Small GTPase-Dependent Regulation of Leukocyte-Endothelial Interactions in Inflammation

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Abstract

Inflammation is a complex biological response that serves to protect the body's tissues following harmful stimuli such as infection, irritation or injury and initiates tissue repair. At the start of an inflammatory response, pro-inflammatory mediators induce changes in the endothelial lining of the blood vessels and in leukocytes. This results in increased vascular permeability and increased expression of adhesion proteins, and promotes adhesion of leukocytes, especially neutrophils to the endothelium. Adhesion is a prerequisite for neutrophil extravasation and chemoattractant-stimulated recruitment to inflammatory sites, where neutrophils phagocytose and kill microbes, release inflammatory mediators and cross-talk with other immune cells to coordinate the immune response in preparation for tissue repair. Many signalling proteins are critically involved in the complex signalling processes that underpin the inflammatory response and cross-talk between endothelium and leukocytes. As key regulators of cell-cell and cell-substratum adhesion, small GTPases act as important controls of neutrophil-endothelial cell interactions as well as neutrophil recruitment to sites of inflammation. Here we summarise key processes that are dependent upon small GTPases in leukocytes during these early inflammatory events. We place a particular focus on the regulation of integrin-dependent events and their control by Rho and Rap family GTPases as well as their regulators during neutrophil adhesion, chemotaxis and recruitment.

List of abbreviations

GTP – guanosine trisphosphate

GTPase – guanosine trisphosphatase

TNF-α - tumour necrosis factor α

EC – endothelial cell

TEM – transendothelial migration

GEF – guanine nucleotide exchange factor

GAP – GTPase activating protein

GDI – GDP dissociation inhibitor

PIP3 - phosphatidylinositol-(3,4,5)-trisphosphate

PI3K – phosphoinositide 3-kinase

LAD - leukocyte adhesion deficiency

Inflammation as an innate, immuno-vascular response

Classical inflammation is a complex immuno-vascular response that is triggered by harmful stimuli such as pathogens, tissue injury or irritants. Inflammation precedes and sets the foundation for healing. The induction of inflammation is triggered by the release of vasoactive pro-inflammatory mediators. These mediators trigger rapid vascular changes, including vessel dilation, decreased blood flow and increased vascular permeability [1, 2]. The extent of these events is dependent upon the vascular bed, with e.g. high permeability in post-capillary venules and very little in brain vessels. These vascular changes permit blood plasma and antimicrobial proteins, such as complement factors and antibodies contained within it to enter the surrounding tissue, leading to oedema in a process that is referred to as 'vascular leakage' (Fig 1).

Pro-inflammatory mediators also activate circulating leukocytes. In the interest of space we will focus this discussion on the neutrophil, a particularly important innate immune cell in early inflammation [3, 4]. Slowed blood flow leads to leukocyte margination, initiating close mechanical contact with endothelial cells (ECs). Meanwhile, endothelial exposure to pro-inflammatory cytokines induces increased display of adhesion molecules and their ligands on the luminal side of the vessel and on the neutrophil (Fig 1), enabling the leukocyte adhesion cascade (reviewed in [5]). In a selectin and integrin-mediated process, leukocytes develop increasingly strong and long-lived interactions with endothelial cells. Individual stages include selectin-mediated tethering and rolling, selectin and integrin-mediated slow rolling and crawling, until integrin-mediated firm adhesion of the leukocyte. The process culminates in integrin-dependent diapedesis (extravasation; Fig 1). Neutrophils breach several barriers, the endothelial cells, basement membrane and pericytes. Of these, neutrophil transendothelial migration (TEM) is most studied. TEM can occur by two routes, through endothelial

junctions (paracellular) or through EC bodies (transcellular), with paracellular TEM observed most frequently in areas with weak endothelial junctions [6]. Intravital imaging has indicated that leukocytes scan the endothelium and the underlying pericytes for transmigration sites, which are used repeatedly [7].

Many of the seminal findings in leukocyte-EC interactions were made by studying leukocyte adhesion in flow chambers *in vitro*, as well as by intravital imaging of post-capillary venules in the cremaster muscle, a site that is amenable to exteriorization and intravital imaging. With the advent of major advances in intravital imaging, other, less accessible vascular beds are also being analysed, e.g. lung and liver. A growing body of work indicates that specialisation of individual vascular beds dictates their requirement for individual adhesion proteins on both leukocyte and EC and for the occurrence of individual steps of the cascade (reviewed in [8]). For example, neutrophil recruitment in ICAM-1/P-selectin doubly deficient mice is affected in the peritoneum, but not in the lungs [9]. The current thinking is therefore that leukocyte-EC interactions are not uniform across all sites in the body.

