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COMMENTARIES

Leukocyte adhesion and polarization: Role of glycosylphosphatidylinositol-anchored proteins

Dion D Richardson and Mar Fernandez-Borja*

Deptartment of Molecular Cell Biology; Sanquin Research and Landsteiner Laboratory; University of Amsterdam; Amsterdam, Netherlands

ABSTRACT. Leukocyte traffic out of the blood stream is crucial for an adequate immune response. Leukocyte extravasation is critically dependent on the binding of leukocyte integrins to their endothelial counterreceptors. This interaction enables the firm adhesion of leukocytes to the luminal side of the vascular wall and allows for leukocyte polarization, crawling and diapedesis. Leukocyte adhesion, polarization and migration requires the orchestrated regulation of integrin adhesion/de-adhesion dynamics and actin cytoskeleton rearrangements. Adhesion strength depends on conformational changes of integrin molecules (affinity) as well as the number of integrin molecules engaged at adhesion sites (valency). These two processes can be independently regulated and several molecules modulate either one or both processes. Cholesterol-rich membrane domains (lipid rafts) participate in integrin regulation and play an important role in leukocyte adhesion, polarization and motility. In particular, lipid raft-resident glycosyl-phosphatidyl-inositol-anchored proteins (GPI-APs) have been reported to regulate leukocyte adhesion, polarization and motility in both integrin-dependent and independent manners. Here, we present our recent discovery concerning the novel role of the GPI-AP prion protein (PrP) in the regulation of β1 integrin-mediated monocyte adhesion, migration and shape polarization in the context of existing literature on GPI-AP-dependent regulation of integrins.

KEYWORDS. GPI-anchored protein, lipid raft, ERM, leukocyte, integrin, uropod, polarization, migration, diapedesis

*Correspondence to: Mar Fernandez-Borja; Email: m.fernandez@sanquin.nl
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INTRODUCTION

In order to fulfill their effector and patrolling functions, leukocytes traffic through the body and need to adapt to the adhesive properties of the different tissue environments. First, differentiated leukocytes egress the bone marrow into the vascular system. Later, circulating leukocytes will exit the vasculature and penetrate into the tissues, either for patrolling in search for pathogens (i.e. resident monocytes, T cells) or to eliminate infection and activate the adaptive immune response (i.e., inflammatory monocytes, neutrophils).[1,2] Leukocyte extravasation depends on the presence of chemoattractants on the luminal side of the vascular endothelium. At first, leukocytes contact the endothelium by establishing weak and transient selectin-dependent interactions, which lead to leukocyte capture and rolling. Binding of chemoattractants to their receptors on leukocytes triggers signaling that leads to the activation of leukocyte $\beta1$ and $\beta2$ integrins and their subsequent interaction with their endothelial counter-receptors VCAM-1 and ICAM-1, respectively. These interactions enable firm adhesion, polarization and crawling of leukocytes on the vascular wall and lead to leukocyte diapedesis across the endothelial layer.[3]

Arrested leukocytes rapidly change shape and initiate crawling on the endothelial surface. They become flattened and develop a leading edge rich in lamellipodial and finger-like protrusions, and a trailing edge with a single membrane protrusion, the uropod (**Fig. 1**). This transformation is initiated by integrin-mediated adhesion and subsequent polarization of the actin and microtubule cytoskeletons, leading to the asymmetric distribution of membrane lipids and proteins as well as cytosolic proteins into either the leading edge or the uropod. Shape and function are closely linked in leukocyte front-rear polarity. Chemokine sensing takes place at the leading edge, where chemokine receptors are located, whereas the uropod concentrates a plethora of adhesion receptors, including integrins, CD44, CD43, ICAMs and PSGL-1, and has primarily an adhesive function both to the substratum and to other cells.[4,5] Similarly, the composition of lipid rafts differs between front and rear: leading edge rafts contain the ganglioside GM3, whereas the ganglioside GM1 and the raft scaffolding proteins flotillin-1 and -2 segregate into uropod rafts.[6]

