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Crossroads of PI3K and Rac pathways

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Rac and PI3Ks are intracellular signal transducers able to regulate multiple signaling pathways fundamental for cell behavior. PI3Ks are lipid kinases that produce phosphorylated lipids which, in turn, transduce extracellular cues within the cell, while Rac is a small G protein that impacts on actin organization. Compelling evidence indicates that in multiple circumstances the 2 signaling pathways appear intermingled. For instance, phosphorylated lipids produced by PI3Ks recruit and activate GEF and GAP proteins, key modulators of Rac function. Conversely, PI3Ks interact with activated Rac, leading to Rac signaling amplification. This review summarizes the molecular mechanisms underlying the cross-talk between Rac and PI3K signaling in 2 different processes, cell migration and ROS production.

The Rho Family GTPases

Rho GTPases constitute one of the 5 distinct families of the Ras superfamily.[1] Members of the Rho GTPase family are found in all eukaryotic organisms and have been implicated in the regulation of several aspects of intracellular actin dynamics. Rho, Rac and Cdc42 members show common intracellular functions as they promote cell growth, inhibit apoptosis and regulate gene expression.[2] They also promote actin cytoskeleton reorganization, although each isoform affects distinct features of cell shape and movement.[3]

Different plasma membrane receptors, including tyrosine kinase (RTKs), G protein-coupled (GPCRs) and cytokine receptors can initiate the signaling cascade engaging Rho GTPases. Rho GTPase activation is mediated by a multistep reaction involving guanine exchange factor proteins (GEFs) that favor the exchange between GDP and GTP on the RhoGTPase.[4] GEF proteins do not stimulate the direct binding of the GTPase to GDP/GTP, but stabilize the nucleotide-free state of the enzyme. Since the free nucleotide-binding site of the GTPase has similar affinity for GTP and GDP, the binding of the GTPase to GTP is thus only determined by the high intracellular concentration of this nucleotide which is 10-fold more abundant than GDP.[5] Subsequently, the binding of GTP displaces the GEF and frees the GTPase to interact with effectors.[6-8] Similar to many other signaling enzymes, the activation status of Rho GTPases is finely regulated. Rho GTPases possess an inefficient intrinsic GTPase activity that is enhanced by GTPase Activating Proteins (GAPs) which promote the formation of the inactive GDP-bound form.[6,9,10] In this context, GEFs and GAPs represent key determinants and regulators of Rho GTPase activity. Another level of regulation of Rho GTPases involves a class of inhibitory proteins defined GDP dissociation inhibitors (GDIs). GDIs negatively regulate Rho GTPases through 2 different mechanisms. Firstly, they bind to the GTPase thus impeding the dissociation of GDP. Secondly, since the membrane localization of Rho GTPases is essential for their biological function, GDIs can inhibit Rho GTPases by interacting with prenylated Rho GTPases and subsequently sequestering them in the cytosol.[11,12]

Based on the primary amino acid sequence, the structural motifs and the biological function, the Rho family can be divided into 6 subfamilies, and among them, the RhoA-related, the Rac1-related and the Cdc42-related subfamilies are the best characterized.[3] Rac GTPases are intracellular signal transducers implicated in controlling actin cytoskeleton organization, cell migration, proliferation and survival in mammalian cells.[3,13] The Rac subfamily includes different members, encoded by distinct genes: the ubiquitously expressed Rac1, the widely expressed RhoG, the haematopoietic -specific Rac2, and Rac3 which is predominantly expressed in the brain. Although they appear to have redundant functions, in some circumstances they play isoform-specific roles.[2] Rac1 predominantly regulates actin organization of cytoskeleton and cell adhesion and its genetic ablation results in embryonic lethality during gastrulation, due to a defective formation of the 3 germ layers.[14] Conversely, the absence of Rac2 expression is compatible with life, but results in deficiencies of haematopoietic cells, including severe neutrophilic, phagocytic and lymphocytic defects[15-18] Rac3 isoform is not strictly required for normal development in utero, but may be relevant to later events in the development of a functional nervous system.[19] RhoG deficiency produces a mild phenotype in lymphocytes where it is abundantly expressed, indicating a functional redundancy of RhoG with other Rac proteins in these cells.[20]

In its active state, Rac signals by recruiting and activating numerous proteins, including, protein kinases, lipid kinases, hydrolases, phosphatases, actin-binding proteins and the actin cytoskeleton.[21] Some of these Rac effectors are bound directly by Rac-GTP and, among them, PAK and MLK[22] regulate actin