Small GTPases

The Ras superfamily of small GTPases comprises several families, including Ras, Rho, Arf and Rab. Put very simply, Rap GTPases, which are part the Ras family, regulate cell-cell and cell-substratum interactions, Rho GTPases are most famous for regulating dynamic actin rearrangements, whilst Arf and Rab small GTPases regulate intracellular transport. They are all required for complex cellular processes such as single/collective cell migration that are dependent on the actin cytoskeleton and the dynamic generation and dissolution of adhesive contacts [10-12]. As such small GTPases are key regulators of the neutrophil, a highly

specialised cell that is able to change rapidly between circulating in the blood stream, adhering to the vessel wall under conditions of blood flow and migrating through tissue.

Small GTPases cycle between an active, GTP-bound and an inactive GDP-bound state (Fig 2). Small GTPases rely on their regulators, GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and GDP dissociation inhibitors (GDIs) [13]. GAPs increase the endogenous GTPase activity of the GTPase, inactivating it. In contrast, GEFs catalyse the exchange of GDP for GTP, thereby moving the small GTPase into the active state. Finally, GDIs sequester a subset of mostly Rho and Rab family small GTPases in the cytosol by shielding their lipid modification and protecting them from being activated. The number of Rho and Rap GEFs and GAPs outweighs that of the small GTPases themselves. These regulators fine-tune the precise timing and location of the GTPase activity. Each GEF/GAP is itself activated only under specific circumstances using defined upstream regulators at the right time and place [13]. Modes of regulation of small GTPases and their regulators are diverse, and can depend amongst other mechanisms, on the formation of protein complexes, phosphorylation events and second messengers, such as the phosphoinositide 3-kinase (PI3K) lipid product phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) [14].

Small GTPases and neutrophils

Neutrophil biology and their regulation in health and disease have been the subject of several excellent recent reviews (e.g. [3, 4]). Neutrophils are terminally differentiated, short-lived abundant circulating innate immune cells. These highly specialised leukocytes chemotax (move towards a chemoattractant) to sites of inflammation with exquisite speed and directionality. They efficiently and quickly phagocytose opsonised and non-opsonised

bacteria and yeasts, and produce reactive oxygen species and degranulate (releasing a range of potent proteases and cytotoxic compounds that are stored in their specialised granules) to kill the ingested microbes inside the phagosome. These dynamic functions are all subject to regulation by small GTPases (Table 1 and [15-18]). In the following we concentrate on the function of Rho and Rap small GTPases in leukocyte-endothelial cell interactions.

Integrins

Integrins are extracellular receptors comprised of two chains (α/β) that are expressed by all nucleated cells [19]. Using their extracellular domains, integrin bind to their ligands. Whereas integrin ligands are normally extracellular matrix proteins, ligands of leukocyte integrins are often expressed on the surface of other cells, e.g. endothelial ICAMs and VCAMs. Integrin intracellular tails form dynamic links to the cellular actin cytoskeleton. In this way integrins mediate cell adhesion to anchor cells and to partake in dynamic cellular functions requiring such contacts, such as cell migration.

Integrins signal bidirectionally [19] with ligand binding-induced signalling referred to as 'outside-in signalling', whereas 'inside-out signalling', refers to intracellular signalling that regulates integrin activation. This allows inactive integrins in their bent conformation to adopt intermediate and finally the active (extended) conformations (see Fig 3 for a simplified drawing). Mechanistically, interactions with intracellular activators, notably talin and kindlin, convert the inactive integrin to the active conformation [20]. Integrin ligand binding activity is furthermore regulated by clustering, which promotes ligand binding avidity. The regulation of the integrin ligand binding avidity and affinity states are tightly interconnected, although the precise molecular mechanism underpinning integrin avidity remains controversial. In addition to regulation of ligand binding activity, integrins are subject to dynamic

internalisation and recycling events that are regulated by a number of different pathways which employ Rab and/or Arf family small GTPases [12]. Our understanding of the regulation of integrin trafficking is currently growing at a rapid pace, with experiments typically performed cultured adherent cell lines. Intracellular trafficking events are at times integrin receptor and also cell line dependent. Neutrophils are known to express numerous Rab and Arf GTPases [21, 22]. The Arf6 GEF cytohesin1 has been reported to regulate Mac-1 in neutrophils [23], but for the most part, specific roles of Arf/Rab-dependent integrin trafficking remain to be elucidated in the neutrophil.

