WD40-repeat proteins control the flow of G\(\beta\gamma\) signaling for directional cell migration

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The ability of cells to generate a highly polarized intracellular signal through G protein-coupled receptors (GPCRs) is essential for their migration toward chemoattractants. The G\(\beta\gamma\) subunits of heterotrimeric G proteins play a critical role in transmitting chemotactic signals from GPCRs via the activation of diverse effectors, including PLC\(\beta\) and PI3K, primarily at the leading edge of cells. Although G\(\beta\gamma\) can directly activate many of these effectors through protein-protein interactions in vitro, it remains unclear how G\(\beta\gamma\) spatially and temporally orchestrates the activation of these effectors in vivo. A yeast two-hybrid screen for G\(\beta\) interacting proteins identified two WD40-repeat domain containing proteins, RACK1 and WDR26, which are predicted to serve as scaffolding/adaptor proteins. Previous data indicates that RACK1 negatively regulates G\(\beta\gamma\)-mediated leukocyte migration by inhibiting G\(\beta\gamma\)-stimulated PLC\(\beta\) and PI3K activities. In contrast, recently published work by Sun et al. indicates that WDR26 promotes leukocyte migration by enhancing G\(\beta\gamma\)-mediated signal transduction. These findings reveal a novel mechanism regulating G\(\beta\gamma\) signaling during chemotaxis, namely through the positive and negative regulation of WDR26 and RACK1 on G\(\beta\gamma\) to promote and fine tune G\(\beta\gamma\)-mediated effector activation, ultimately governing the ability of cells to polarize and migrate toward a chemoattractant gradient.

Directed cell migration along chemoattractant gradients, termed chemotaxis, plays a crucial role in normal physiological and pathological processes. Chemotaxis is required for embryonic development, wound healing, immune surveillance and immune response. However, excessive or dysregulated chemotaxis promotes disease, including inflammatory disorders such as asthma and rheumatoid arthritis, formation of arterial plaques during atherosclerosis and cancer metastasis. Thus, understanding chemotaxis has important ramifications for both physiology and pathology, and has been the subject of intense study.

Motile cells have an amazing ability to navigate effectively in the direction of a very shallow chemoattractant gradient, with as small as 1–3% concentration difference between either end of the cell. This has been attributed to their ability to translate the shallow extracellular gradient into a much steeper intracellular gradient, which acts as an internal “compass” to steer cell migration. Recent studies from model systems such as Dictyostelium amoeba and neutrophils have begun to uncover the underlying molecular mechanisms responsible for this development of an intracellular “compass.” These include spatial and temporal activation of diverse signaling pathways and numerous positive and negative feedback loops acting at the front and back of cells. Nevertheless, despite these advances, many components of the signaling pathways activated by chemoattractants remain to be defined, and the mechanistic details of many feedback regulatory pathways on the chemotactic signal remain to be elucidated.
Chemoattractants act on cell surface receptors to regulate chemotaxis. Among these are G protein-coupled receptors (GPCRs) that transmit chemotactic signals through heterotrimeric G proteins. In leukocytes, chemotaxis is initiated by the binding of a diverse family of small proteins, called chemokines, or the bacterial by-product fMLP to their cognate receptors and the activation of two classes of G proteins, Gi/o and G12/13. Activated Gi/o proteins release free Gβγ to activate diverse effectors that transmit chemotactic signals, ultimately facilitating actin polymerization at the leading edge to drive forward movement. In contrast, activation of G12/13 mediates actomyosin complex formation via a Rho guanine exchange factor (RhoGEF) and RhoA dependent pathway at the posterior of cells to facilitate tail retraction. The Gβγ-mediated signaling mechanisms controlling leading edge formation are highly conserved in many types of motile cells, including Dictyostelium amoeba and leukocytes, although the mechanisms underlying actomyosin formation differ among different cell types.

Following Gβγ activation, an intracellular signal gradient is established that likely acts as an internal “compass” for directing cell polarization and migration. Gβγ-mediated phosphatidylinositol 3-kinase (PI3K) activation was the first pathway found to play a critical role in generating this intracellular signal. PI3K activation releases phosphatidylinositol (3,4,5) phosphate (PIP3), which accumulates asymmetrically at the leading edge of chemotaxing cells. This in turn recruits RhoGEFs such as DOCK2 and αPix, which activate Rac and Cdc42 to promote F-actin polymerization and cell polarization. In recent years, much work has gone into identifying the mechanisms underlying spatially localized activation of PI3Ks. In Dictyostelium and neutrophils, amplification of the PI3 signal is mediated by the reciprocal translocation of PI3K to the leading edge and phosphatases, such as phosphatase and tension homology (PTEN), to the lateral and trailing edge of cells. Moreover, the subsequent activation of Rac by PI3K creates a positive feedback loop which further enhances PI3K activation at the leading edge via F-actin polymerization. Additionally, G12/13-mediated RhoA activation antagonizes Gβγ-mediated PI3K activation at the posterior of neutrophils, reinforcing PI3K activity at the leading edge. In addition to the PI3K pathway, several other pathways, including those mediated by PLCβ2, PLA2, p38 MAPK, pRex1 and mTORC2 have now been shown to act in parallel or in concert with the PI3K pathway to regulate leukocyte migration. These pathways are all activated downstream of Gβγ. However, beside PI3Kγ, PLCβ2/3 and pRex1, which are directly activated by Gβγ, the biochemical nature of the other pathways involved in leukocyte chemotaxis remains poorly defined because the direct binding targets of Gβγ have not been identified.

