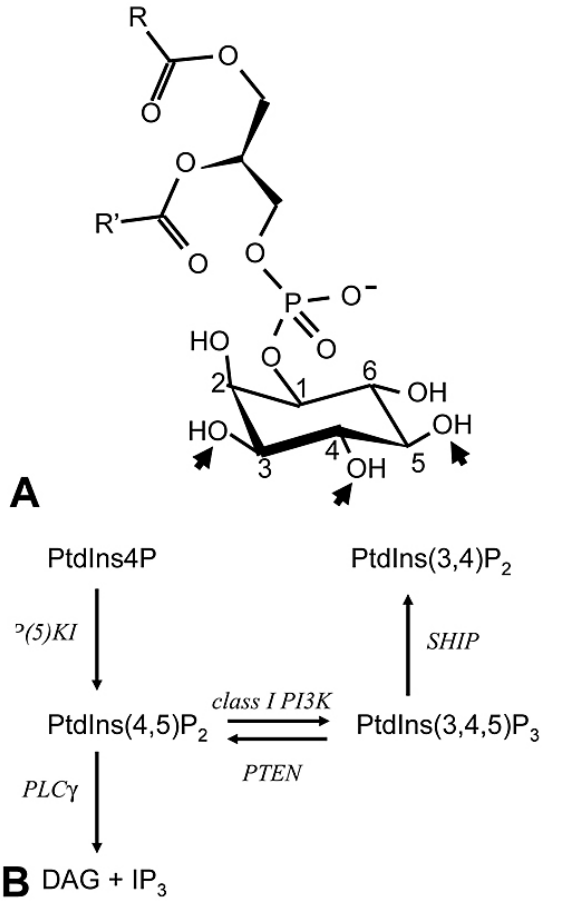

Figure 2. Phosphoinositide metabolism. (A) Structure of Phosphatidylinositol (PtdIns). R and R' represent fatty acyl chains. Arrows indicate positions on the inositol ring at which phosphorylation can occur. (B) Enzymes involved in metabolism of phosphoinositides. Only a subset of the total complement of phosphoinositides, kinases and phosphatases is illustrated.

phosphoinositides appear to play a major role in initiating or orchestrating this signaling.

2.2. Phosphoinositides

Phosphatidylinositol (PtdIns) is a phospholipid that can be phosphorylated on multiple different positions of the inositol headgroup, resulting in a variety of different phosphoinositides (Figure 2). In all, seven different such species of phosphoinositide are found *in vivo*, corresponding to all possible combinations of single, double, and triple phosphorylation of positions 3,4, and 5 of the inositol ring. These phosphoinositides are key signaling molecules implicated in a plethora of cellular processes (reviewed in (4)). They function in large part by virtue of their interaction with a diverse collection of lipid-binding domains found in a wide range of proteins; these include pleckstrin homology (PH) domains, band 4.1-ezrin-radixin-moesin (FERM) domains, Phox (PX) domains, epsin amino-terminal homology (ENTH) domains, and Fab1-YOTB-Vac1p-EEAI (FYVE) domains. Thus, different phosphoinositides contribute to recruitment and activation of distinct downstream effector molecules at different locations within the cell. In this review, the focus will be on PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (henceforth termed PIP_3), which have been the most extensively studied phosphoinositides in the context of FcgammaR signaling.

2.2.1. PtdIns(4,5)P_2

PtdIns(4,5)P_2 is present in resting cells largely in the plasma membrane, and constitutes less than 1% of total membrane lipid (4). It is synthesized primarily via phosphorylation of PtdIns4P by the type I phosphatidylinositol 4-phosphate 5-kinase (PIP(5)KI) (Figure 2). PtdIns(4,5)P_2 can be consumed through several different routes. Hydrolysis by phospholipase C (PLC) gives rise to the products diacylglycerol (DAG) and inositol (3,4,5) *tris*-phosphate, key signaling messengers. PtdIns(4,5)P_2 can also be dephosphorylated at the 5 position of the inositol headgroup by type II 5-phosphatases (5), and phosphorylated at position 3 of the inositol headgroup to give rise to PIP_3.

