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Structural, biochemical, and functional properties of the Rap1-Interacting Adaptor Molecule (RIAM)

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ABSTRACT

Leukocytes, the leading players of immune system, are involved in innate and adaptive immune responses. Leukocyte adhesion to endothelial cells during transmigration or to antigen presenting cells during T cell activation, requires integrin activation through a process termed inside-out integrin signaling. In hematopoietic cells, Rap1 and its downstream effector RIAM (Rap1-interacting adaptor molecule) form a cornerstone for inside-out integrin activation. The Rap1/RIAM pathway is involved in signal integration for activation, actin remodeling and cytoskeletal reorganization in T cells, as well as in myeloid cell differentiation and function. RIAM is instrumental for phagocytosis, a process requiring particle recognition, cytoskeletal remodeling and membrane protrusion for engulfment and digestion. In the present review, we discuss the structural and molecular properties of RIAM and the recent discoveries regarding the functional role of the Rap1/RIAM module in hematopoietic cells.

Structural insight into RIAM and its molecular impact

The human Rap1-interacting adaptor molecule (RIAM) was first identified in T cells bound on the small guanosine triphosphatase (GTPase) Rap1 via a yeast two-hybrid assay [1]. The Ras superfamily small GTPase Rap1, is involved in secretion, cell

proliferation and migration. In vitro data have indicated that RIAM interacts with guanosine triphosphate (GTP)-bound Rap1, but not with guanosine diphosphate (GDP)-bound Rap1, and exhibits weak binding to other Ras GTPases [1,2]. RIAM was also found to interact in T cells with Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP), which participates in cell motility and actin polymerization [1]. In mouse fibroblasts,

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RIAM (also named proline-rich EVH1 ligand 1 or PREL1) was found to colocalize with Ena/VASP at the tips of lamellipodia and focal adhesions under epidermal growth factor (EGF) treatment, suggesting that RIAM might be a potential link for Ras signaling to cytoskeletal remodeling during cell migration and spreading [3]. Through an independent line of research, RIAM was found to bind to amyloid b (A4) precursor protein-binding family B member1 (APBB1; also known as Fe65) via interaction between its proline rich regions and the WW (tryptophan–tryptophan) domain of APBB1, and was named amyloid b (A4) precursor protein-binding, family B, member 1 interacting protein (APBB1IP) [4].

RIAM protein is 665 amino-acids long, containing an RA (RalGDS/AF-6 or Ras-association) domain, a PH (pleckstrin homology) domain, and two proline-rich regions (PRRs), all of which participate in its molecular functions. In addition, there are two putative coiled-coil regions at RIAM N-terminus [Fig. 1A] [1]. Protein homology studies revealed the existence of several RIAM homologous genes, collectively termed the MRL (Mig-10/RIAM/Lpd) family [5] [Fig. 1A]. These include human lamellipodin (Lpd) (also known as KIAA1681 and AY494951) and Lpd-S (a short isoform of human lamellipodin, also known as ALS2CR9 and BAB69020) [1,6], CG11940 (AAF49029) in *Drosophila melanogaster* and Mig-10 (P34400) in *Caenorhabditis elegans*.

Structural examination of homologs identified the C-terminal PRR and possibly the 27-amino acid N-terminal coiled-coil region as evolutionarily conserved domains. Additionally, several regions of the RA-PH domain are found to be conserved among homologous proteins [1]. As a proline-rich (12.9%) protein, RIAM contains six putative profilin binding motifs (XPPPPP) and six putative Ena/VASP homology 1 (EVH1) protein-binding motifs (D/E) (F/L/W/Y)PPPPX(D/E) (D/E) [7]. RIAM also contains binding motifs for Src homology 3 (SH3) domain- and WW domain-containing proteins [Fig. 1B] [8]. These protein-protein interaction domains are essential for its role as a signaling platform.

Besides the structural similarities among the MRL proteins, there are also significant differences that impact their molecular functions. The N-terminal part of RIAM differs from other MRL family proteins as it includes a short PRR with two putative EVH1-binding sites and one additional coiled-coil region. Even though RIAM and Lpd share conserved RA and PH domains, the N- and C-terminal regions are more divergent (29.1% and 23.2% amino acid identity, respectively). Also, the Lpd C-terminal region is 500 amino acids longer than that of RIAM [Fig. 1A] [1,6].