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polymerization, PAK[23] and p70S6K[24] control cell proliferation and transcription, and p67phox[25] controls ROS production.[21,26,27] In other contexts, Rac-GTP promotes the activation of indirect targets such as MEKK1[28] and WAVE.[29] Finally, inactive GDP-bound Rac is also able to indirectly trigger downstream effectors, such as IQGAP1/2.[30]

Class I PI3K

The PI3K family includes 3 different classes of enzymes able to phosphorylate the D3 position of the inositol ring of phosphatidylinositol (PtdIns) as well as its phosphorylated derivatives. Among them, class I PI3K represents the most characterized group of enzymes that produce PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2. They operate downstream of different cell surface receptors and, upon activation, they exert their role through the recruitment of signaling effectors carrying phosphoinositide-recognition modules, such as the FYVE zinc finger (Fab-1, YGL023, Vps27, and EEA1), the PH (pleckstrin homology), the GRAM (Glucosyltransferases, Rab-like GTPase activators and Myotubularins), the GLUE (GRAM-like, ubiquitin-binding on Eap45) and the PX (phosphoinositide-binding) domains.[31]

Class I PI3Ks are dimeric enzymes composed by a 110 kDa catalytic subunit and a regulatory adaptor. On the basis of the different regulatory subunit, class I is further divided into class IA, represented by PI3Kα, PI3Kβ and PI3Kδ, and class IB whose unique member is PI3Kγ. Class IA catalytic subunits, p110α, p110β and p110δ associate with 5 different regulatory subunits, p85β, p55γ, p85α and its splice variants p55α and p50α. All catalytic subunits can form dimers with any of the type IA regulatory subunit.[32] Class IA enzymes exist in a heterodimeric form uniquely, since monomeric p85 is unstable and loss of p110α and p110β results in a concomitant reduction of p85 expression levels.[33-35] PI3K regulatory subunits can affect the activity of the p110 catalytic subunit by promoting its stabilization, by inhibiting its enzymatic activity and by recruiting it to phosphorylated tyrosine residues on RTKs or on adaptor proteins, such as the insulin receptor substrate (IRS). The full length p85 subunit is structurally characterized by distinct domains, including an N-terminal SH3 (Src homolory-3) region, a BCR (breakpoint cluster region) and 2 SH2 domains, respectively at the N-terminus (n-SH2) and C-terminus of the protein (c-SH2), separated by an intervening coiled-coil domain (iSH2). On the contrary, the p50α, p55α and p55γ are shorter variants of the regulatory subunits and they maintain only the 2 SH2 domains and the iSH2.[36,37] Class I catalytic subunits share common domains represented by an N-terminal adaptor binding domain (ABD), a Ras binding domain (RBD), a C2 domain (C2), a helical domain and a C-terminal kinase domain. During the formation of the heterodimer, the regulatory subunit contacts, with high affinity, the ABD domain of the p110 catalytic subunit through the iSH2.[38,39]

Among class IA PI3Ks, the PI3Kβ isoform is the unique member to be recruited downstream of GPCR and to be activated by the direct binding of Gβγ.[40,41] The Gβγ subunit directly binds p110β within a region located between the C2 and the helical domains.[42] Intriguingly, Gβγ is able to stimulate p110β activity even in the absence of p85 and phosphotyrosine.[42]

Differently from class IA enzymes, the single class IB isotype, p110γ, interacts with either a p101 or a p84/87 regulatory subunit.[43-45] Both p101 and p84/87 facilitate the activation of the p110γ enzymatic activity downstream of GPCRs and determine the substrate specificity in vitro.[46] The N- and C-terminal regions of p101 are critically involved in the binding to p110γ and to Gβγ subunits of GPCRs, respectively, and the residues required for this interaction have been recently characterized.[47]

Another important mechanism of PI3K activation involves the small GTPase Ras. Activation of PI3K by Ras was demonstrated for p110α and p110γ and was shown to be critical for regulation of cell growth, survival, cytoskeleton reorganization, and metabolism.[48,49] Notably, Ras binds to the p110 catalytic subunit directly and independently of the p85 adaptor.[50,51]

Class II PI3Ks are 3 monomeric enzymes, PI3KC2α, PI3KC2β and PI3KC2γ, composed by a Ras binding domain, 2 C2 domains, a PX domain and a catalytic domain. Recently, this class has emerged as a key regulator of vesicle trafficking and signal transduction downstream of RTKs. In particular, PI3KC2α[52,53] and PI3KC2β[54,55] activate Rho family GTPases at the plasma membrane and in endosomes, through a lipid-dependent mechanism. Class III PI3Ks comprise only one protein, Vps34, that is composed by a C2 and a catalytic domain, associated with a regulatory subunit named p150, and is