2.2.2. PIP_3

In contrast to PtdIns(4,5)P_2, PIP_3 is present in very small amounts in resting cells, but is synthesized in response to cell activation by numerous receptors, including activating FcgammaR as discussed below. PIP_3 is synthesized by phosphorylation of PtdIns(4,5)P_2 by Class I and Class II phosphatidylinositol 3-kinases (PI3K) (5). Class I PI3K are composed of a catalytic p110 subunit associated with a regulatory subunit. The Class IA PI3K subgroup consists of multiple isoforms; there are three catalytic subunits (p110alpha, p110beta and p110gamma), each encoded by separate genes, and multiple regulatory subunits generated by expression and alternative splicing of three different genes (p85alpha, p85beta, and p55gamma). These regulatory subunits are activated in response to tyrosine kinase signaling, and it is Class IA PI3K that are primarily thought to be involved in FcgammaR signaling. The Class IB PI3K consists of a p110gamma catalytic subunit and a regulatory p101 subunit that is activated by G proteins, while the Class II PI3K are at this point relatively poorly understood. Wortmannin (a fungal metabolite) and the synthetic compound LY294002 are, at appropriate concentrations, specific inhibitors of PI3 kinases that have

been extensively used in studying the involvement of PI3K in various processes (6). The appearance of PIP_3 in response to a stimulus is typically transient. PIP_3 can be turned over by dephosphorylation at the 3 position by the phosphatase PTEN and at the 5 position by the phosphatases SHIP1 and SHIP2.

2.2.3. Other phosphoinositides

While this review will focus on PtdIns(4,5)P_2 and PIP_3, other phosphoinositides may prove to have important roles in FcgammaR-mediated events as well. In particular, there are multiple distinct species of 3-phosphorylated phosphoinositide. *In vivo,* type I PI3K predominantly phosphorylates PtdIns(4,5)P_2 to generate PIP_3 (5). However, FcgammaR engagement is also associated with appearance of PtdIns(3,4)P_2 (7,8,9), which is likely generated as a secondary consequence of dephosphorylation of PIP_3 by 5-phosphatases (5). It can not be excluded that this PtdIns(3,4)P_2 may share some of the functions of PIP_3 or in fact play its own unique roles, since PH domains from several proteins recognize both PIP_3 and PtdIns(3,4)P_2 (10), and at least one (in TAPP1) is specific for PtdIns(3,4)P_2 (11).

In addition, the singly phosphorylated phosphoinositide PtdIns3P (PI3P) is found in endosomes where it is generated by the phosphorylation of PtdIns by Class III PI3 kinase (hVPS34 in humans). PI3P is not generally thought of as a signaling molecule *per se*, but rather is involved in the homeostasis of intracellular compartments (12). Nonetheless, by virtue of this important function PI3P can affect FcgammaR-driven processes. An example of this is that PI3P plays a critical role in maturation of phagosomes subsequent to FcgammaR-mediated phagocytosis ((13), see below). Notably, the inhibitors wortmannin and LY294002 typically used to target PI3K activity inhibit all classes of PI3K. Thus, it should be borne in mind that effects of these inhibitors on FcgammaR-mediated events may result from disruption of PI3P-dependent intracellular trafficking events, rather than (or in addition to) reflecting a direct role of activation of class I PI3K in FcgammaR signaling.

3. ROLES OF PHOSPHOINOSITIDES IN FCgammaR-MEDIATED PROCESSES

3.1. Phagocytosis

The activating FcgammaR are phagocytic receptors; their presence in professional phagocytes such as macrophages and neutrophils allows these cells to engulf antibody-coated particles many microns in size through a process triggered by FcgammaR aggregation (14). Phagocytosis is an FcgammaR-mediated process for which the roles of phosphoinositides have been extensively studied, and will therefore be discussed in some detail. It is likely that many of the insights obtained from studies of phagocytosis regarding phosphoinositide function will apply to other FcgammaR-mediated signaling functions, and *vice versa*.