The β2 integrins $\alpha_M\beta_2$ (also known as CD11b/CD18, Mac1 or CR3) and $\alpha_L\beta_2$ (also known as CD11a/CD18 or LFA1) are the major neutrophil integrins, but neutrophils also express other leukocyte integrins such as $\alpha_4\beta_1$ (CD49d/CD29; VLA4) as well as RGD binding integrins, e.g. $\alpha_5\beta_1$ (CD49e/CD29, fibronectin receptor) $\alpha_v\beta_3$ (CD51/CD61, vitronectin receptor). β2 integrin function is probably best characterised in neutrophil firm adhesion to endothelial ICAM-1 and TEM in post-capillary venules, thanks to a large body of work by many investigators who employed blocking and/or affinity status specific antibodies as well as knock-out mice as well as neutrophils from leukocyte adhesion deficiency (LAD) patients. In recent years, use of ever advancing imaging techniques has helped to visualise these processes in increasingly fine detail. Such investigations have built onto older observations to illustrate how neutrophils flatten their bodies [24, 25] and employ catch-bonds [26], long tethers [24], and slings [27] to enable and stabilise rolling even under conditions of high sheer stress. In contrast, neutrophil recruitment to the alveoli of the inflamed lung, and extravascular migration have been shown to depend on β_1 integrins (e.g. [28, 29]). $\alpha_5\beta_1$ and $\alpha_L\beta_2$ integrins were shown to be subject to recycling from the rear towards the front of the migrating neutrophil [30, 31].

Small GTPases in the regulation of (leukocyte) integrins

Rap small GTPases represent the best understood enzymatic regulators of the activity status of integrins. Rap1A-deficient mice are characterised by integrin-dependent leukocyte adhesion defects [32, 33]. Three distinct Rap effectors, RAPL [34], RIAM [35] and RADIL [36] have all been shown to function as links to integrin affinity and avidity. The mechanism involving RIAM-mediated integrin regulation is best characterised. Formation of Rap1-RIAM-talin complexes allows the recruitment of talin to integrin, inducing integrin activation [37]. Genetic experiments indicate that RIAM is particularly important for the activation of leukocyte β2 integrins [38, 39], whilst alternative mechanisms appear to operate in other cell types such as platelets [40]. Several Rap GEFs were shown to be involved in integrin activation. The cAMP activated Rap GEFs Epac1/2 [41-43] were shown to regulate integrin-mediated adhesion in many cell types, including in some leukocytes (e.g. [44-46]). Although expressed by neutrophils, Epac activation is not thought to be sufficient for neutrophil integrin activation [47]. A rare mutation in CalDAG GEF1 (also known as RasGRP2) was found to results in LAD type III [48], and neutrophils from CalDAG GEF1-deficient mice have adhesion and recruitment defects in keeping with this disease [49].

The Rho GTPases, RhoA, Rac and Cdc42, are also involved in dynamic cell-matrix adhesion [50]. The function of Rho GTPases in integrin outside-in signalling has been clearly documented (reviewed in [51]), but there is little evidence for Rho-dependent integrin inside-out signalling from experimentation with cultured adherent cell lines. In contrast, analyses of leukocytes under flow conditions have shown that Rap and Rho small GTPases are involved in the activation of leukocyte integrins (Fig 4). The first leukocyte-EC interaction, neutrophil capture, relies on display of stored endothelial P-selectin. This is followed by rolling