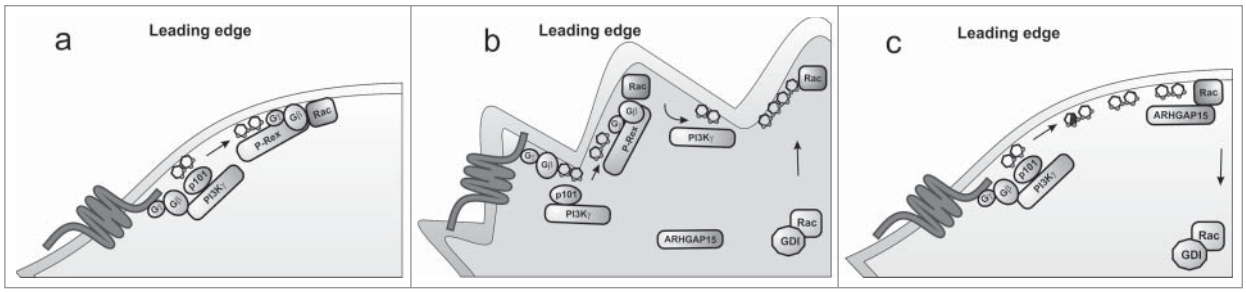

Figure 1. Schematic representation of signaling events activated by PI3K leading to cell polarization. GPCR activation stimulates the lipid kinase activity of PI3K to activate Rac through the GEF protein P-Rex (**A**) A positive feedback loop mediated by the interaction between PI3K and activated Rac sustains cell polarization (**B**) ArhGAP15 binds to PtdIns(3,4,5)P_3 produced by PI3K to terminate Rac signaling at the leading edge (**C**).

responsible for the production of PtdIns(3)P in endosomes to stimulate vesicle transport and autophagy.[56]

PtdIns(3,4,5)P_3 as a Linker for Rac Activation

The understanding of the role of class I PI3K, and of its lipid product PtdIns(3,4,5)P_3, in Rac signaling is mainly due to studies using selective PI3K inhibitors. In particular, the treatment with the fungal metabolite Wortmannin and the expression of dominant negative enzymes have allowed the identification of a functional link between PI3K- and Rac-mediated signaling pathways in response to extracellular cues.[57-59] Once activated, class I PI3Ks produce PtdIns(3,4,5)P_3 which functions as a second messenger that recruits proteins containing phosphoinositide-binding modules, like the PH domain. The PH domain was also identified in several members of the GEF family, including Dbl proteins.[60,61] All members of the Dbl family share structural similarities and, in particular, they possess common modules such as the DH (Dbl homology) domain, a 240 residue region involved in the GTP exchange reaction, and a C-terminal PH domain.[62-64] The identification of a PH domain, able to bind membrane phosphoinositides in different GEFs, has initially provided a possible mechanism whereby cytosolic proteins like GEFs are translocated to the plasma membrane upon receptor activation.[65] For instance, the T-cell lymphoma invasion and metastasis 1 protein (TIAM1) presents 2 different PH domains, one at the N terminus and the other one at the C terminus, associated with the DH domain. The N terminal PH region interacts with PtdIns(3,4,5)P_3 and enhances Rac1 activation, both in vitro and in vivo. However, TIAM1 localization on the plasma membrane appears to be independent of PtdIns(3,4,5)P_3 as the presence of PtdIns(3,4,5)P_3 is not sufficient to recruit to the plasma membrane an isolated TIAM1 PH domain.[66] On the contrary, the class III PI3K lipid product, the PtdIns(3)P, seems to be sufficient for membrane recruitment of TIAM to the early endosomes.[67] Accordingly, further analysis demonstrates that the PH domain of GEFs possesses only a micromolar affinity for phosphorylated lipids (summarized in **Table 1**) compared to the PH domains found in other proteins,[68] thus suggesting a lipid-independent mechanism for membrane recruitment and a different role for the PH domain of GEFs.[69] The membrane localization