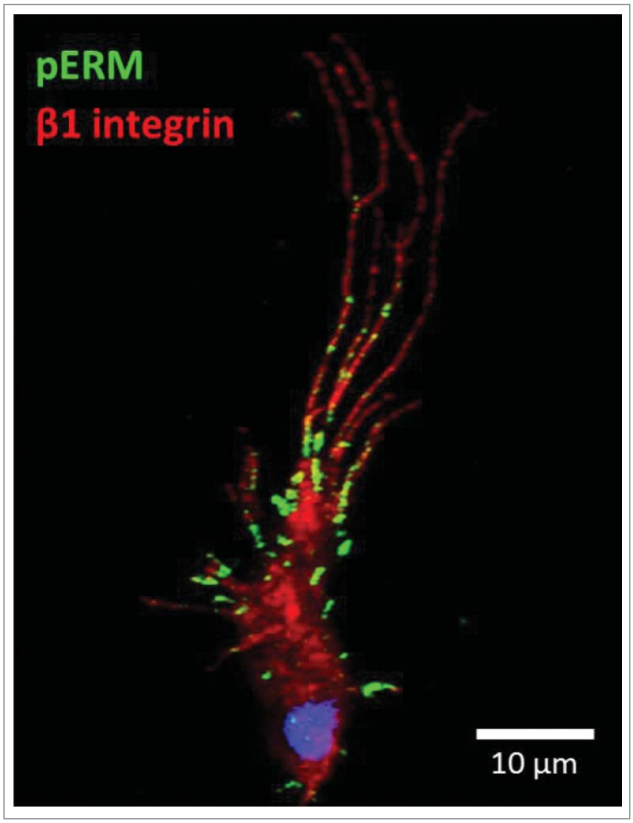
Leukocytes are a striking example on the functional relationship between cell shape and cell behavior: from round cells in the circulation to a flattened polarized shape when crawling on the luminal side of blood vessels. Basically, effective migration depends on the coordinated regulation of integrin adhesion/ de-adhesion dynamics, the polarized distribution of lipids and proteins, the reorganization of the cytoskeleton and the linkage between plasma membrane and cytoskeleton.

GPI-APs and integrin adhesion

Integrins are heterodimeric complexes formed by one α and one β subunit. Primarily, leukocytes express $\alpha L\beta2$ and $\alpha M\beta2$ heterodimers (LFA-1 and Mac-1, respectively), as well as $\alpha4\beta1$ and $\alpha5\beta1$ heterodimers (VLA-4 and VLA-5, respectively). These integrins are fundamental for leukocyte anchorage to the vascular wall as well as for leukocyte motility where they mediate force transduction between the substrate and the cytoskeleton enabling cell displacement.

Integrin adhesiveness is regulated by changes in integrin affinity and/or valency. Integrin affinity is induced by a conformational change that consists in the extension of the extracellular domains of $\alpha\beta$ integrin heterodimers.[7,8] This conformational switch is induced by inside-out signaling triggered by chemokine receptors leading to the binding of talin and kindlin-3 to the β chain cytosolic domain.[9] Valency refers to the extent of multivalent binding to ligand. Increases in valency are induced by (polyvalent) ligand binding and depend on the lateral diffusion of integrins and subsequent microclustering.[7,10,11] Detachment of integrins from the actin cytoskeleton is required for integrin lateral mobility, enabling ligand-induced clustering and adhesion reinforcement.[10,11] Although integrin mobility is important for the onset of leukocyte adhesion, integrins must re-attach to the cytoskeleton to

FIGURE 1. Monocyte polarization on the b1 integrin ligand VCAM-1. Migrating human monocytic U937 leukocytes plated on immobilized VCAM-1 and CXCL12 acquire a polarized shape consisting of a leading edge followed by the cell body and a rear end formed by the uropod, where b1 integrins and phosphorylated ERM proteins accumulate.[20] (Color figure available online.)