mediated by endothelial P-selectin / E-selectin and leukocyte L-selectin each binding their carbohydrate ligands on their counterparts. Leukocyte rolling induces a shift in the leukocyte integrin $\alpha_L\beta_2$ activation to an intermediate state, which in turn promotes slow rolling. Chemokines encountered during rolling induce further leukocyte integrin activation to the fully extended conformation. Cal-DAG-GEF1-mediated Rap1A activation was shown to be required for slow rolling of neutrophils to occur, due to Rap's role in rolling-mediated leukocyte integrin activation [52]. Rac2-deficient mouse neutrophils, and those isolated from a patient who carried a dominant negative Rac2 mutation were characterised by defective rolling on P-selectin [53, 54]. It remains unclear whether this defect might have been secondary to a defect in conveying $\alpha_L\beta_2$ activation. Neutrophils deficient in the Rac GEF P-Rex1 also displayed a defect in slow rolling, which was shown to be due to a role of P-Rex1 in the selectin-mediated activation of leukocyte integrins [55]. Intravital imaging of airway postcapillary venules in inflamed lungs suggested that the Rac GEFs P-Rex1 and Vav GEFs together regulate Rac-mediated $\alpha_L\beta_2$-activation in this context [56]. RhoA and Rac were also shown to mediate rapid chemokine-induced $\alpha_L\beta_2$ activation (inside-out signalling) in T lymphocytes under flow conditions [57, 58]. In contrast to neutrophils, primary T cells are amenable to being cultured and transfected. Careful analysis of signalling events in primary human lymphocytes identified CXCL12-induced $\alpha_L\beta_2$ inside-out signalling involved $G\alpha_I$-JAK2/3-Vav1-Rho-PLD1-Rap1 signalling [59, 60]. Additional knock-down studies with primary human lymphocytes identified that further Rho GEFs, SOS1, ArhGEF1 and DOCK2 are also involved in the chemokine-mediated affinity regulation of $\alpha_L\beta_2$ [61]. In contrast, Cdc42 was shown to counteract chemokine-driven leukocyte integrin activation under flow [59] and to interfere with chemoattractant-driven Rap activation [62].

As is often the case with switching off biological processes, our understanding of integrin inactivation lags far behind that of integrin activation. It stands to reason that such mechanisms not only exist, but, given the importance of integrin signalling, will be subject to tight regulation. Neutrophils from mice deficient in the PI3K and Rap-regulated RhoA and Arf6 GAP ARAP3, or those in which ARAP3 was uncoupled from activation by PI3K, were characterised by increased β_2 integrin ligand binding, by increased adhesion under static and flow conditions and by increased outside-in signalling *in vitro*. *In vivo,* these neutrophils were characterised by reduced crawling and increased firm adhesion and by a recruitment defect in sterile inflammation in the context of bone marrow chimeras [63, 64]. Collectively, this is suggestive of a function of small GTPases also in the regulation of integrin inactivation. Moreover, two mechanisms that compete with integrin activation have recently been described (Fig 3). First, SHARPIN binding to integrin α subunits was shown to interfere with talin and kindlin binding to β-subunits and integrin activation [65]. SHARPIN was subsequently shown to bind the α subunit of lymphocyte $\alpha_L\beta_2$, and to colocalise with $\alpha_L\beta_2$ to the trailing end of migrating T lymphocytes; *in vivo*, *Sharpin*-deficient T lymphocytes were deficient in homing to lymph nodes [66]. Second, sequestration of GTP-Rap by SHANK1/3 proteins has recently been shown to interfere with Rap-RIAM-talin mediated integrin activation in cancer cells, promoting the inactive integrin state ([67]; Fig 3).

Further information on small GTPase-dependent functions in the regulation of integrins in inflammation is bound to emerge in the future. It will be exciting to watch this space.

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Declarations of interest

The authors have no interests to declare.

Author contribution statement

JC and SV wrote the initial version and drafted the figures; all authors wrote the final version of the paper.

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Figure Legends

Figure 1. Vascular leakage and leukocyte-endothelial cell interactions in inflammation.

With the induction of an inflammatory response, the endothelium is stimulated and remodeled by the cytokines released by tissue resident immune cells such as macrophages, resulting in the upregulation of P and E selectins and vascular leakage. At the same time, the leukocyte recruitment cascade is initiated which involves several sequential stages, tethering, rolling, firm adhesion and transendothelial migration. Circulating neutrophils are captured by

the endothelium through the binding the selectin glycoprotein ligand 1 (PSGL-1) by the P- and E-selectins on the endothelial cells. Loosely tethered neutrophils then start rolling on the endothelium, and the engagement of the selectins and glycoproteins induces inside-out signaling in the neutrophil, shifting the conformation of β2 integrins, initially $\alpha_L\beta_2$ /LFA1 and later $\alpha_M\beta_2$/Mac1 from inactive to intermediate with higher ligand binding affinity to ICAM ligands expressed by the endothelium. This mediates crawling along, and subsequently firm adhesion of neutrophils to the endothelium. To reach the site of inflammation, neutrophils transmigrate across the endothelium in a process that requires leukocyte integrins and endothelial junctional proteins such as PECAM-1.
The paracellular mechanism, where leukocytes use EC junctions is shown here. Please note that the selectin and integrin usage and dependency for leukocyte extravasation does not appear to be universal across all endothelial beds.