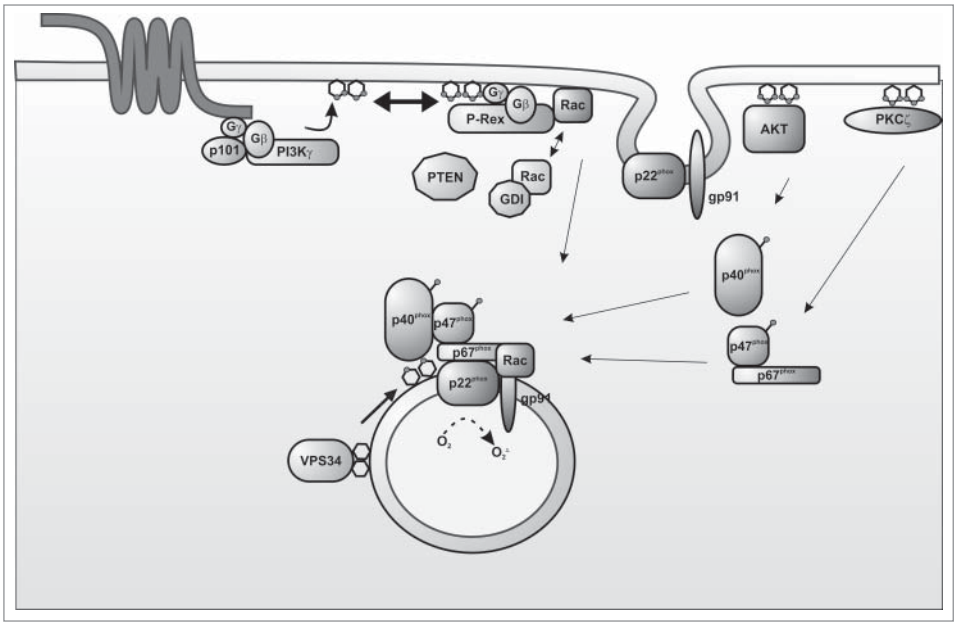

Figure 2. Schematic representation of signaling events activated by PI3K leading to ROS production. GPCR activation stimulates the production of PtdIns(3,4,5)P_3 at the plasma membrane to activate Rac through the GEF protein P-Rex. The phosphorylated lipids produced by PI3K activate $p40^{phox}$ and $p47^{phox}$ through Akt and PKC phosphorylation. The assembly of the NADPH oxidase complex on the phagosomal membrane is favored by the PtdIns(3)P produced by Vps34.

Table 1. Molecular interactions between membrane lipids and RacGEFs

GEF	Lipid Binding Domain(s)	PI3K lipid product	Effect(s)
Tiam 1	DH-PH, PH N-ter and PH C-ter	PtdIns3P[144–146]	No effect on GEF activity[146]
		PtdIns(3,4)P_2[144]	Enhanced Guanine Nucleotide Exchange activity in vitro [66]
		PtdIns(3,4,5)P_3[144]	Stimulated GDP/GTP exchange on Rac1 in vivo [147]
Intersectin	DH-PH	PtdIns3P[145]	No effect[145,148]
		PtdIns(3,4)P2[145]	
Dbs	DH-PH	PtdIns3P[145,149]	PH domain is required for efficient exchange in vivo[69]
		PtdIns(3,4)P_2[145]	PH-DH domains may promote guanine nucleotide exchange of Rho GTPases[150]
		PtdIns(3,4,5)P_3[145]	The PH domain contributes independently to membrane targeting[151]
Vav1	FL	PtdIns(3,4,5)P_3[132]	PtdIns(3,4,5)P_3 enhances phosphorylation and activation of Vav by Lck[132]
			Recruitment to the plasma membrane[152]
P-Rex1	FL and DH-PH	PtdIns(3,4)P_2	Stimulate the GEF activity[153,72]
		PtdIns(3,4,5)P_3	
Dbl	PH	PtdIns(3,4,5)P_3	The binding to PtdIns(3,4,5)P3 inhibits the GEF activity [154]
			The PH domain contributes to the membrane recruitment of Dbl[154]