It is now well established that Gβγ functions as a master switch to transmit chemotactic signals during the migration of various cell types, including Dictyostelium and leukocytes. Nonetheless, it remains unclear how Gβγ orchestrates the activation of diverse effectors to generate the spatiotemporal intracellular signal that directs cell polarization and migration. Studies in vitro have shown that Gβγ can directly activate a number of downstream effectors, including PI3Kγ and PLCβ2/3, by direct protein-protein interactions, but how Gβγ actually regulates these effectors in vivo remains to be resolved. For example, both PI3Kγ and PLCβ2/3 are known to reside in the cytosol of resting cells and are rapidly translocated to the leading edge of chemotaxing cells upon stimulation with chemoattractants. The mechanism regulating the asymmetric accumulation of these proteins at the leading edge remain unclear, but is unlikely due to anchoring through Gβγ because Gβγ and chemoattractant receptors remain largely uniformly distributed in the cell membrane. Thus, there may be other regulatory mechanisms acting upon Gβγ and/or downstream effectors of Gβγ to control PI3Kγ and PLCβ2 activation.

In an attempt to identify novel Gβγ interacting proteins, a yeast two-hybrid screen was performed using Gβ as bait. Interestingly, this screen identified a number of WD40-repeat containing proteins as interacting with Gβγ, including RACK1 (receptor for activated C kinase 1) and WDR26. This finding was intriguing, as Gβγ is considered to be the prototypical WD40 protein, and has never before been shown to interact with other WD40 proteins.

WD40-repeat containing proteins are a highly conserved family of proteins, characterized by the presence of a 40 to 60 repeated amino acid sequence containing Trp-Asp dipeptides, which form a circular β propeller. The WD40 repeat domain does not appear to have any intrinsic enzymatic activity. Rather, the multi-sided propellers may serve as a scaffold to mediate protein-protein interactions. Indeed, Gβ is considered to be a scaffold as it has a long list of interacting proteins involved in diverse signaling networks. Although RACK1 was initially identified as a receptor for activated protein kinase C, it was later shown to be an adaptor/scaffold for a variety of proteins, including enzymes such as Src, FAK and PDE4D5, and membrane proteins such as receptors for NMDA, IP3, insulin-like growth factor 1, thromboxane and type 1 interferon. Not surprisingly, RACK1 was found to be involved in diverse physiological and pathological functions including development, cell migration, circadian rhythm and cancer. Previous studies suggest WDR26 may also function as a scaffold/adaptor as it was identified as one of many WD40 proteins associated with the cullin 4(CUL4)-DNA damage-binding protein 1 (DDB1) ubiquitin E3 ligase complex. However, the exact function of WDR26 remains elusive. Gβγ is known to recruit scaffold proteins such as Ste5p to promote the MAPK signaling cascade for pheromone response in yeast. The findings that RACK1 and WDR26 interact with Gβγ raise the possibility that they may serve as scaffold/adaptor proteins for orchestrating the signaling specificity and efficacy of Gβγ.

Initial studies have characterized the Gβγ-RACK1 interaction and have shown that RACK1 acts as a negative regulator in Gβγ-mediated signal transduction and leukocyte migration. Downregulation of RACK1 by siRNAs enhanced SDF1α- and fMLP-stimulated chemotaxis of Jurkat T cells and differentiated HL60 cells, whereas overexpression of RACK1...
translocate from the cytosol to the membrane and accumulate at the leading edge of leukocytes.\textsuperscript{29,31} Given that activation of PLC\(\beta\) and PI3K by G\(\beta\gamma\) at the leading edge is part of the “compass” mechanisms that generate the internal gradient of signaling molecules required for leukocyte polarization and chemotaxis, the exact role of RACK1 in the formation of the signal gradient at the leading edge is not immediately clear. Nevertheless, the fact that a negative regulator is recruited to the leading edge by G\(\beta\gamma\) raises the intriguing possibility that a positive regulator may be also recruited by G\(\beta\gamma\) to augment its signal during chemotaxis.