RA-PH domain

The RA domain of RIAM was demonstrated to interact with Ras and with Rap1 by pull-down assays using a recombinant glutathione S-transferase (GST)-tagged RIAM RA domain and lysates from NIH 3T3 cells transfected with Myc-tagged RasV12. Although both Rap1-GTP and Ras-GTP were shown to interact in vitro with RIAM with comparable affinities, in intact cells only Rap1 was able to regulate RIAM translocation to the plasma membrane, where Rap1 is recruited

subsequently to its geranylgeranylation [1,2]. RIAM translocation to the plasma membrane requires both RA and PH domains. Specifically, interaction of RIAM RA domain with Rap1-GTP and interaction of RIAM PH domain with PI(4,5)P₂ (see below) are essential for RIAM translocation to the plasma membrane upon T cell receptor (TCR)-mediated cell activation [1,2]. RIAM colocalizes with Rap1-GTP only at the plasma membrane but not in any intracellular membrane compartments, which is important for its specific functional role. As a consequence, RIAM subcellular translocation and function are linked to Rap1-GTP, as a molecular partner, but not to Ras-GTP [2].

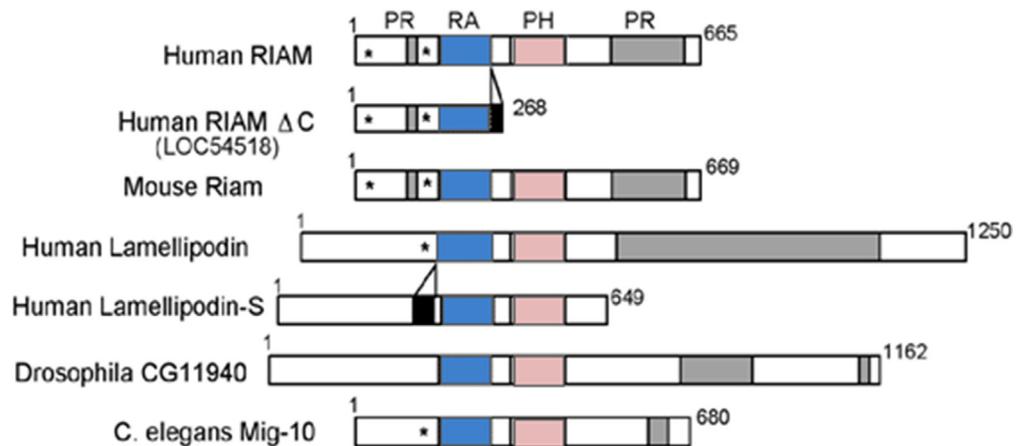
These in vitro findings are supported by the crystal structure of Rap1 GTPase-RIAM RA domain complexes [2] showing that Lys²¹³ in the α1 helix of the RIAM RA domain and Asp³³ of Rap1 are likely the interacting sites, since a mutation of Lys²¹³ blocks RIAM recruitment to the plasma membrane. An additional salt bridge between Rap1 Lys³¹ and RIAM Glu²¹² has a critical role for Rap1-RIAM association, as its disruption results in loss of co-clustering and cell adhesion, consistent with the mandatory role of the Rap1-RIAM module during inside-out integrin activation [2].

Through the use of lipid-coated membranes and GST-fused RIAM constructs, RIAM PH was shown to have high affinity for phosphatidylinositol monophosphates (PI(3)P, PI(4)P and PI(5)P), but even higher affinity for phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂; usually named PIP₂) [2,3,6]. Since PI(4,5)P₂ is most abundant in the plasma membrane and a substrate for phospholipase C-γ1 (PLC-γ1), it is the most probable partner of the PH domain of RIAM in terms of physiological role. As revealed by structural studies, this interaction with PI(4,5)P₂ is mediated through a K-X_n-(K/R)-X-R sequence in the β1-β2 loop region of the PH domain of RIAM [2]. The crystal structure of the RIAM RA-PH also showed that these two subdomains form a single structural unit through an extensive RA-PH domain interface. Lys³³¹, Arg³³³, and Lys³²⁷ mutations within the β1-β2 loop region of the PH domain of RIAM restrained RIAM recruitment to the plasma membrane [2]. Several side-chain interactions have also been identified which mediate the specificity of RIAM recognition by Rap1-GTP [2]. Thus, the interaction of both components of the integrated RA-PH unit with their binding partners and their natural structural integrity are essential for RIAM translocation to the plasma membrane.

Proline rich regions

RIAM has multiple proline rich regions [Fig. 1A] [1]. Proline-rich motifs constitute protein interaction sites for SH3 domain- and WW domain-containing proteins [8]. The interaction of RIAM with formin-binding protein 11 (FBP11) and growth arrest-specific protein 7 (Gas7) are mediated via a WW domain present in each of the latter two proteins [9]. The proline-rich regions of RIAM also bind the regulators of the actin cytoskeleton, profilin and the EVH1 domain of Ena/VASP proteins, as demonstrated by yeast two-hybrid assays, in vitro association of recombinant proteins, coimmunoprecipitation and pull-