of the GEF upon GPCR stimulation can be instead mediated by the interaction between the Gβγ subunits of the activated G protein and the nucleotide exchanger, such as in the case of the PtdIns(3,4,5)P_3-dependent Rac exchanger 1 protein (P-Rex).[70] The analysis of the crystal structure of the GEF-Rho GTPase complex, demonstrates a role for the PH domain not only as a docking module, but also as an intra-molecular inhibitor of the DH domain activity. This model was demonstrated for TIAM and for the dual Rho-Ras GEF Son of Sevenless (SOS), also acting as a GEF for Rac, and it is supported by the finding that deletion of the PH domain within these proteins leads to constitutive in vitro and in vivo activation of Rac.[47,53,71] Relief of this autoinhibition is a major mechanism of GEF regulation and is accomplished by phosphorylation of specific residues and/or protein-protein interactions that in certain conditions can involve phosphoinositide binding. The ability of PtdIns(3,4,5)P_3 to release the inhibitory effect of the PH domain has been demonstrated for the protein P-REX. The binding of PtdIns(3,4,5)P_3 to this GEF results in the inhibition of the DH-PH interaction and increases the GEF activity by 10 folds.[72] Notably, a synergistic cooperation between PtdIns(3,4,5)P_3 and the Gβγ subunits of the GPCR is able to induce a 50-fold change in GEF activity.[72] Another example is represented by the Vav protein whose activity is regulated by phosphorylation of the Tyr174 residue,[73,54] and in some circumstances can be potentiated by the binding of PtdIns(3,4,5)P_3 and inhibited by the interaction with PtdIns (4,5)P_2.[39] In particular, the binding of PtdIns(3,4,5)P_3 to the PH domain of Vav releases the inhibitory action and, at the same time, induces the exposure of the Tyr174 residue that is promptly phosphorylated by Src-family kinases, with consequent activation of the GEF activity.[74] Alternatively, the activity of some GEFs can be modulated by specific protein-protein interactions: for instance, the nucleotide exchange of SOS is enhanced when PI3K is recruited to the Eps8-Abi1-Sos complex.[75,76] Non-universal mechanisms of GEF activation are also reported. For instance, the non-canonical GEF protein DOCK180 is recruited to the plasma membrane by a PtdIns(3,4,5)P_3-dependent mechanism, but its full activation is lipid-independent and strictly relies on a protein-protein interaction with the PH domain of ELMO.[77,78] Another example is provided by the atypical GEF protein Swap-70 whose PH domain is required for the membrane translocation of the protein after EGF stimulation, through a Ras-independent mechanism. In primary fibroblasts, the binding of Swap-70 to membrane PtdIns(3,4,5)P_3 stimulates nucleotide exchange and eventually results in increased membrane ruffling.[79] Altogether, these data are consistent with a model where PtdIns(3,4,5)P_3 can concomitantly favor the membrane translocation of GEFs and positively regulate the exchange reaction. In addition, PtdIns(3,4,5)P_3 can promote GEF activity also by interacting with the GEF itself and driving a conformational change of the protein which ultimately allows the binding with Rac and the ensuing stabilization in the nucleotide-free conformation.

The analysis of knockout mouse models of different GEFs demonstrated a functional redundancy between these proteins. For instance, the absence of Dbl, Vav1/2/3, Tiam1 and P-Rex1 expression does not result in embryonic lethality or developmental defects.[80-85] Likewise, genetic inactivation of all 3 Vav genes induces alteration in the haematopoietic lineage[83] and only the simultaneous loss of Vav1 and Vav2 leads to B cell defects.[81,86]

While compelling evidence supports the role of PtdIns(3,4,5)P_3 as positive regulator of Rac activity, several studies demonstrate an additional function as inhibitor of Rac signaling. On these grounds, PtdIns(3,4,5)P_3 has been involved in the activation of Rac-GAPs, since some isoforms present a lipid-binding domain able to activate and localize the enzyme at the plasma membrane. A prototype of GAP proteins regulated by lipid interaction is represented by the cdGAPs family. cdGAPs contain a polybasic region (PBR) able to interact with PtdIns(3,4,5)P_3 and this binding is required for full inactivation of Rac1 signaling.[87,88] PtdIns(3,4,5)P_3-binding domains have been also identified in other GAPs, such as p190 Rho-GAPs that possess a PBR (polybasic region) domain able to recognize phosphorylated lipids,[89,90] and the ArhGAP family, characterized by a PH domain.[91] In particular, ArhGAP15 has been suggested to be activated downstream of PI3Kγ and to negatively regulate Rac activity in C5a-stimulated bone marrow-derived macrophages.[92]

All these data thus support a fundamental interplay between PI3K lipid kinase activity and Rac signaling, with the PI3K lipid product affecting crucial modulators of Rac activity.