Phagocytosis proceeds through a series of stages. Binding of antibody-opsonized particles via Fcgamma receptors leads to accumulation of F-actin at the site of particle attachment, forming a “phagocytic cup”. This localized actin remodeling contributes to extension of pseudopods around the particle; pseudopods are tightly apposed to the surface of the particle by zippering between the FcgammaR and the opsonizing antibodies (15). Exocytosis from internal compartments may also be important for providing sufficient membrane for this process. Pseudopods ultimately fuse around the particle, leading to its delivery into a vacuole termed the phagosome. The phagosome then undergoes a maturation process, fusing with endosomal and lysosomal compartments and so developing into a microbicidal compartment, the phagolysosome. The phagocytic engulfment process thus requires coordinated rearrangements of the actin cytoskeleton accompanied by changes in membrane dynamics. Phosphoinositides appear to play key roles in regulating these processes through their accumulation in highly temporally and spatially restricted patterns during the process of particle engulfment (16).

3.1.1. PtdIns(4,5)P_2 in phagocytosis

A significant advance for understanding phosphoinositide function has been the development of probes for visualization of phosphoinositides in living cells. These probes consist of chimeras of fluorescent proteins with specific phosphoinositide-binding domains taken from a variety of proteins. Recruitment of the probe from the cytosol to a cellular membrane provides a measure of local concentration of the recognized phosphoinositide. Botelho *et al* used such an approach to examine the localization of PtdIns(4,5)P_2 in macrophages during phagocytosis, using a probe consisting of green fluorescent protein (GFP) fused to the PH domain of PLCdelta (17). While PtdIns(4,5)P_2 is localized homogeneously in the plasma membrane in resting cells, during phagocytosis localized changes in its concentration occur at sites of phagocytosis (Figure 3). PtdIns(4,5)P_2 accumulates at the site of particle binding and in the extending pseudopods. This initial accumulation parallels the local recruitment of the enzyme PIP(5)KIalpha that mediates PtdIns(4,5)P_2 synthesis (18). As phagocytosis proceeds, however, PtdIns(4,5)P_2 is rapidly lost, disappearing from the base of the phagocytic cup even before the phagosome has sealed. By the time the phagosome has sealed, PtdIns(4,5)P_2 is completely absent from the resulting phagosomal membrane. This disappearance seems to be at least in part due to cleavage of PtdIns(4,5)P_2 by PLC to form DAG and IP_3; PLCgamma is recruited to the phagocytic cup, and a probe consisting of GFP fused to the C1 domain of PKCdelta reveals that DAG forms at exactly the time and site that PtdIns(4,5)P_2 disappears, appearing first at the base of forming phagosomes, and being maximally present on newly sealed phagosomes (17).

These localized changes in PtdIns(4,5)P_2 are suggestive of an important role for this phosphoinositide in phagocytosis, which has been confirmed in a number of ways. Overexpression of the PLCdelta-PH probe (to sequester PtdIns(4,5)P_2), expression of a plasma membrane-targeted phosphoinositide 5’ phosphatase (to degrade PtdIns(4,5)P_2) or overexpression of a dominant-negative PIP(5)KIalpha (to prevent its synthesis) all inhibit

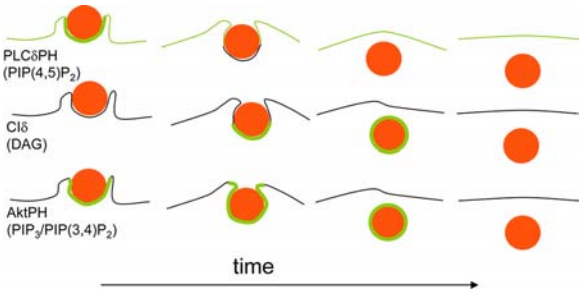

Figure 3. Localization of fluorescent protein probes during phagocytosis in macrophages. Black lines indicate the macrophage membrane; green lines indicate approximate positions of the indicated fluorescent protein fusion constructs; red circle indicates particle being engulfed (from Refs. 17 and 36). PLCdelta1PH-GFP localizes to the plasma membrane in the resting state, accumulates in phagocytic cups and pseudopods, is lost from the base of the cup during phagocytosis, and is completely absent from sealed phagosomes. C1deltaGFP begins to appear at the base of phagocytic cup before closure, and then is present on the formed phagosome before disappearing within minutes. Akt-PH-GFP appears in phagocytic cups and extending pseudpods, and is present on newly sealed phagosomes before disappearing within minutes.

phagocytosis (17,18). Several possibilities, which are not mutually exclusive, can be considered for the function(s) of PtdIns(4,5)P_2 in phagocytosis.