A



B

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1  MGESSEDIDQMFSTLLGEMDLLTQSLGVDTLPPDPNPPRAEFNYSVGFK
51  DLNESLNALEDQDLDMADLVADISEAEQRTIQAQKESLQNQHHSASLQ
101 ASIFSGAASLGYGTNVAATGISQYEDDLPPPADPVLDLPLPPPPPEPLS
151 QEEEEAQAKADKIKLALALEKLKEAKVKKLVVKVHMNDNSTKSLMVDERQLA
201 RDVLDNLFEKTHCDCNVDWCLYEIYPELQIERFFEDHENVEVLSDWTRD
251 TENKILFLEKEEKYAVFKNPQNFYLDNRGKKESKETNEKMNAKNKESLLE
301 ESFCGTSIIVPELEGALYLKEDGKKS WKRRYFLLRASGIYVPGKGTKTS
351 RDLACFIQFENVNIYYGTQHMKYKAPTDCFVLKHPQIQKESQYIKYLC
401 CDDTRTLNQWVMGIRIAKYGKTLYDNYQRAVAKAGLASRWTNLGTVNAAA
451 PAQPSTGPKTGTTPNGQIPQATHSVSAVLQEAQRHAETSKDKKPALGNH
501 HDPVAVPRAPHAPKSSLPPPPVRRSSDTSGSPATPLKAKGTGGGGLPAPP
551 DDFLPPPPPPPLDDPELPPPPDFMEPPPDFVPPPPPSYAGIAGSELPP
601 PPPPPAPAPAPVPDSARPPPAVAKRPPVPPKRQENPGHPGGAGGGEQDFM
651 SDLMKALQKGRNVS

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Fig. 1 Structural characterization of RIAM and its homologs. (A) Subunits of human RIAM and homology with MRL family proteins are schematically presented. The proline-rich (PRR), RA, and PH domains are shown. The coiled-coil regions are indicated with an asterisk. (B) Human RIAM is a proline-rich protein and has six putative profilin-binding motifs (highlighted in gray) and six EVH1-binding motifs (underlined).

down assays [1]. RIAM interacts with non-phosphorylated VASP as determined by immunoprecipitation of endogenous VASP in lysates from mouse fibroblasts isolated from wild-type and β_3 integrin-deficient animals where VASP is found in a non-phosphorylated form because of the impaired activation of protein kinase A (PKA) [10].

Profilin and Ena/VASP family proteins are mediators of actin cytoskeleton polymerization [11]. Profilin-G-actin-adenosine 5'-triphosphate (ATP) complexes are produced by profilin binding to G-actin and nucleotide exchange factor. Association with profilin promotes addition of actin monomers to F-actin ends [12]. Mena, VASP, and Evi are members of the Ena/VASP family that are brought to regions such as lamellipodia, filopodia, focal contacts, phagocytic cup and the T cell-APC contact site where actin cytoskeleton remodeling takes place [13,14]. However, Ena/VASP family proteins do not play an active role in the T cell-APC conjugate formation [15]. All of these proteins have an EVH1 domain that associates with the proline-rich motifs found in proteins such as zyxin and vinculin that direct Ena/VASP proteins to focal adhesions [7], or in FYN binding protein ADAP (adhesion- and degranulation-promoting adapter protein), and SLAP-130 (SLP-76-associated phosphoprotein of 130 kDa) that bring Ena/VASP family members to the T cell-APC interface [11,14].

The Ena/VASP family members also contain proline-rich regions which interact with SH3 domain-containing proteins and profilin. In addition, they have EVH2 domain that regulates their tetramerization and association with both G- and F-actin. Due to these properties, Ena/VASP proteins function as actin elongators and regulators of cell migration [13]. Therefore, RIAM interaction with profilin and Ena/VASP family proteins represents a mechanistic link underlying its significant role in cytoskeletal remodeling.

Talin binding site

RIAM has a functional talin binding site (TBS) at its N-terminal region. Upon its recruitment to the plasma membrane in a Rap1-dependent manner, RIAM interacts with talin via its TBS region, and these two proteins colocalize at the plasma membrane. The talin-RIAM interaction was discovered by coimmunoprecipitation assays using lysates of Chinese hamster ovary (CHO) cells stably expressing integrin $\alpha_{IIb}\beta_3$ and transiently cotransfected with hemagglutinin (HA)-tagged talin and GFP-tagged RIAM constructs of various lengths [16]. Subsequent studies showed that even though the N-terminal domain of RIAM has two distinct TB sites (TBS1 and TBS2), TBS1 is the one that can recruit talin to the plasma membrane [17].