Figure 2. The small GTPase cycle. Small GTPases are molecular switches that regulate many cellular functions. They cycle between an active, GTP-bound (drawn in green) and an inactive, GDP-bound form (drawn in red). Guanosine phosphate groups are indicated by blue dots, and lipid modifications are indicated in brown. Guanine nucleotide exchange on small GTPases is catalysed by GEFs. The frequently slow intrinsic GTPase activity of small GTPases is activated by GAPs. Some small GTPases (especially Rho and Rab families) are in addition regulated by GDIs. GDI-dependent sequestration of the lipid modification of the small GTPase in the cytoplasm prevents degradation, nucleotide exchange and membrane association. Both GEFs and GAPs are subject to regulation by a variety of mechanisms, including, phosphorylation, second messengers and formation of protein complexes. Together, these mechanisms achieve the correct spatiotemporal activation of small GTPases in any given situation.

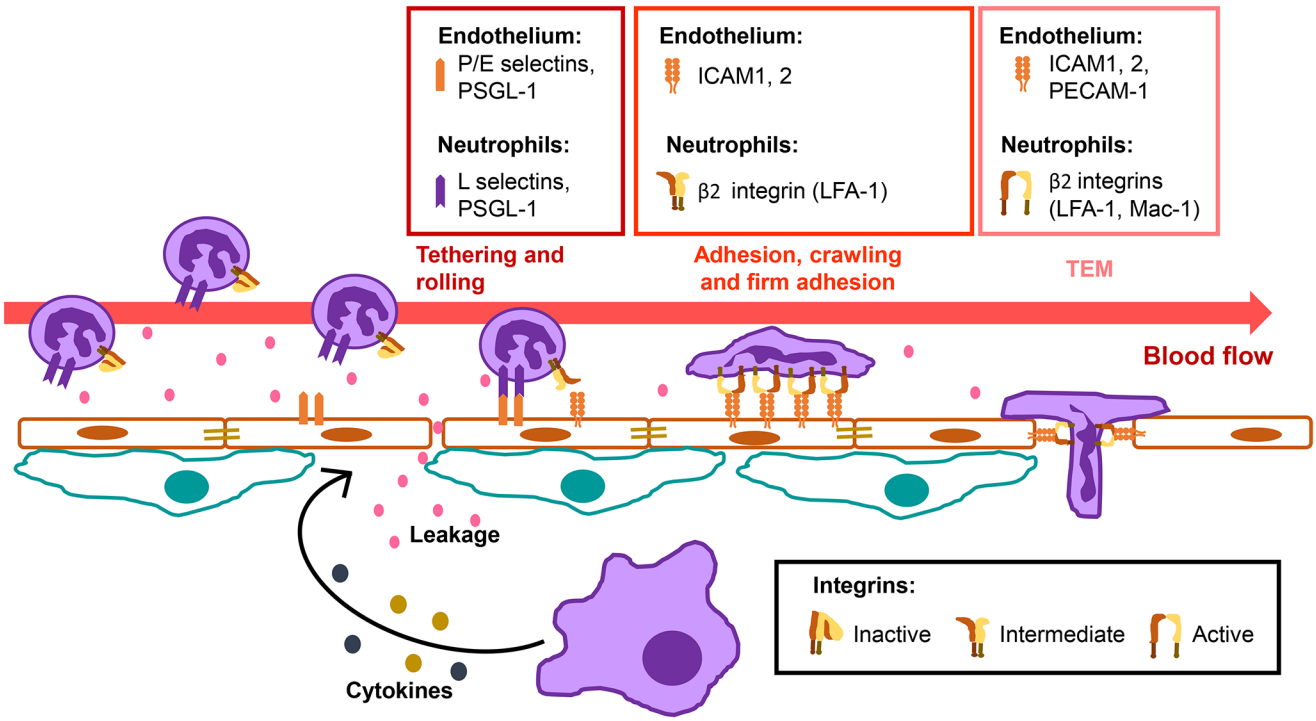
Figure 3. Rap-dependent regulation of integrin inside-out signalling. The shift from the inactive (bent) to the active (extended) conformation is regulated by integrin inside-out signalling. Talin (and kindlin, not shown) binding to the cytoplasmic tail of the integrin β subunit is required for integrin activation, and regulated by the small GTPase Rap in combination with its effectors, the best understood of which is RIAM. Formation of a Rap-RIAM-talin complex that binds integrin mediates integrin activation. Several GEFs upstream of Rap have been identified in integrin inside-out signalling, of which CalDAG-GEF1 is thought to be particularly important in neutrophils. Two negative regulators have been described that interfere with Rap/talin-mediated integrin activation. SHANK proteins can sequester Rap and interfere with the formation of the Rap-RIAM-talin complex, whereas SHARPIN binding to the integrin α subunit cytoplasmic tail interferes with the complex binding to the β subunit.