One likely role of PtdIns(4,5)P_2 is a direct function in modulation of actin polymerization. Indeed, inhibition of PIP(5)KIalpha leads to inhibition of F-actin accumulation at the phagocytic cup (18). PtdIns(4,5)P_2 is known to interact with a wide variety of actin-regulating proteins (reviewed in (19,20)). Among these are a number of actin capping and severing proteins, including gelsolin, CapG, and cofilin. PtdIns(4,5)P_2-dependent removal of capping proteins or increase in severing activity can result in a local increase in the concentration of barbed ends, leading to actin polymerization. Notably, gelsolin accumulates at phagocytic cups and both gelsolin and CapG are necessary for efficient phagocytosis (21,22). Furthermore, PtdIns(4,5)P_2 may stimulate *de novo* actin polymerization by indirectly activating the actin nucleating activity of the Arp2/3 complex. It is known that Arp2/3 can be activated through the cooperative action of the small GTPase Cdc42 and PtdIns(4,5)P_2 on WASP (Wiskott-Aldrich syndrome protein) family members (23,24,25). Moreover, Cdc42, WASP and Arp2/3 have all been shown to be both localized to phagocytic cups and necessary for efficient FcR-mediated phagocytosis (26,27,28). PtdIns(4,5)P_2 may also play an additional role in phagocytosis regulating the localization of proteins that are thought to couple F-actin to the plasma membrane, such as talin and vinculin, both of which are found in the phagocytic cup (29,30,19).

PtdIns(4,5)P_2 may also be important in phagocytosis by virtue simply of its role as the substrate for production of DAG and IP_3 by PLC. While phagocytosis in macrophages does not appear to require calcium signaling (31), DAG may play a role through activation of PKCs (32) or other effectors. Inhibition of PtdIns(4,5)P_2 hydrolysis by PLC inhibits phagocytosis (17). However, a consequence of PLC action that may be equally or more important than the generation of these products is the coordinated *removal* of PtdIns(4,5)P_2 from the base of the phagocytic cup. This removal may be crucial for the progression of phagocytosis, by allowing for a local disassembly of actin necessary for phagosomal sealing. Consistent with this idea, actin disappears from the forming phagosome in a similar spatial and temporal pattern to the disappearance of PtdIns(4,5)P_2, and preventing the disappearance of PtdIns(4,5)P_2 through inhibition of PLC or overexpression of PIP(5)KIalpha prevents actin disassembly and the final sealing of phagosomes after pseudopod extension (33).

3.1.2. PIP_3 in phagocytosis

FcgammaR-mediated phagocytosis is accompanied by production of PIP_3 (34). This is likely due largely to activation of class IA PI3K (35), which is recruited to phagocytic cups (36). The functional importance of PI3K in phagocytosis has been shown by several approaches. Inhibitors of PI3K impair FcgammaR-mediated phagocytosis in neutrophils, U937 monocytic cells, and macrophages (35,34). Overexpression of the phosphatases PTEN or SHIP inhibits phagocytosis (37,38,39), while conversely macrophages deficient in PTEN or SHIP display an enhanced phagocytic ability (40,37,41). Fibroblasts deficient in most of the class IA PI3K regulatory subunits upon transfection with FcgammaRIIA exhibit strongly impaired phagocytosis of antibody-coated particles relative to wild type fibroblast transfectants (13). Microinjection of macrophages with antibodies to the p110beta catalytic subunit also substantially inhibits FcgammaR-mediated phagocytosis (42).