GTPases as Linkers for PI3K Activation

Although a large body of evidence identifies the PI3K lipid product as a key activator of Rac signaling, several works define PI3K as a possible direct effector of activated Rho-GTPases. The first evidence that PI3K can be activated by small GTPases comes from the finding that PI3K interacts with the GTP-bound forms of CDC42Hs and Rac1, via the p85 regulatory subunit, and serves as an effector of these 2 GTPases.[93] Another study demonstrates that Rac1 and Rac2 specifically bind to PI3K in an equimolar complex. Rac interacts with the BCR homology domain of the PI3K regulatory subunit p85[93] and such interaction is markedly enhanced when Rac is in the GTP-bound state, thus suggesting that PI3K is a direct target of activated Rac.[94] However, evidence indicates that PI3K cannot be directly triggered by Rho-GTPases as GTP[S]-Cdc42 fails to activate recombinant PI3K in vitro and thus suggests that other mechanisms underlie the interaction between Rho-GTPase and PI3K signaling.

Intriguingly, Rho-GTPase and PI3K are causally linked by a feedback loop that is critically involved in the establishment and the maintenance of leukocyte polarity. In particular, through the binding with p85, Rac contributes to the recruitment of PI3K and the ensuing production of PtdIns(3,4,5)P_3 at the leading edge of migrating cells. This increase in PtdIns(3,4,5)P_3 levels induces GEF-Rac activation that in turn sustains the formation of directional protrusions.[95] Experiments in differentiated HL60 cells demonstrate that Rac activity is necessary and sufficient for the accumulation of PtdIns(3,4,5)P_3 at the leading edge.[96] Likewise, both genetic and pharmacological ablation of

different PI3K isoforms results in defective leukocyte migration. For instance, PI3Kγ-deficient neutrophils show loss of directionality during N-formyL-Met-Leu-Phe-(fMLP)-induced chemotaxis.[97] In addition, PI3Kδ inhibitors impair PtdIns(3,4,5)P_3 accumulation at the leading edge of migrating neutrophils and eventually result in defective chemotaxis.[98] Nevertheless, in particular conditions, PI3K activity is dispensable for directional cell migration. The emerging view is that, although PI3K is necessary to accelerate neutrophil migration, PI3K is instead not required for long-term fMLP-driven neutrophil chemotaxis, thus indicating that alternative pathways overcome PI3K and critically control late events during cell migration.[99] The feedback loop linking PI3Ks and RhoGTPase signaling pathways appears to be cell-specific as in Hela and NIH3t3 cells, conditional activation of individual members of RhoGTPases, including Rac1, Cdc42 and RhoG, is not sufficient to increase PI3K activity.[100] In addition, in Zebrafish, the expression of a genetically-encoded photoactivable version of Rac, namely PA-Rac, rescues the protrusion defects but not the migration defects induced by PI3K inhibition, thereby demonstrating that PI3K activity is essential for neutrophil polarity and motility in vivo.[101] Altogether, these works suggest that in vivo other components are required to allow the feedback loop between Rac and PI3K. For instance, simultaneous activation of Rac1, Cdc42 and RhoG, triggers PI3K through a Grb2 and Ras-independent mechanism[96,102,103] Therefore, combined engagement of multiple small GTPases is necessary for the establishment of the feedback loop culminating in PI3K activation.[100]

Activation of the Rac/PI3K feedback loop also involves the process of actin polymerization as cells expressing constitutively active Rac display elevated levels of PtdIns(3,4,5)P_3 which can be reduced by actin depolimerization. In addition, experiments using a pharmacological cocktail that inhibits actin dynamics while preserving cell function and cytoskeleton integrity, demonstrate that actin remodeling is required for the spatial persistence of Rac activity.[104]

Although compelling evidence indicates that the interaction between PI3K and Rac is primarily mediated by the PI3K regulatory subunit p85, the emerging view is that Rac can also interact with PI3K catalytic subunits. A recent report demonstrates that Rac1 and Cdc42, but not Ras, directly associate with the Ras binding domain (RBD) of the PI3K catalytic subunit, p110β. Such association occurs even in the absence of the BH domain of p85, while it is abrogated by point mutations in the RBD of p110β. This work elegantly demonstrates that GPCRs couple to PI3K via Dock180/Elmo1-mediated Rac activation and binding to p110β and that the interaction between Rac and p110β is a crucial determinant of fibroblast migration. Accordingly constitutively active Rac stimulates migration in absence of chemoattractants, but in presence of stimuli triggering p110β, while mutations in the RBD domain of p110β abolish the effect of constitutively active Rac.[105]

Altogether, these findings clearly support a key role of GTP-bound Rac in the activation of the PI3K signaling via feedback mechanisms, but this activation is context-dependent and requires the action of actin dynamics. In the following sections, we will discuss how Rac and PI3K signaling pathways cooperate in the regulation of essential functions of immune cells, such as migration and ROS production.