Figure 4. Regulation of leukocyte integrin inside-out signalling by Rho GTPases. Chemokine-driven integrin activation in the leukocyte adhesion cascade depends on Rho GTPase signalling in neutrophils and thymocytes. In thymocytes, CXCL12 binding causes Vav (and other) RhoGEF activation, inducing RhoA activation. This drives Rap activation (and Rap-mediated integrin activation) by making use of an indirect mechanism involving PLD1 and an as-yet-undefined Rap GEF.

Neutrophil Function	**Small GTPase involved**	**References**
Adhesion	Rac2 (under flow)	[53]
	RhoA	[68]
	Rap	[69]
	Arf6	[23]
Spreading	Rac2	[53]
	Rap1	[32]
Polarisation	Cdc42	[70, 71]
	Rac2	[72]
	RhoG	[73]
	Rap1	[69]
Chemotaxis	Rac1, Rac2	[53, 72]
	Cdc42	[70, 71]
	RhoA	[68]
	Rap1b	[69, 74]
	Rab27	[75]
	Arf6	[23, 76]
Recruitment	Rac1, Rac2	[53, 77]
	RhoA	[68]
	Rap1b	[74]
	Rab27	[75, 78]
	Arf6	[76]
Phagocytosis	Arf6	[23]
	Rab5a	[79]
	Rap1	[32]

NADPH Oxidase	Rac2 (but not Rac1) RhoG Rap1 Arf6 Rab27	[53, 77] [80] [32] [81, 82] [83]
Degranulation	Arf6 Rab27	[81] [83]
Apoptosis	RhoG Cdc42	[80] [84]
NET release	Rac2 Rab27	[85] [86]