The data presented in our recently published paper by Sun et al. suggests that WDR26 may fulfill the role of a positive regulator of G\(\beta\gamma\) signaling during leukocyte migration.\textsuperscript{32} Similar to RACK1, WDR26 was found to be rapidly translocated to the leading edge of leukocytes upon stimulation with chemoattractants. However, unlike RACK1, knockdown of WDR26 resulted in decreased leukocyte migration in vitro and homing to lymphoid tissues in vivo. By monitoring the migratory behaviors of dHL60 cells deficient in WDR26, it was found that suppression of WDR26 led to reduced cell migration speed and loss of directionality. In contrast to RACK1 inhibition, downregulation of WDR26 by siRNAs alleviated PLC\(\beta\)-mediated calcium mobilization and PI3K-stimulated AKT phosphorylation in both Jurkat T cells and dHL60 cells. The effect of WDR26 was likely mediated by its binding to G\(\beta\gamma\) because overexpression of a WDR26 fragment that mediates G\(\beta\gamma\) binding recapitulated the effect of WDR26 knockdown on G\(\beta\gamma\) signaling and cell migration. Together, these data indicate that WDR26 positively regulates G\(\beta\gamma\) signaling during leukocyte migration.

The molecular mechanisms by which WDR26 facilitates G\(\beta\gamma\) signaling are not yet clear. Our unpublished data indicate that in addition to binding G\(\beta\gamma\), WDR26 also directly interacts with select G\(\beta\gamma\) effectors such as PLC\(\beta\)2. Moreover, WDR26 is able to assemble a signaling complex containing both G\(\beta\gamma\) and PLC\(\beta\)2 and promote PLC\(\beta\)2 activation by G\(\beta\gamma\), both in vitro and in vivo. Additionally, WDR26 is required for membrane translocation and accumulation of PLC\(\beta\)2 at the leading edge of polarized neutrophils induced by chemoattractant stimulation. Together, these findings indicate that WDR26 functions as a scaffolding protein to promote membrane translocation and activation of G\(\beta\gamma\) effectors.

Our data provides a number of new insights into both our understanding of leukocyte migration and the regulation of signaling downstream of G\(\beta\gamma\). The novel regulation of G\(\beta\gamma\) by WDR26 and RACK1 may explain why, despite global activation of GPCRs and the heterotrimeric G protein at the periphery of the cell, activation of G\(\beta\gamma\) effectors selectively occurs at the leading edge.\textsuperscript{31} The rapid translocation of WDR26 to the leading edge after cell stimulation likely facilitates translocation and activation of select G\(\beta\gamma\) effectors to promote leading edge formation (Fig. 1). The recruitment of RACK1 may act in a feedback loop to restrict or fine tune the activation of such effectors, to efficiently allow for gradient formation and subsequent directed migration. Consistent with this hypothesis, co-immunoprecipitation studies showed that upon chemoattractant stimulation, WDR26 rapidly forms a complex with G\(\beta\gamma\) (within five minutes of stimulation), whereas the interaction of RACK1 with G\(\beta\gamma\) was delayed, occurring nearly thirty minutes after stimulation.\textsuperscript{30,32} Moreover, downregulation of WDR26 blocked the interaction of RACK1 with G\(\beta\gamma\) and its ability to negatively regulate G\(\beta\gamma\)-mediated cell migration, indicating that RACK1 functions downstream of WDR26.\textsuperscript{32}

Despite our initial findings on the importance of WDR26 and RACK1 in regulating leukocyte migration, a number of key questions remain unanswered. For example, it remains unknown what causes the membrane translocation and accumulation of WDR26 and RACK1 at the leading edge. Given that G\(\beta\gamma\) forms a shallow gradient from leading to trailing edge in a polarized leukocyte, direct binding with G\(\beta\gamma\) is unlikely to be sufficient for the accumulation of WDR26 and RACK1 at the leading edge. Nevertheless, our findings that pretreatment of cells with pertussis toxin to block Gi/o activation abolished WDR26 and RACK1 inhibited cell migration.\textsuperscript{31} The effect of RACK1 on chemotaxis may be due to its regulation of G\(\beta\gamma\) signaling, because SDF1\(\alpha\)- and fMLP-stimulated PLC\(\beta\) and PI3K activities were significantly augmented by RACK1 downregulation, whereas RACK1 overexpression had the opposite effect. Importantly, overexpression of a fragment of RACK1 that retained the G\(\beta\gamma\)-binding mimicked the inhibitory effect of the full-length RACK1 on G\(\beta\gamma\) signaling and leukocyte chemotaxis.\textsuperscript{31} In vitro studies with purified proteins showed that RACK1 can directly inhibit G\(\beta\gamma\)-mediated PLC\(\beta\) and PI3K activation.\textsuperscript{30,31} This inhibition was probably a result of competitive binding of RACK1 with PLC\(\beta\) and PI3K for a common binding site located on the side-surface of G\(\beta\gamma\).\textsuperscript{30,31}

Intriguingly, following stimulation by chemoattractants, RACK1 was found to
translocation suggest that active Gαi/o and/or Gβγ signaling is required for translocation. However, it remains to be delineated what downstream signaling pathways of Gβγ dictates translocation, and whether active Gαi/o signaling contributes to translocation.