Localization of PIP_3 in macrophages during phagocytosis has been assessed using a fusion of GFP to the PH domain of Akt (36). As for PtdIns(4,5)P_2, a transient accumulation on forming phagosomes of PIP_3 (and also possibly PIP(3,4)P_2, since the probe can also recognize this phosphoinositide) is observed, paralleling the recruitment of p85 to phagocytic cup (36). In contrast to the pattern seen for PIP(4,5)P_2, the Akt-PH probe remains associated with the forming phagosome until a few minutes after the phagosome has sealed (Figure 3). Notably, the phosphatase SHIP is also recruited during phagocytosis (37,36), where it may regulate phagocytosis by dephosphorylating PIP_3 at the 5 position.

There are several possible functions for PI3K in phagocytosis. It is noteworthy that the dependence of phagocytosis on PI3K is dependent on the size of the particle, with phagocytosis of particles less than 3 microns in diameter being relatively unaffected by inhibition of PI3K (34). Moreover, focal accumulation of F-actin in phagocytic cups is not inhibited upon PI3K inhibition, suggesting that PI3K is not essential for initiating actin polymerization (43,34,44); rather, it is the complete

extension and sealing of pseudopods around particles that appears to be impaired. It was determined that PI3K activity is required for exocytosis of internal membranes during phagocytosis (34,44), though contradictory results have been reported (45). Focal exocytosis at the sites of phagocytosis may serve to provide extra membrane necessary for the engulfment of large particles (46,34,44). The mechanism of PI3K involvement in such exocytosis is not established. Small GTPases are activated upon their loading with GTP by guanine nucleotide exchange factors (GEFs). Several GEFs are known to be regulated by PIP_3, including GEFs for the GTPase ARF6 (47). ARF6 is required for phagocytosis (48), and in particular plays a role in the focal exocytosis of membranes (44). Thus, a PI3K-dependent activation of a GEF for ARF6 may lead to delivery of membrane to the cell surface during phagocytosis. It is possible that this effect of ARF6 might in turn involve ARF6-mediated activation of PIP(5)KIalpha ((49), see section 3.2).

In addition to a potential role of PIP_3 in membrane delivery, closure of phagosomes requires a PI3K-dependent contractile activity (50). This likely is supplied at least in part by myosin X (51). Myosin X contains a PH domain that interacts with PIP_3, and is recruited to phagocytic cups in a wortmannin-inhibited manner. Expression of a truncated Myosin X inhibited phagocytosis and spreading on IgG-coated surfaces (51). PI3K is also important for signaling that can lead to cooperation of non-Fc receptors in phagocytosis; mobilization of the integrin Mac-1 upon FcgammaR engagement, which augments FcgammaR-mediated phagocytosis, is inhibited by LY294002 (52).

Strikingly, expression in COS cells of chimeric molecules consisting of the extracellular and transmembrane domains of FcgammaRI fused to the p85 subunit of PI3K is sufficient to confer on these cells the ability to perform phagocytosis of antibody-coated particles (53). This indicates that while other signaling components downstream of FcgammaR can mediate actin assembly when PI3K is inhibited, strong localized PI3K activity may nonetheless be sufficient to trigger all the steps, including actin assembly, required for phagocytosis.

PI3K activity also appears to be involved in the maturation of phagosomes. Inhibition of PI3K impaired phagosome acidification and acquisition of late endosomal markers LAMP-1 and lysobisphosphatidic acid in macrophage cell lines (54,13). In this case, however, it appears to be PI3P that is the relevant phosphoinositide. PI3P is synthesized on phagosomal membranes shortly after phagosomes seal, as revealed by GFP fusions to PI3P-binding FYVE and PHOX domains (55,13). Injection of antibodies against the class III PI3K hVPS34 was used to confirm that it is this PI3K that is involved in maturation, whereas the class IA enzymes are required for particle engulfment (54,13). Interestingly, while levels of PI3P are typically rather constant in cells (12), during phagocytosis the total cellular amount of PI3P increases significantly (13,55). This may result from an increased availability of the substrate PtdIns to hVPS34 upon the formation of phagosomes; whether recruitment of hVPS34 to phagosomes requires specific elements of FcgammaR signaling *per se* or is rather a default outcome of early phagosome maturation is unclear.