Table 1. Neutrophils are subject to regulation by multiple small GTPases. Neutrophils carry out a range of specialised functions which allow them to ingest and kill pathogens and to generate inflammation. These functions are regulated by small GTPases as well as their regulators (not shown here). Please note that regulators identified in other model systems (e.g. macrophages in the case of phagocytosis) have not been included in this table. We apologise to the authors of many primary research papers that could not be cited here.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Neutrophils:
L selectins, PSGL-1
Endothelium:
ICAM1, 2
Neutrophils:
β2 integrin (LFA-1)
Endothelium:
ICAM1, 2, PECAM-1
Neutrophils:
β2 integrins (LFA-1, Mac-1)
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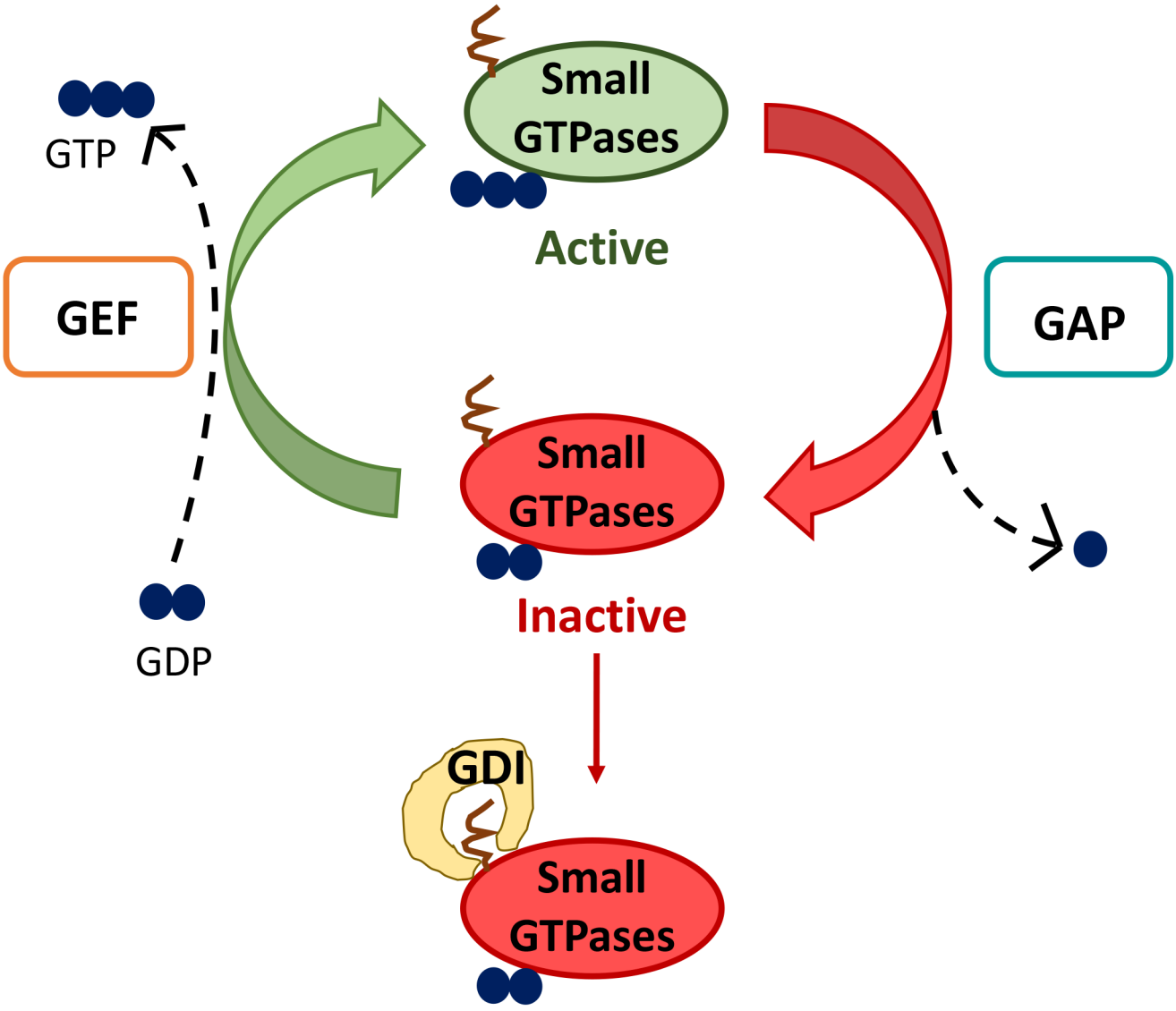
Adhesion, crawling and firm adhesion
TEM
Blood flow
Leakage
Cytokines
Integrins:
Inactive
Intermediate
Active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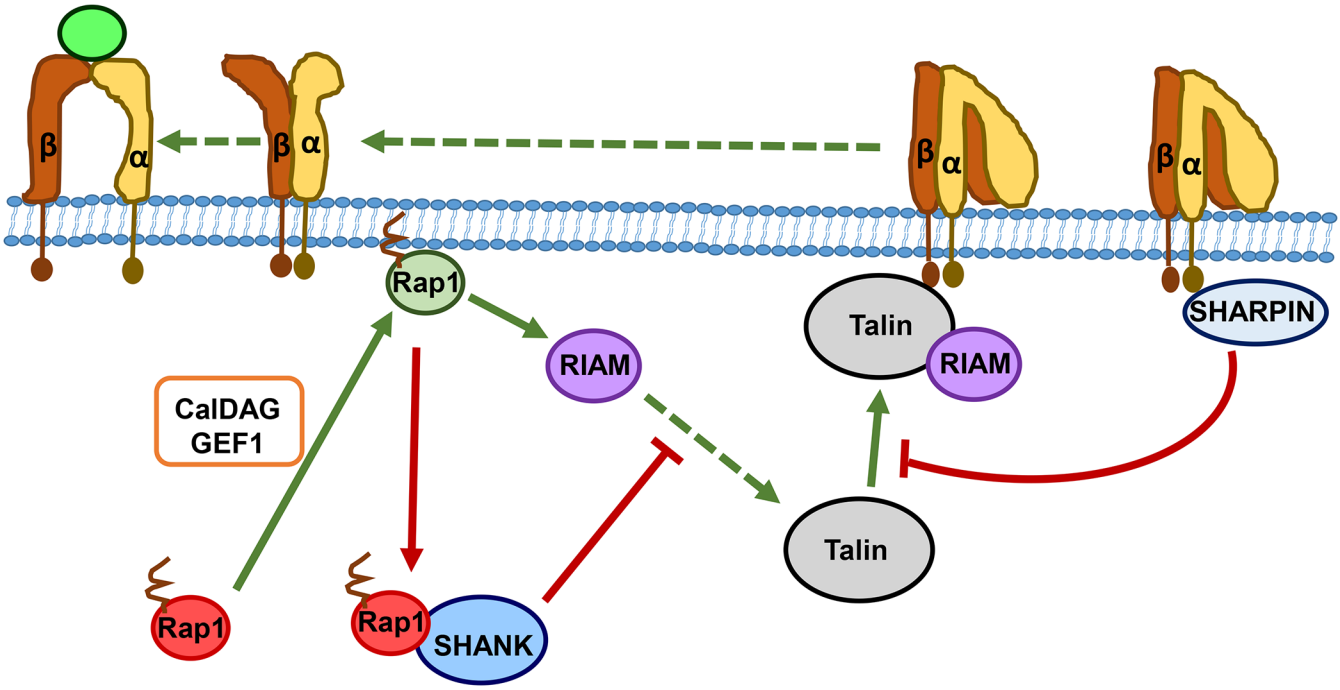
β
α
β
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β
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β
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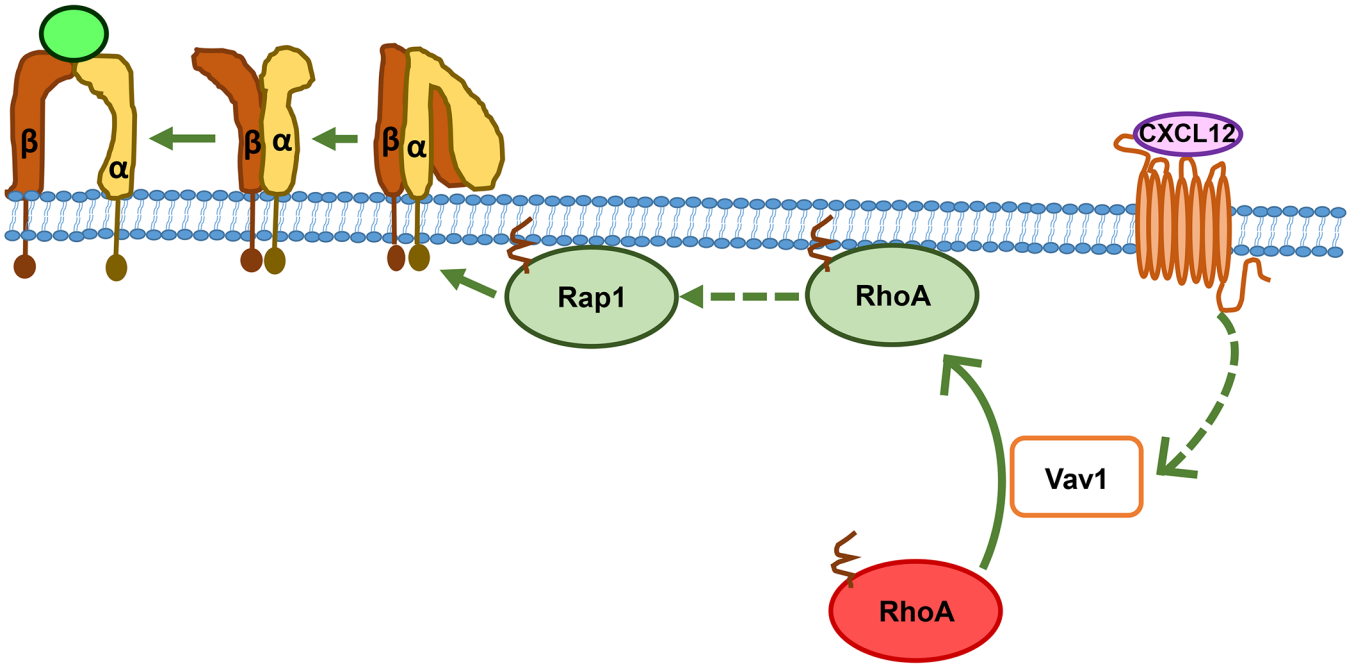
Rap1
RIAM
Talin
RIAM
SHARPIN
CalDAG
GEF1
Talin
Rap1
Rap1
SHANK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α
β
α
CXCL12
Rap1
RhoA
Vav1
RhoA