Talin is a multidomain protein composed by a talin-R (rod) and talin-H (head) [Fig. 2A]. RIAM TBS1 and TBS2 can interact with different sites in talin-R and talin-H at the F2, F3 domains. For this reason, it has been presumed that more than one RIAM molecule can bind to a single talin molecule. Structural studies revealed that RIAM TBS1 interacts with the strongest binding site of talin, the R8 domain. However, RIAM TBS1 can also bind next to the integrin site on talin-F3 [Fig. 2B] [17]. RIAM binding to talin-F3 competes with the talin autoinhibitory interaction maintained between its F3 and R9

domains and results in the unmasking of the integrin binding site on talin F3, thereby allowing talin F3-integrin interaction [17,18]. The TBS region in RIAM N-terminus is also involved in RIAM autoinhibition by inhibiting the association of the RA and PH domains of RIAM with their interacting partners at the plasma membrane. Based on the combination of structural and functional studies, it had been initially predicted that this inhibition might be alleviated by the interaction of talin with this region at the N-terminus of RIAM [2]. Subsequently, it was determined that this autoinhibition is mediated by binding of an inhibitory (IN) segment in RIAM N-terminus with the RA domain, thereby preventing interaction with Rap1. Phosphorylation of Tyr45 in RIAM IN mediated by focal adhesion kinase (FAK), releases this intramolecular interaction thereby alleviating RIAM autoinhibition and allowing the binding of Rap1 [19]. Furthermore, a conserved intermolecular interface was identified that promotes oligomerization of two RIAM molecules via the PH domain and masks the PI(4,5)P₂-binding site, thereby mediating an additional autoinhibition mechanism. Phosphorylation of Tyr267 and Tyr427 in the RIAM PH domain by Src family kinases releases this intermolecular interaction [20] and promotes PH domain interaction with PI(4,5)P₂, localization of RIAM at the plasma membrane and integrin activation [Fig. 2C] [21].

The Rap1/RIAM pathway on platelets and myeloid differentiation and function

The role of the Rap1/RIAM pathway in myeloid cell differentiation and maturation was studied initially in megakaryocytes. Thrombopoietin-induced maturation requires extracellular signal-regulated kinases (ERK) signaling and relies on both Ras and Rap1 [22,23]. This pathway is inhibited during coculture with stromal cells, where inhibited or delayed Rap1 but not Ras activation prevented megakaryocytic differentiation [24]. The importance of Rap1 in megakaryopoiesis has also been demonstrated by the deletion of Rasa3 (also known as GAP11P4BP, R-Ras GAP), a GTPase-activating protein (GAP). Loss of GAPs leads to uncontrolled activation of the relevant targets. By binding of its PH domain to the cytosolic leaflet of the plasma membrane, Rasa3 mediates inactivation of Ras and Rap1 [25,26]. Catalytic inactivation of Rasa3 in mouse hematopoietic cells resulted in the generation of undifferentiated and functionally defective megakaryocytes, impaired platelet production, and predisposition to preleukemia, eventually leading to a fatal outcome. These effects in Rasa3^{-/-} megakaryocytes, were mediated by aberrant activation of Rap1, leading to constitutive inside-out $\alpha_{IIb}\beta_3$ integrin activation and signaling, and cell adhesion [27].

When RIAM was identified as a Rap1 effector molecule, it was investigated whether RIAM might be involved in Rap1-mediated activation of platelet $\alpha_{IIb}\beta_3$ integrins [28]. In primary megakaryocytes, RIAM knockdown blocked $\alpha_{IIb}\beta_3$ activation induced by thrombin protease-activated receptors, which depends on Rap1-GTP and talin. Reconstruction of this pathway in a CHO cell-based system demonstrated that RIAM knockdown abrogated $\alpha_{IIb}\beta_3$ activation in the presence of