To conclude, both PtdIns(4,5)P_2 and PIP_3 are regulated in a temporally and spatially organized manner during phagocytosis, and both appear to play crucial roles in orchestrating the complex dynamic remodeling of cytoskeleton and membranes that underlies particle engulfment. An intriguing model recently put forward by Swanson and Hoppe (3) proposes that the local accumulation of phosphoinositides allows for threshold detection, integrating signals from multiple simultaneously engaged FcgammaR bound to an opsonized particle. Only when phosphoinositides reach a threshold concentration would particular effectors necessary for uptake of large particles be recruited. This would allow for the coordination of signaling over the substantial area of cell surface engaged in particle uptake. If such a model holds, it may also apply for other spatially coordinated events initiated by FcgammaR, considered below.

3.2. Antibody Dependent Cellular Cytotoxicity

Natural killer (NK) cells play an important role in the killing of virus-infected and tumor cells. This killing can occur either via direct recognition of target cells or through the coating of the targets with antibodies. In the latter case, antibody-dependent cellular cytoxicity (ADCC) depends on recognition of the antibody-coated cells by FcgammaRIIIA, the sole FcgammaR expressed on NK cells (1). FcgammaRIIIA stimulation induces activation of PI3K (9), and inhibition of PI3K leads to a block of cytotoxic granule release and ADCC (56). Recently, it has been shown that stimulation of FcgammaRIIIA on NK cells induces a PI3K-dependent activation of ARF6 (49). ARF6 is required for ADCC, and specifically for granule secretion; this may be analogous to its role in supporting focal exocytosis during FcgammaR-mediated phagocytosis (44). Notably, FcgammaRIIIA stimulation also induces membrane translocation and activation of PIP(5)KIalpha in a manner dependent on ARF6. Activation of PIP(5)KIalpha is due in part to its direct interaction with ARF6, and is likely amplified by a concomitant ARF6-mediated activation of phospholipase D, leading to generation of phosphatidic acid, a known activator of PIP(5)KIalpha (49). Thus, PI3K may function in ADCC in part through an effect on PtdIns(4,5)P_2 production.

3.3. Platelet activation

Human platelets express FcgammaRIIA as their sole FcgammaR. Clustering of FcgammaRIIA in platelets triggers platelet activation, with associated shape changes, degranulation, and platelet aggregation. Clustering of FcgammaRIIA triggers an rapid increase in FcgammaRIIA-associated PI3K activity, coincident with recruitment to the receptor of p85 (57,8). Moreover, FcgammaRIIA-mediated platelet aggregation is inhibited by wortmannin, indicating a functional requirement for PI3K in platelet activation. Recruitment of PLCgamma2 to the plasma membrane is blocked upon PI3K inhibition (8). The role of PI3K in platelet shape change has recently been examined in detail

(7). In contrast to the situation for phagocytosis in macrophages, PI3K appears to be essential for actin assembly in response to FcgammaRIIA activation in platelets, since wortmannin treatment leads to a complete inhibition of actin filament assembly. Activation of the small GTPase Rac and recruitment of the Arp2/3 complex to the cytoskeleton are blocked by wortmannin or LY294002, and addition of exogenous $PI(3,4)P_2$, PIP_3, or PI3K to permeabilized platelets is sufficient to increase actin filament barbed end exposure. These results led to a model wherein PI3K leads to Rac activation, which leads to both uncapping of barbed ends and activation of Arp2/3 (7).

3.4. Cytokine production

An important outcome of cross-linking of FcgammaR in myelomonocytic and NK cells is the production of inflammatory cytokines such as TNF-alpha, IL-1 and IL-8. There is evidence that the signaling required involves PI3K activity. NF-kappaB activation is required for transcription of many of these cytokines. Activation of NF-kappaB downstream of FcgammaR depends on MAPK activation (58). In THP-1 monocytes both MAPK and NF-kappaB activation upon FcgammaR cross-linking were inhibited by wortmannin and LY294002 (59). Moreover, PTEN-deficient macrophages show increased FcgammaR-induced production of TNF-alpha, IL-6, and IL-10 (40). SHIP-1 was also shown to inhibit NF-kappaB activation in THP-1 cells upon FcgammaR clustering (60).