ensure strong cell adhesion to the substrate. This could be achieved by the recruitment of activated integrins to specialized domains of the plasma membrane where stable cytoskeletal anchorage takes place. Consistent with this notion, integrin clustering at lipid raft domains has been shown to be important for integrin-mediated adhesion.[12,13] Lipid rafts are cholesterol- and sphingolipid-rich membrane nanodomains that harbor a specific set of proteins, both transmembrane and glycosyl-phosphatidyl-inositol-anchored proteins (GPI-APs). Also specific proteins associate to the cytoplasmic leaflet of lipid rafts, including Src family kinases and the scaffolding proteins caveolin and flotillin.[14] Lipid raft connection to the cytoskeleton has a prominent role in both domain formation and signaling, and takes place through several membrane-actin linkers such as filamin, annexins and ERM (ezrin-radixin-moesin) proteins.[15] In line with this, GPI-APs have been found to form nanoclusters anchored to the actin cytoskeleton in steady-state conditions.[16] These nanoclusters could function as preformed platforms for the cytoskeletal linkage, clustering and immobilization of integrins moving in by lateral diffusion. In support of this idea, Garcia-Parajo and colleagues[13] have shown that nanoclusters of β2 integrins (LFA-1) on the membrane of quiescent monocytes reside close to GPI-AP nanodomains. 'Outside-in' activation of LFA-1 by ICAM-1 binding induces the 'fusion' of GPI-AP nanodomains with LFA-1 nanoclusters and the further recruitment of mobile integrins, which results in the formation of integrin microclusters and adhesion strengthening[13]. In line with these data, there is evidence of a functional

association between specific GPI-APs and integrin-mediated adhesion.[17-20] In particular, the GPI-APs uPAR, CD157 and PrP (prion protein) have been found to have a role in the regulation of leukocyte adhesion and migration *in vivo* and *in vitro*.[19-23] These GPI-APs can establish lateral associations with integrins: uPAR interacts with β2 integrins in monocytes and with β1 and β3 integrins in adherent cells[24-26] and CD157 associates to β1 and β2 integrins in monocytes.[19] A physical association between PrP and β1 integrins has so far only been reported after the proteomic analysis of the PrP interactome in neuroblastoma cells.[27] Antibody-mediated ligation of CD157 promotes the clustering of β1 and β2 integrins into lipid raft domains and leads to increased activity of signaling pathways that control monocyte transendothelial migration.[19,28] Similarly, PrP was found to colocalize with β1 integrins on membrane caps in polarized monocytic cells and to regulate β1 integrin-mediated monocyte adhesion to fibronectin and VCAM-1.[20] PrP-deficient monocytes display a reduced ability to bind polyvalent VCAM-1, without significant alterations in β1 integrin affinity, suggesting that PrP silencing reduces the valency of integrin adhesion. In agreement with the fact that increases in integrin valency are important to resist blood flow shear stress forces, PrP-deficient monocytes displayed reduced firm adhesion to the endothelium under shear flow conditions.[20] These data suggest that, similarly to other GPI-APs, PrP may contribute to integrin microclustering. However, no direct interaction between PrP and β1 could be demonstrated by conventional biochemical methods, arguing that PrP may modulate integrin adhesion by alternative pathways. Interestingly, a functional association between PrP and β1 integrins has also been identified in neuronal cells, where PrP was proposed to regulate integrin signaling through the regulation of the production of the β1 ligand fibronectin.[29] Excessive extracellular deposition of fibronectin by PrP-deficient neurons was proposed to enhance β1 integrin clustering and signaling, leading to reduced focal adhesion turnover and defective neuritogenesis.[29] However, this mechanism cannot explain the effects of PrP silencing in monocytes since these cells do not express fibronectin (at least not in detectable amounts). Interestingly though, both studies show increased levels of polymerized actin in PrP-deficient neurons and monocytes as well as enhanced activation of the RhoA-cofilin pathway leading to cofilin inactivation. Cofilin is crucial for the spatiotemporal regulation of actin dynamics. It induces actin filament depolymerization and severing, which provides free barbed ends for further actin polymerization at the leading edge and it is hence essential for leukocyte migration.[30,31] Cofilin activity is controlled by an inactivating phosphorylation at Ser3 by LIMK downstream of RhoA.[30] It is therefore possible that altered actin dynamics in PrP-deficient cells reduces the lateral mobility and clustering of integrins in leukocytes as well as focal adhesion turnover in adherent cells. Interestingly, uPAR induces cofilin phosphorylation in prostate cancer cells.[32] Collectively, these studies suggest that cofilin plays a role in the regulation of actin dynamics by GPI-APs.