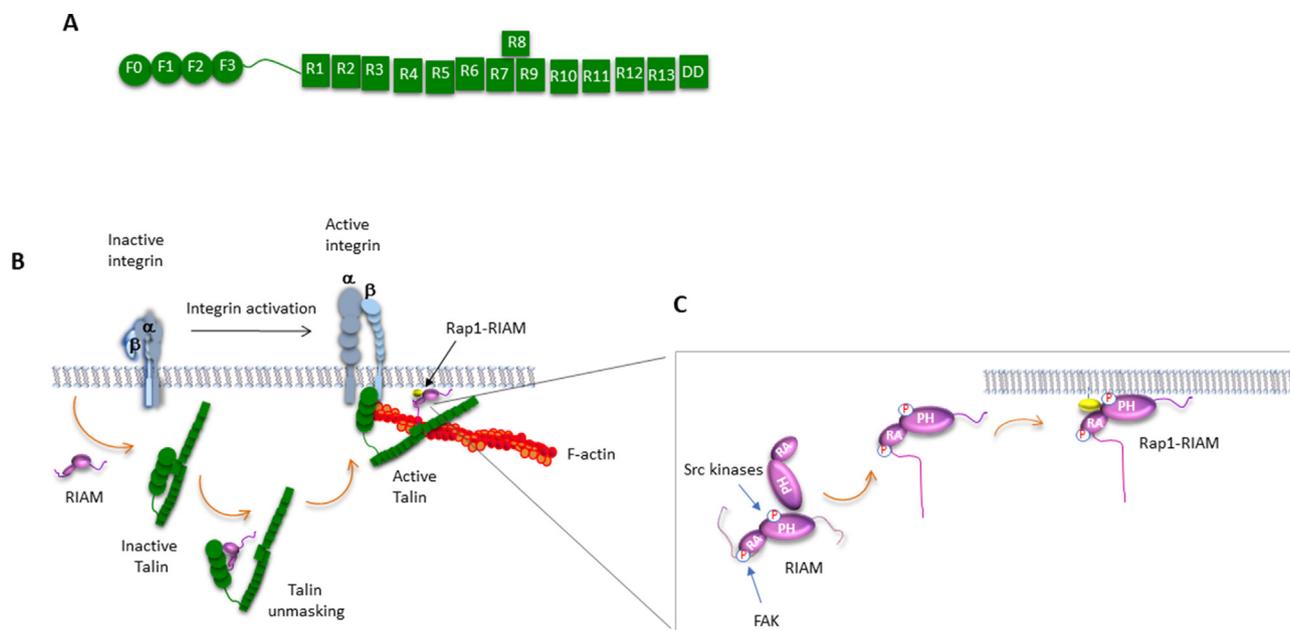


Fig. 2 RIAM implication in the integrin activation. (A) The composition of talin subunits. F0, F1, F2, and F3 subdomains are located at the N-terminus. The C-terminus talin-R includes 13 helical bundles (R1 to R13) followed by a dimerization domain (DD). (B) Although R8 is the strongest binding talin domain, the N-terminus talin binding region of RIAM also binds on talin F3 next to the integrin site, and competes with the talin autoinhibitory intramolecular interaction maintained between its F3 and R9 domains, thereby unmasking the integrin binding site on talin F3 and allowing talin F3-integrin binding. (C) RIAM itself is also subject to autoinhibition induced by binding of its N-terminus to the RA domain. Phosphorylation of Tyr45 in RIAM N-terminus, mediated by focal adhesion kinase (FAK), releases this intramolecular interaction thereby alleviating RIAM autoinhibition and allowing the binding of Rap1. In addition, RIAM autoinhibition is induced by oligomerization of two RIAM molecules via their PH domains masking the PI(4,5)P₂-binding site. Phosphorylation of Tyr267 and Tyr427 in the RIAM PH domain by Src family kinases releases this intermolecular interaction and promotes PH domain interaction with PI(4,5)P₂, localization of RIAM at the plasma membrane and integrin activation.

Rap1-GTP. Despite these compelling *in vitro* findings indicating an important role of the Rap1-RIAM-talin module in $\alpha_{IIb}\beta_3$ integrin activation, genetic deletion of RIAM in mice did not affect platelet differentiation or function, suggesting that recruitment and activation of talin *in vivo* could be mediated by alternative pathways [29]. Indeed, Rap1-independent mechanisms of integrin activation have been identified in platelets [30]. Moreover, Rap1B (encoded by a different gene) is expressed predominantly in platelets and regulates activation of $\alpha_{IIb}\beta_3$ integrin [31].

Rap1 activation during differentiation of hematopoietic progenitors undergoes a strict regulation by Rap1-GAPs. The G-CSF-induced granulocytic differentiation of the IL-3-dependent promyeloblastic cell line 32D correlates with a progressive decline of signal-induced proliferation-associated gene-1 (SPA-1), a Rap1-GAP normally expressed in immature lineage negative bone marrow cells. Simultaneously, RapGAP, the Rap1-GAP of the lineage positive bone marrow cells, is upregulated [32]. The same shift in favor of RapGAP expression is described during differentiation of the promyelocytic cell line HL-60 [33], in which RapGAP was first identified [34]. Notably, both RIAM and Rap1GAP are upregulated during all-trans retinoic acid (ATRA)-induced neutrophilic differentiation [35,36]. Furthermore, SPA-1 deletion in mice causes a wide spectrum of myeloid disorders indicating an abnormal differentiation of myeloid lineage cells [32]. Together these

findings point to the entire Rap1/RIAM pathway possessing a critical role in myeloid cell differentiation and further studies are required to dissect its role in this process.

The Rap1/RIAM module in T cell activation and function

In T cells, Rap1 regulates integrin activation via its downstream effector molecules, RapL and RIAM, thus coupling TCR activation to integrin-mediated signaling [1,37]. While RapL mediates activation of integrin $\alpha_L\beta_2$ (or LFA-1) by interacting with the integrin α_L chain, RIAM, promotes integrin activation by targeting the cytoskeletal protein talin to the cytoplasmic tail of the β_2 subunit [1,37,38].