PtdIns(3,4,5)P_3 Delimits Actin Polymerization at the Leading Edge

Cell migration strictly depends on the ability of cells to polarize. During polarization cells respond to chemoattractants by forming a leading edge where actin is rapidly polymerized and a trailing edge where actin is more stable. This process promotes the formation of protrusions and focal adhesions at the leading edge which, in turn, allow cells to attach to the substrate matrix and to contract the trailing edge.[106-108]

PI3Ks cooperate with Rac to regulate cell polarization in response to different stimuli and, in particular, to identify and organize the leading edge of migrating cells (**Fig. 1**).[108] The functional interaction between PI3Ks and Rac has a major role in neutrophils, a subset of immune cells that need to rapidly respond to extracellular cues. In general, chemoattractants such as CXCL12, CXCL2 and fMLP activate neutrophil GPCRs leading to the dissociation of the G protein into Gαi and Gßγ subunits. While historically Gαi appeared to be not necessary for cell migration,[109] recent evidence points to a key role of this protein in the regulation of directionality and termination of neutrophil chemotaxis.[110,111] On the other hand, Gßγ activates major intracellular pathways governing migration, via downstream modulation of PI3Ks and Rac. More recently, a Gβγ effector, named ElmoE, was shown to associate with Dock-like proteins in Dictyostelium to activate Rac and promote actin polymerization. In these cells, ElmoE associates with both Arp2/3 and F-actin, thus serving as a molecular link between GPCR, G proteins and actin cytoskeleton, at least in Dictyostelium, although it is plausible to envisage a similar mechanism in mammalian cells.[112,113]

In unstimulated neutrophils, the lipid product of PI3Ks, PtdIns(3,4,5)P_3, is nearly absent, but increases rapidly and dramatically after stimulation with chemoattractants.[114] Notably, the increase of PtdIns(3,4,5)P_3 concurs to enhanced actin polymerization. This indicates that the burst of PtdIns(3,4,5)P_3 is coupled with Rac activation and the ensuing actin polymerization. Consistent with this view, manipulation of PI3K activity, using pharmacological inhibitors or dominant-negative PI3K proteins, results in defective actin polymerization and cell polarization.[59,115,116] Conversely, enhancement of intracellular amount of PtdIns(3,4,5)P_3, using membrane-permeant lipids, promotes migration and cell polarization.[117] Experiments with fluorescent probes for PI3K and Rac activity (AKT-PH domain and PAK-PBD, respectively) demonstrate that PtdIns(3,4,5)P_3 production colocalizes with sites of Rac activation at the leading edge during cell polarization.[96,118] The asymmetric distribution of phosphorylated lipids between the leading and the trailing edge of polarizing cells is due, at least in part, to the ability of Gßγ subunits to localize and activate class IB PI3Kγ at the leading edge.[92,119,120] PI3Kγ is the major responsible for PtdIns

$(3,4,5)P_3$ synthesis in neutrophils after stimulation with tripeptide formyL-Met-Leu-Phe (fMLP), interleukin 8 and C5a. Accordingly, neutrophils lacking PI3Kγ have reduced migration, both in vitro and in vivo.[121-123] Similarly, the use of a PI3Kγ selective inhibitor (AS252424) reduces directional migration of mouse neutrophils.[124,125] Other PI3K isoforms can cooperate with PI3Kγ in regulating neutrophil migration. Both human and mouse cells treated with PI3Kβ inhibitors (TGX-115 and TGX 221) show impaired chemotaxis, while PI3Kδ inhibition with IC87114 affects migration only in human neutrophils.[98,125]

Two different proteins, which antagonize PI3K-dependent activation of Rac signaling, further contribute to the establishment of an asymmetric distribution of $PtdIns(3,4,5)P_3$ in migrating cells: PTEN and SHIP1. PTEN and SHIP1 act as phosphatases to dephosphorylate the 3′ and the 5′ phosphate, respectively, of $PtdIns(3,4,5)P_3$, leading to the formation of $PtdIns(4,5)P_2$ or $PtdIns(3,4)P_2$.[126,127] Neutrophils lacking PTEN display normal migration in response to a single chemoattractant, but fail to respond to gradients of multiple chemoattractants, indicating that PTEN functions to 'prioritize' chemotactic cues and prevent 'distraction' in migrating neutrophils.[128] On the contrary, ablation of SHIP1 dramatically impairs neutrophil chemotaxis toward a single chemoattractant, as a result of unpolarized $PtdIns(3,4,5)P_3$ localization.[129] The different phenotypes observed in SHIP1- and PTEN-knockouts are due to the fact that the 2 phosphatases affect $PtdIns(3,4,5)P_3$ distribution through different mechanisms. While PTEN localizes to the rear of neutrophils and facilitates the accumulation of phosphorylated lipids at the leading edge, SHIP1 is active at the cell–matrix interface to abolish the gradient of $PtdIns(3,4,5)P_3$ induced by integrin activation.[130]