3.5. Negative signaling by FcgammaRIIB

A special case of the importance of phosphoinositides in FcgammaR signaling is negative signaling from the FcgammaRIIB family of receptors. FcgammaRIIB1 is preferentially expressed in lymphoid cells and FcgammaRIIB2 in myeloid cells. Coligation of FcgammaRIIB with activating receptors represses ITAM-mediated cell activation. In large part this is due to the FcgammaRIIB-mediated recruitment of SHIP phosphatases, which hydrolyze the PIP_3 produced by activating receptors (61). This has been studied most thoroughly in the context of B cell activation, where FcgammaRIIB1 has proved to play a critical negative regulatory role (62). In the course of an immune response, when antibody concentration reaches a critical level, immune complexes containing antigen and antibody will be formed. These complexes can simultaneously engage the B cell receptor (through the antigen) and FcgammaRIIB1 (through the Fc portion of the antibodies) on the surface of the antibody-producing cells. This coligation of FcgammaRIIB1 inhibits the BCR-triggered calcium mobilization that is required for driving B cell proliferation. The Tec family kinase Btk is a crucial component in PLCgamma-activation leading to the calcium signals downstream of BCR engagement. Recruitment of Btk to the plasma membrane via interaction of its PH domain with PIP_3 is an important step in this signaling (63). SHIP-1 antagonizes BTK recruitment via hydrolysis of PIP_3 (64). An additional component in inhibitory signaling by SHIP-1 is its recruitment of RasGAP via binding of p62dok (65). SHIP-2, which is inducibly expressed in monocytes and B cells, can also contribute to negative signaling (66,67).

Given the importance of PIP_3 in FcgammaR-mediated cell activation described above, it is not surprising that FcgammaRIIB can also antagonize signaling from activating FcgammaR. This leads to the somewhat counterintuitive situation in which cells such as monocytes express both activating and inhibitory FcgammaR, receptors that mediate opposing effects and yet are simultaneously engaged upon binding of immune complexes. As a general paradigm, it is the balance between levels of activating and inhibitory receptors that determines the extent of phagocytosis and other FcgammaR-mediated functions (62,68). For example, phagocytosis is inhibited by overexpression of FcgammaRIIB and enhanced in FcgammaRIIB-deficient cells (69,70). Physiological conditions such as the cytokine milieu that affect the balance of expression of activating and inhibitory receptors thus affect the overall degree of FcgammaR-mediated cell activation (71,72). It is likely that this balancing act is coordinated at the level of PIP_3.

4. REGULATION OF PHOSPHOINOSITIDE METABOLISM BY FCgammaR

Several possible downstream effectors of phosphoinositides have been discussed in the context of specific FcgammaR-mediated functions above. An additional question that arises is what lies upstream of the enzymes controlling synthesis and degradation of these PtdIns during FcgammaR signaling, i.e. what connects the receptors to modulation of phosphoinositides? The first step in signaling from activating FcgammaR upon receptor clustering is thought to be activation of Src family kinases such as Lyn and Hck, which phosphorylate the tyrosine residues in the ITAM (73). The doubly phosphorylated ITAM then recruits the tyrosine kinase Syk through binding of its dual SH2 domains. Syk is crucial for phagocytosis and other downstream responses (74,75). How the enzymes of phosphoinositide metabolism are engaged in response to these early events is only partially clear. We will consider what is known about the regulation of PIP(5)KI, PI3K, and SHIP-1/2.

4.1. PIP(5)KI and PI3K

As discussed above, PIP(5)KIalpha is recruited to phagocytic cups during FcgammaR-phagocytosis, concomitant with a localized increase in $PtdIns(4,5)P_2$ (17,18). The mechanism of PIP(5)KI recruitment and activation is unclear at this point. Two possible activators are the small GTPases Rac (76) and ARF6 (77), both of which are recruited during phagocytosis (26,44).