In contrast to the finding that germline deletion of RIAM does not impair hemostasis and integrin-mediated platelet function *in vivo*, RIAM deletion leads to altered migration/homing dynamics in leukocytes [39]. T and B lymphocyte development remained intact when Rap1 or RIAM were depleted in leukocytes using Rap1a/b floxed and Lck-Cre system or a universal germline RIAM knock-out mouse model, respectively [40]. These surprising findings prompted researchers to generate additional conditional knock-out mouse models to further interrogate the role of the Rap1/RIAM module in lymphocyte biology. As integrins mediate not only

lymphocyte trafficking but also immunological synapse formation between antigen-presenting cells and lymphocytes, through which lymphocyte activation is accomplished [41,42], the contribution of Rap1/RIAM module to this key cellular event was investigated. Indeed, compartmentalization of RIAM to the immunological synapse was first described in Jurkat T cells and subsequently in primary mouse CD4⁺ T cells [Fig. 3A] [43,44].

Apart from the contribution to the integrin activation machinery, certain T cell signaling events were also reported to be fine-tuned by Rap1/RIAM node. TCR signaling triggers phosphorylation of RIAM, itself, which functions as a bridging molecule within the TCR downstream signalosome complex [21]. The mechanism through which RIAM mediates TCR downstream signaling was further illuminated by studies showing that RIAM is directly involved in TCR signaling, independently of LFA-1, by mediating membrane localization and activation of PLC- γ 1 as well as subsequent intracellular Ca⁺² release in primary human and Jurkat T cells [45]. Therefore, knocking-down RIAM in T cells resulted in profound defects in T cell signaling, such as reduction in the levels of active calcium- and diacylglycerol-regulated guanine nucleotide exchange factors (CalDAG-GEFs) and Ras guanyl releasing protein 1 (RasGRP1). The impaired TCR-mediated calcium release compromised the activation of calcineurin which dephosphorylates the nuclear factor of activated T cells

(NFAT) allowing its nuclear translocation. As a consequence, RIAM knock-down resulted in defective NFAT nuclear translocation and impaired production of IL-2, which is well-known to have critical implications in conventional T cell and regulatory T cell biology [45]. By taking advantage of CD4-Cre knockout system, it was later determined that T cell-specific deletion of RIAM impaired immune synapse formation and diminished contact-mediated cell proliferation, which abrogated autoreactive capacity of T cells in a mouse type-I diabetes disease model [46]. Together these findings suggested that the capabilities of the Rap1/RIAM module were not restricted to the regulation of lymphocyte trafficking and provided evidence that T cell activation requires intact Rap1/RIAM signaling module due to its direct effects on signal transduction and generation of second messengers.

Regulatory T cells (Treg), a CD4⁺ T cell subset, are responsible for keeping the immune system in check, counteracting effector functions of conventional T cell subsets [47]. Despite exerting opposite tasks, Treg cells share a great deal of similarities with conventional T cells (Tconv) in terms of the signaling and adhesion molecules required for their function, activation, and maintenance [48]. Quantitative or qualitative defects in Treg function result in systemic inflammation and multiorgan autoimmunity [49,50]. The integrin activation