$PtdIns(3,4,5)P_3$ Burst ROS Production

In professional phagocytes, superoxide production from oxygen and NADPH by activated NADPH oxidase is an obligated step in destruction of invading microorganisms. The NADPH oxidase complex is composed by a catalytic core, including gp91 and $p22^{phox}$, and by a regulatory part composed by $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac. Upon cell stimulation, the degranulation of intracellular granules brings the catalytic subunits to the plasma membrane and concomitantly induces the translocation of the regulatory elements from the cytosol to the plasma membrane in order to form an active complex.[131] Phosphoinositides regulate the assembly of the regulatory components of the NADPH oxidase in different ways, such as phosphorylation of $p47^{phox}$ by phosphoinositide-dependent kinases, activation of Rac via phosphoinositide-dependent GEF or direct interaction of cytosolic subunits ($p40^{phox}$, $p47^{phox}$ and Rac) with phosphoinositides (**Fig. 2**).

$p67^{phox}$ is one of the cytosolic members of the NADPH oxidase complex and is strictly required for O_2 radical production in a reconstituted cell-free system.[132,133] The activation of $p67^{phox}$ is regulated in vitro by the interaction between $p40^{phox}$ and $p47^{phox}$, which localize the heterotrimeric complex to the phagosomal membrane. In vitro $p40^{phox}$ associates with $p67^{phox}$ with high affinity[134,135] and it is possible that, in vivo, this interaction is regulated by PI3K-dependent phosphorylation of $p40^{phox}$.[136] A similar scenario has been described for the interaction between $p47^{phox}$ and $p67^{phox}$. $p47^{phox}$ presents 4 different domains, a PX in the N-terminal region, followed by 2 SH3 and a prolin rich region that binds to $p67^{phox}$. The SH3 domains interact with both the PX and the proline-rich domains within $p47^{phox}$, thereby driving the auto-inhibition of the protein that is retained in the cytosol and does not associate with $p67^{phox}$. The interaction with both phosphorylated lipids at the plasma membrane and $p67^{phox}$ is promoted by phosphorylation events controlled by several kinases, such as PKC, MAPK as well as the PI3K effector, Akt.[137,138] In line with a fundamental role of PI3K in NADPH oxidase activation, inhibition of PI3K decreases ROS production in neutrophils stimulated with immune complexes.[122,139] In addition, class III PI3Ks critically contribute to the localization of the NADPH cytosolic complex to phagosomal membrane by producing PtdIns(3)P that functions as a docking site for $p40^{phox}$. Accordingly, neutrophils expressing a mutant $p40^{phox}$, carrying a point mutation in the lipid-binding domain, display defective ROS production.[140]

Besides PI3K-mediated mechanisms, the assembly and activation of the NADPH complex is strictly controlled by Rac, at least in response to serum-opsonized Staphylococcus Aureus.[141] The mechanism whereby Rac controls NADPH assembly and ROS production is still debated. The current view is that Rac, activated via a PI3K-dependent mechanism, either stabilizes the interaction between the regulatory complex and the catalytic core of the oxidase or participates to the electron transfer.[142]

Concluding Remarks

PI3K and Rac are tightly connected due to the ability to regulate each other in a lipid-dependent (PI3K upstream) or -independent (Rac upstream) manner. However, the comprehension of the functional impact of these processes in vivo appears more complicated. The major drawback of genetic approaches is that inactivation of distinct Rac isoforms in mouse models may lead to compensatory upregulation of other isoenzymes, thus confusing the resulting phenotype.[143] A further level of complexity is provided by the intricate network of feedback regulatory mechanisms whereby PI3K controls both positive and negative regulators of Rac which in turn fosters PI3K activity. In recent years, the availability of inducible systems to control PI3K-Rac axis have dramatically improved our understanding of the spatial and temporal regulation of the connection between PI3K and Rac signaling pathways and, of feedback mechanisms of regulation in isolated systems. If the lessons learnt in cells could be applied to the tight control of cell behavior in intact organisms will be investigated in the near future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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