In addition to changes in cytoskeletal dynamics, PrP silenced monocytes contain lower levels of active ERM proteins.[20] ERM proteins are cytoplasmic proteins recruited to the cytoplasmic domain of surface adhesion receptors and link them to the actin cytoskeleton. For this to occur, ERM proteins need to be activated by phosphorylation of a C-terminal threonine residue leading to the disruption of intramolecular interactions.[33] ERM proteins have been involved in leukocyte adhesion and migration: ERM protein silencing impairs lymphocyte adhesion to the vascular endothelium,[34] whereas the expression of constitutively active ezrin reduces T cell diapedesis, possibly through increasing membrane rigidity and tension.[35]

In addition to their regulation of integrins, GPI-APs can induce cell adhesion by means of their binding to different extracellular matrix proteins including vitronectin (uPAR and PrP), fibronectin (CD157) and laminin (PrP).[36-39] GPI-AP binding to these matrix ligands is sufficient to induce cell adhesion and spreading independently of integrin engagement.[38,40,41] Recently, a mechanism was described for the

uPAR-dependent, integrin-independent cell adhesion in which binding of uPAR to vitronectin increases membrane tension leading to ligand-independent integrin signaling and cell spreading in the absence of uPAR/integrin interactions.[40] However, it is unlikely that this is the only mechanism underlying adhesion regulation by GPI-APs since the specific matrix protein ligand is absent in *in vitro* assays of leukocyte adhesion to immobilized VCAM-1 and ICAM-1.

GPI-APs in leukocyte migration and uropod formation

Uropod formation requires uropod-substrate adhesion as well as the forward pulling of the leading edge[42] and the absence of any of these 2 events prevents shape polarization. In contrast, excessive integrin-mediated adhesion at the uropod induces the formation of long tails and hampers cell motility.[43,44] Consistent with their role as adhesion

FIGURE 2. PrP silencing prevents shape polarization of monocytic cells following arrest on the endothelium. Snapshots extracted from time-lapse movies showing U937 cell adhesion to and transmigration across endothelial monolayers (TNFa-activated and coated with CXCL12) under laminar flow conditions. After U937 cell arrest on the endothelial surface (t = 3 min), control cells polarize into a leading edge (a) and a uropod (b) (t = 6 min) before transmigrating through the endothelial monolayer. In contrast, PrP-deficient cells fail to acquire a flattened polarized shape and remain round (t = 6 min). However, both control and PrP-deficient cells are able to diapedese between two adjacent endothelial cells (t = 9 min) suggesting that shape polarization is not essential for leukocyte transmigration.[20] (Time points correspond to the time lapsed from the perfusion of monocytes over the endothelial monolayer) (Color figure available online.)