Moreover, we do not know how WDR26 and RACK1 work in conjunction to balance the activation and inhibition of Gβγ signaling. Our data suggests that the interaction of RACK1 with Gβγ is subsequent to WDR26 binding to Gβγ, but we do not know about the dynamics of their interactions, nor do we know what regulates their alternate interactions with Gβγ. Based on our co-immunoprecipitation data, we find that WDR26 and RACK1 interact with Gβγ within 5 and 30 min of stimulation, respectively. However, the slow dynamics of complex formation likely reflects the lack of sensitivity of the assay rather than the actual dynamics in vivo, because membrane translocation and co-localization of WDR26 and RACK1 with Gβγ can be detected within one minute of stimulation, as revealed by immunofluorescence staining. More sensitive assays such as fluorescence resonance energy transfer (FRET) based approaches are required to further discern the exact dynamics of their interactions.

In addition to PI3K and PLCβ, additional Gβγ effectors may contribute to leukocyte migration, including PLA2, p38 MAPK, pREX1, Radil and mTORC2.15-20 It remains to be seen if WDR26 and RACK1 influence Gβγ-mediated activation of these effectors, and whether this regulation contributes to directional migration in leukocytes.

Finally, the molecular basis for the different functions of WDR26 and RACK1 in regulating Gβγ signaling is not clear. Crystal structure determination has shown that like Gβ, RACK1 adopts a seven-bladed β-propeller structure.30 However, it has a short N-terminus (~10 aa) and does not require a GY-like protein to stabilize its structure.33 WDR26 shares little sequence homology with either RACK1 or Gβ, and is a much larger protein (~75 kDa vs. 36 kDa for RACK1 or Gβ). Based on sequence alignments with Gβ and other WD40 repeat proteins, WDR26 is predicted to contain six to seven WD40 repeat motifs at its C-terminus. However, it has an extensive N-terminus (~352 aa) that contains two distinguishable domains, Lis-homology (LisH) and C-terminal to LisH (CTLH) domains, which are implicated in protein-protein interactions and protein dimerization. Our studies have shown that RACK1 and WDR26 have distinct binding sites on Gβγ. While the RACK1-binding sites are located on one side-surface of Gβγ that has little overlapping with the Gα-contact surface, the WDR26-binding sites are contained in the region of Gβγ that interacts with Gα.30,32 Intriguingly, although the Gα-contact surface of Gβγ is required for the activation of many Gβγ-effectors, the binding of WDR26 to Gβγ enhances rather than inhibits effector activation. The exact mechanisms for the action of WDR26 is not yet clear, but may be related to its ability to interact with both Gβγ and its effectors. By forming a complex with Gβγ and its effectors, WDR26 may force Gβγ to use alternative residues for more efficient activation of its effectors. Such a mechanism has been reported for one Gβγ-interacting protein, AGS8, which directs Gβγ to activate PLCβ2 through alternative activation sites.34 Alternatively, WDR26 may interact sequentially with Gβγ and its effectors to bring them in close proximity, thereby facilitating more efficient activation. Gβγ has been found to bind to the N-terminal domain of RACK1 that comprise the first two WD40 repeats, whereas the binding sites of Gβγ on WDR26 have been mapped to a much more extensive region that are located in the C-terminal fragment of WDR26 between the LisH and the WD40 domains. Further work will be needed to discern the precise sites of WDR26 and RACK1 interactions with Gβγ and identify the structural determinants that are responsible for the different function of WDR26 and RACK1 in regulating Gβγ signaling.

In addition to regulating leukocyte migration, recent work has shown that Gβγ also plays a critical role in transmitting signals from multiple GPCRs to promote tumor growth and metastasis.35,36 Conceivably, aberrant regulation of Gβγ signaling by WDR26 and RACK1 may contribute to tumor progression. Indeed, our preliminary data show that WDR26 is overexpressed in human breast cancer tissue, and contributes to Gβγ-mediated tumor cell growth and migration. The role of RACK1 in promoting or suppressing tumor growth and metastasis has also been reported, but its functions in the context of Gβγ-mediated tumor progression remain unknown.37,38 Future work will be important to determine how altered expression of WDR26 and RACK1 results in aberrant Gβγ signaling and how this in turn contributes to various diseases.

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