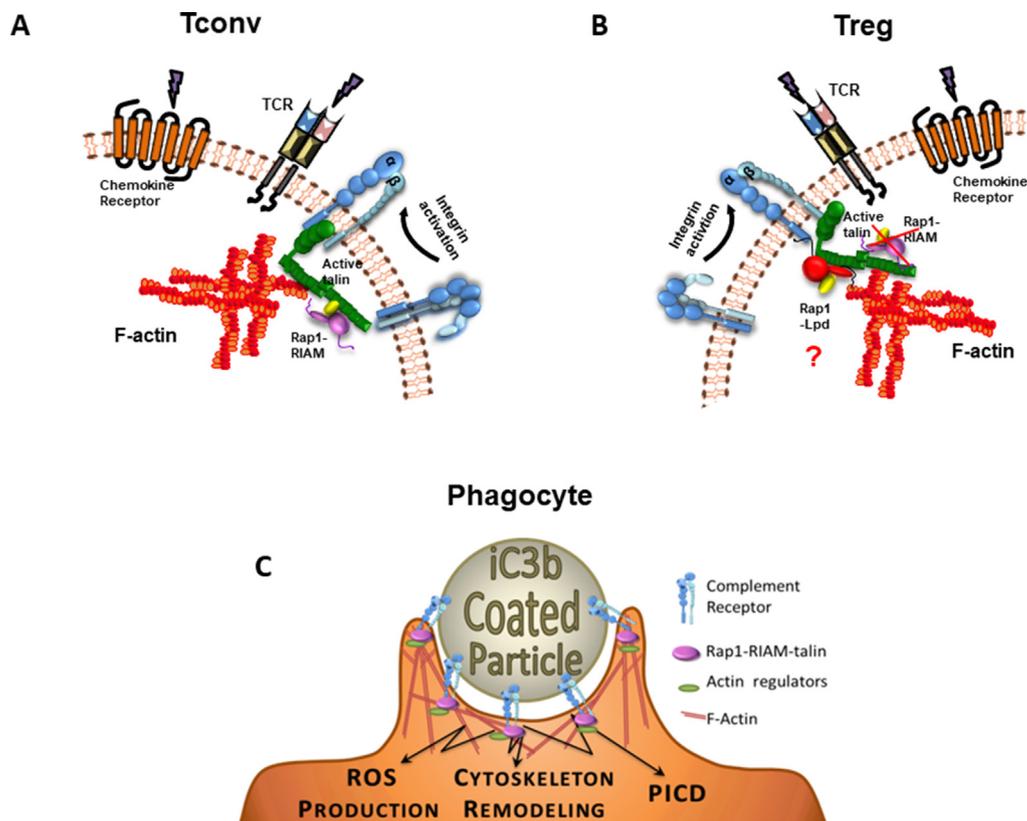


Fig. 3 RIAM is involved in inside-out signaling dynamics in T cells and myeloid cells. Upon stimulation via TCR or chemokine receptors, activated talin is recruited to the membrane where it mediates conformational change and activation of integrins. This process couples receptor signaling to downstream cellular events such as migration and chemotaxis. (A) In conventional T cells this process requires the Rap1/RIAM module. (B) In Treg cells, Lamellopodin (Lpd), a RIAM paralogue, might contribute to integrin activation, rendering RIAM partially redundant for such function. (C) RIAM is localized in the phagocytic cup and enhances pathogen clearance through complement-mediated phagocytosis and production of ROS.

machinery is crucial for Treg function and maintenance. Prior studies performed in mice lacking either the α_L (CD11a^{-/-}) or β_2 (CD18^{-/-}) subunit of LFA-1 revealed an increased propensity to auto-inflammation and autoimmunity. Likewise, LFA-1^{-/-} Treg cells exhibited impaired suppressive capacity compared to wild-type counterparts when assessed in an experimental colitis model [51,52]. These findings were supported by studies which provided evidence that Rap1 controls Treg generation through LFA-1-dependent and independent mechanisms [53]. Consistently, conditional deletion of Rap1a and Rap1b in T cells led to spontaneous colitis [54]. Although the authors attributed this phenotype to the altered trafficking of effector and naïve T cells, it was not fully examined whether the Treg compartment was affected in these mice.

Further research also showed that talin1 plays an indispensable role in Treg homeostasis. T cell- as well as Treg cell-specific deletion of talin1 resulted in dysregulated IL-2 signaling, leading to impaired effector differentiation and maintenance of Treg cells, break of immune tolerance and spontaneous lymphocyte activation [55,56]. Given the important role of upstream and downstream molecules linked to RIAM in Treg cells, it remained elusive how RIAM-deficiency in T cells led to an opposite phenotype, as mice were protected against autoimmune diseases. This intriguing phenomenon was investigated by a recent study using Foxp3-YFP Cre/RIAM-null mice, which concluded that RIAM-null Treg were still able to ameliorate development of experimental inflammatory bowel disease (IBD) secondary to IL-10 deficiency [57]. Further analyses showed that in contrast to Rap1, RIAM has a redundant role in integrin-mediated function, development, and maintenance of Treg, because Lpd was able to compensate downstream signaling in the absence of RIAM in Treg cells [Fig. 3B]. These findings indicate a fundamental dichotomy in the regulation of integrin activation machinery between Tconv and Treg cells. This observation is intriguing because previous studies indicated that unique structural properties of RIAM are responsible for a selective role of RIAM interaction with talin and integrin activation [17]. Specifically, it was discovered that RIAM has two talin-binding sites (TBS1 and TBS2), of which TBS1 is capable of interacting with the strongest binding site of talin, the R8 domain, and recruiting talin to the plasma membrane. Crystal structure of a talin R7R8 and RIAM TBS1 complex revealed a kink in the TBS1 helix of RIAM that is absent from the homologous region of Lpd. This kinked helix conformation is required for the interaction and membrane co-localization of RIAM and talin leading to activation of integrin [17]. Thus, the mechanism by which the proposed Lpd/Talin complex might be formed leading to integrin activation in Treg cells [57] remains elusive.

IL-2 signaling promotes the expression of Foxp3 and is required for the development and integrity of Treg cells in the secondary lymphoid organs [49,58]. While mouse Treg lacking talin [55,56], LFA-1 [51] and another Rap1 downstream effector, Mst1 [59], exhibited diminished IL-2 signaling, this phenomenon was not detected in RIAM deficient Treg [57] suggesting the presence of a potential compensatory mechanism rendering RIAM redundant in IL-2R downstream signaling. Given the fundamental differences and redundancies between conventional and regulatory T cells regarding the role of Rap1 and RIAM in integrin activation and TCR signaling, it is necessary to

illuminate the role of the Rap1/RIAM node in different T helper subsets. Indeed, a relevant study reported that Rap1A-GTP transgenic mice (Rap1-Tg) that express constitutively active Rap1A under the CD2 promoter, displayed an enlarged Treg cell pool whereas the proliferative and activation capacity of non-Treg T helper subsets remained defective [60]. Future studies are required to investigate the contribution of RIAM to the T cell signaling events that occur in distinct T cell subsets in order to understand the physiological importance of the Rap1/RIAM module in T cell biology.