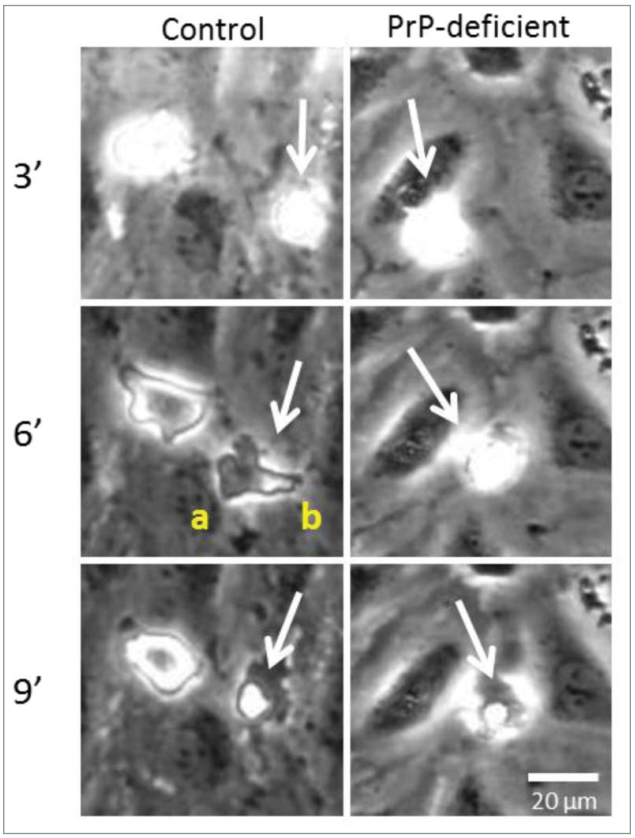
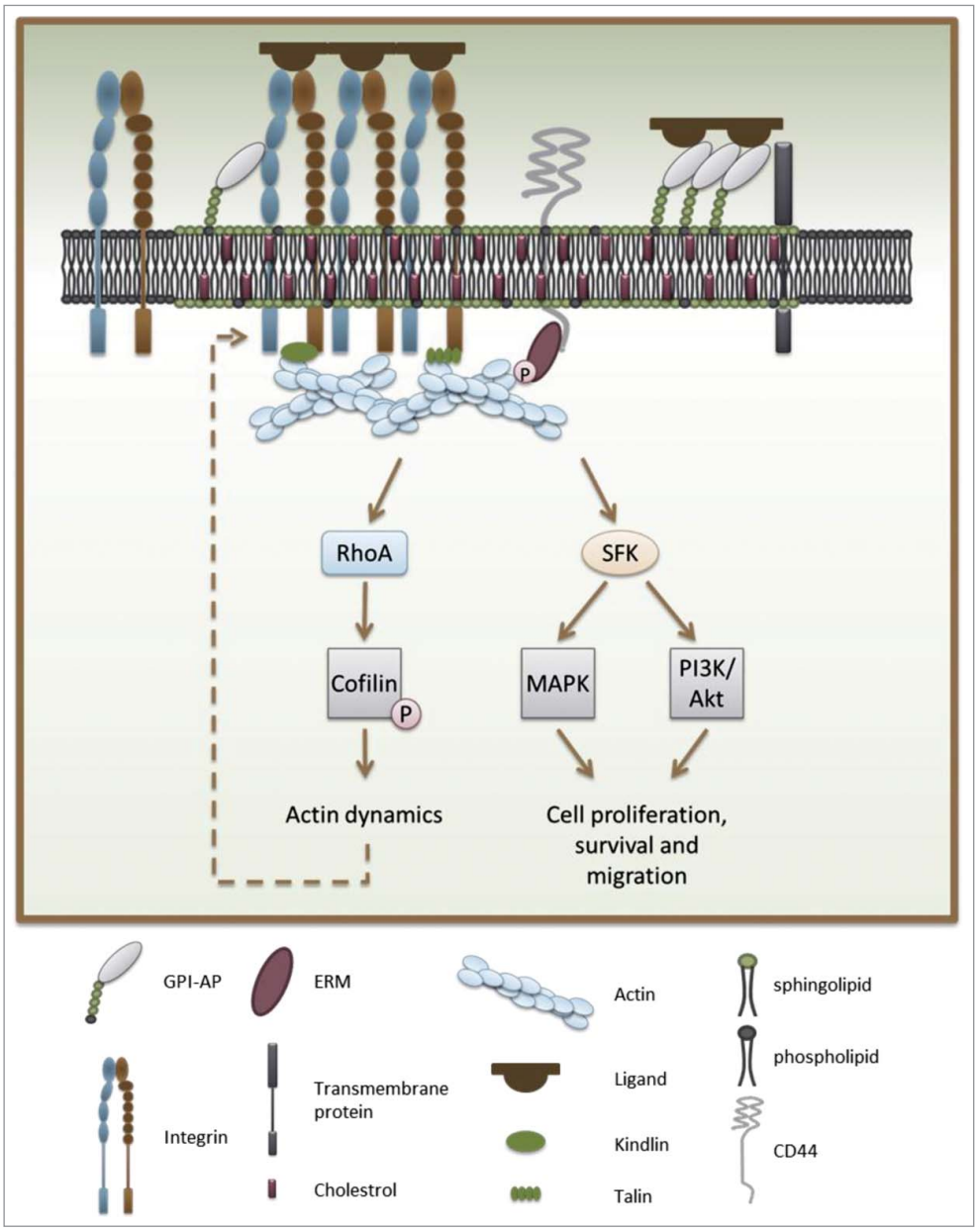