Activation of type IA PI3K by FcgammaR appears to lie downstream of both Src family kinases (78) and Syk (74,79). Several distinct modes of recruitment of the p85 regulatory subunit to the active Fcgamma receptor complex have been proposed. The simplest of these is direct recruitment of p85 via an SH2-mediated binding to the phosphorylated ITAM tyrosines. This has been proposed to occur for both the FcRgamma chain and FcgammaRIIA (80), and direct binding of p85 to phosphopeptides representing the phosphorylated FcgammaRIIA ITAM has been demonstrated (81).

Alternatively, p85 may be recruited indirectly through its binding to Syk, since in other studies binding of p85 from platelet lysates to a doubly phosphorylated FcgammaRIIA ITAM peptide was only seen after platelet activation, which results in phosphorylation of Syk (57). An interaction between PI3K and phosphorylated Syk has recently been shown to occur via binding of the C-terminal SH2 domain of p85 to phosphorylated tyrosine 317 of Syk (82).

Several adaptor proteins that are recruited during FcgammaR signaling may also contribute to the recruitment of PI3K. The adaptor Gab2 binds to PIP_3 via a PH domain, and also to the p85 subunit of PI3K. FcgammaR-mediated phagocytosis is defective in macrophages from Gab2-/- mice, and this correlates with decreased Akt activation, a measure of PI3K activity (83). These results led to the proposition that Gab2 is recruited by PIP_3 and then further recruits p85 to amplify the initial PIP_3 signal. The adaptor protein LAT is constitutively associated with FcgammaRI and FcgammaRIIA, and undergoes an activation-induced association with p85 (84). Overexpression of wild type LAT, but not a mutant form incapable of binding to p85, enhances phagocytosis, suggesting a role for LAT-mediated p85 recruitment. In addition, macrophages from LAT-deficient mice display reduced phagocytosis activity. Finally, the E3 ubiquitin ligase Cbl becomes phosphorylated on tyrosines upon FcgammaR activation (85), and can bind to the SH2 domains of p85 through its phosphorylated tyrosine 731 (86). However, at this point it is unclear whether this interaction would play a positive role (with Cbl acting as an adaptor recruiting p85 (87)) or a negative role (by targeting p85 for ubiquitination (88)) in PI3K signaling.

There is also evidence that secondary activation of Class IB PI3K can occur upon FcgammaRI cross-linking on monocytes, though the molecular mechanism of activation of this G-protein coupled isoform by an FcgammaR is not known (89).

4.2. SHIP

Recruitment of the inositol phosphatases SHIP1/2 to murine FcgammaRIIB has recently been shown to involve multiple distinct molecular interactions (90). SHIP is recruited by binding of its SH2 domain to the tyrosine within the FcgammaRIIB ITIM, which becomes phosphorylated by Src family kinases upon coligation of FcgammaRIIB with an activating receptor. However, interactions of an additional tyrosine-containing motif at the C-terminus of FcgammaRIIB with the adaptors Grb2 and Grap are also important for SHIP recruitment *in vivo*. SHIP may also be recruited by FcgammaRIIA in a manner independent of FcgammaRIIB, either through direct interaction with the phosphorylated ITAM of FcgammaRIIA, or via the adaptor Shc (41,60).

5. CONCLUDING REMARKS

Recent years have seen substantial progress in our understanding of the variety of cellular functions performed by phosphoinositides. While there are commonalities in phosphoinositide usage among different FcgammaR, cell type-specific differences, e.g. dependence on PI3K for actin remodeling, are apparent. One major challenge for understanding molecular mechanisms is that the function of each phosphoinositide can not truly be considered in isolation, since in any given process multiple different phosphoinositides may be involved, and these phosphoinositides are subject to dynamic interconversion through the action of kinases and phosphatases,. The existence of positive feedback effects, such as the stimulation by PI3K via Arf6 (7) or Btk (91) of production of its own substrate, PtdIns(4,5)P_2, complicates any simple notions of upstream and downstream players, as does the formation of large multiprotein complexes scaffolded by interactions with phosphoinositides (63). A further challenge will be better understanding how phosphoinositide signaling relates to lipid rafts, membrane microdomains that appear to play a major role in signaling (92), but whose actual physical properties remain controversial (93).

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