FIGURE 3. GPI-APs in integrin activation and signaling at the uropod. GPI-APs co-cluster with activated integrins following integrin activation and/or ligand binding as well as after antibody-mediated GPI-AP ligation. CD157 and the prion protein (PrP) are two GPI-APs that localize to the uropod of polarized leukocytes and regulate integrin-mediated adhesion and intracellular signaling. These effects might be mediated through lateral associations of GPI-APs with integrins, by which GPI-APs may promote integrin activation and/or recruitment to lipid rafts. Some of the pathways known to be regulated by PrP and/or CD157 include Src-family kinases (SFK), mitogen activated protein kinases (MAPK) and the survival kinase Akt. In addition, PrP regulates actin dynamics by the RhoA-cofilin pathway. Since GPI-APs lack cytoplasmic domains, it has been postulated that they transmit signals by lateral association with transmembrane proteins. In the case of integrin-mediated adhesion, it is unclear whether GPI-AP-dependent signaling is downstream or upstream of integrin activation/clustering. A possibility is that GPI-AP regulate RhoGTPases by integrin-independent pathways to induce local changes in actin dynamics that impact on integrin activation and microclustering. (Color figure available online.)



modulators, GPI-APs also regulate leukocyte shape polarization and motility.[18,20,28,45,46] Polarized leukocytes show an asymmetrical distribution of GPI-APs: uPAR localizes to the leading edge whereas Thy-1, PrP and CD157 colocalize with the uropod markers GM1 and/or flotillin.[6,19,46,47] Antibody-mediated ligation of CD157 induces uropod formation.[28] Conversely, PrP silencing impairs uropod formation in 2D migrating monocytic cells, both on immobilized VCAM-1 and on the surface of endothelial cells[20] (**Fig. 2**). Consistent with an anchoring role of the uropod in leukocytes moving on 2D surfaces as well as during transendothelial migration,[4,42] PrP-deficient cells lacking a uropod migrate faster on VCAM-1 and show increased diapedesis across endothelial monolayers.[20]

How does PrP regulate uropod formation? As explained above, PrP silencing reduces β1 integrin adhesion without significantly decreasing β1 integrin affinity, suggesting that PrP may regulate uropod adhesiveness through integrin valency changes. In support of this idea, Morin et al[48] have shown that uropod adhesion depends on integrin valency, whereas leading edge advance is mediated by increases in integrin affinity. In addition, regulation of ERM protein phosphorylation by PrP could also contribute to uropod formation in leukocytes. Active phosphorylated ERM proteins localize to the rear of migrating leukocytes where they associate to the cytoplasmic tail of several adhesion receptors (i.e. CD44, ICAM3 and PSGL1)[5] and have a critical role in uropod formation.[35,49,50] Silencing of ezrin and moesin abolished uropod extension by T cells migrating on VCAM-1 but not on ICAM-1, suggesting that the role of ERM in uropod formation is dependent on β1 integrin adhesion.[34] It is therefore possible that decreased ERM protein phosphorylation contributes to defective β1 integrin adhesion to the substrate explaining the defects in uropod formation observed in PrP-silenced cells.[20]

In conclusion, GPI-APs are involved in the regulation of several pathways controlling leukocyte adhesion and migration, including integrin activation and signaling as well as actin cytoskeleton dynamics (**Fig. 3**). However, the molecular details of the mechanism involved in the control of integrin adhesion by GPI-APs still need to be elucidated.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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