The Rap1/RIAM pathway in phagocytic processes

In the context of pathogen phagocytosis and apoptotic cell clearance, the complement receptors CR3 or integrin $\alpha_M\beta_2$ and CR4 or integrin $\alpha_X\beta_2$ take special relevance [61]. The implication of RIAM in the activity of these integrin receptors was analyzed in RIAM knockout mice where phagocytes presented strong reduction (70%) in the phagocytic clearance of fluorescently labelled *Escherichia coli*, as well as diminished adhesion and production of reactive oxygen species (ROS) [39].

In human monocyte-derived macrophages and neutrophil-like HL60 cells, RIAM knockdown blocked CR3/ $\alpha_M\beta_2$ activation and complement-mediated phagocytosis downstream of LPS or fMLP activation. Direct activation of EPAC, a Rap1-specific guanosine exchange factor, through the use of the cAMP analogue 8CPT-2Me-cAMP could not rescue the observed defect in phagocytosis [62]. This lack of activation correlated with a significant decrease in talin enrichment at the phagocytic cup, suggesting a similar mechanism to that observed in focal adhesions and immune synapse formation [Fig. 3C].

In addition to integrin activation downstream of Rap1-GTP (inside-out pathway), RIAM involvement in integrin signaling downstream of ligand binding (outside-in) was shown in neutrophils [63]. Integrin signaling can be triggered in vitro using Mn²⁺, which binds to the Ad-MIDAS domain of CR3, emulating the conformational change induced by ligand binding and, therefore, promoting the acquisition of a high-affinity conformation [64]. RIAM knockdown in HL-60 cells treated with Mn²⁺ presented reduced complement-mediated phagocytosis, distorted phagocytic dynamics, and altered downstream ERK signaling [63].

RIAM knockdown also resulted in reduced F-actin and VASP recruitment to phagocytic cup [63], as well as reduced VASP Ser¹⁵⁷ phosphorylation, which is required for membrane localization [65–67], and correlates with a higher in vivo actin polymerization [68,69]. The defect in phagocytosis of RIAM-deficient cells was alleviated by using Forskolin, a PKA activator capable of inducing VASP Ser¹⁵⁷ phosphorylation [63]. This suggests that in addition to recruiting talin and activating integrins, RIAM is responsible for coordinating, via VASP, the actin remodeling necessary for particle engulfment.

Due to its roles in integrin activation and VASP recruitment to the phagocytic cup, RIAM seems to be involved in the early stages of phagocytosis, namely the formation of nascent adhesions and the initiation of VASP-mediated membrane protrusions. Indeed, RIAM has been shown to contribute to the binding of vinculin to talin, as RIAM-talin interaction unmasks

a vinculin binding site in talin [70]. As adhesions mature, vinculin binding to F-actin and α -actinin favors filament bundling and force generation [71], to the partial unfolding of talin, and the loss of RIAM-binding sites [72,73]. Vinculin has been described to form focal complex-like signaling platforms at CR3 phagocytic cups [74]. In light of the previously presented evidence, RIAM may form an early VASP-containing module [63] which favors actin protrusions, whilst by subsequent formation of a vinculin-containing molecular clutch, RIAM might favor force generation and complement-mediated phagocytosis [74].

There are several proposed alternative talin-recruitment mechanisms but their contributions and biological significance are yet to be established. An evolutionarily conserved direct interaction between Rap1 and talin has been demonstrated, where Rap1 cooperates with PI(4,5)P₂ to direct talin towards integrin β tail [75,76]. This pathway has been proposed to have a dominant role for integrin activation in platelets [77], where RIAM deletion has no effect [29], and also plays a role in neutrophil adhesion and extravasation, but not on macrophage adhesion and migration [78]. This pathway might be relevant for fast cell responses or might prime RIAM-mediated activation of integrins. Alternatively, the Rap1-talin interaction might be an evolutionarily older pathway, and RIAM might have evolved subsequently to ensure a more robust integrin activation. However, direct activation of Rap1 with 8CPT-2Me-cAMP indicates that Rap1 activation alone, cannot compensate for RIAM deficiency in complement-mediated phagocytosis [62].

The significance of RIAM phosphorylation is also a subject of intense investigation. Recent reports indicate that Src family kinase-mediated RIAM phosphorylation at Tyr²⁶⁴ and Tyr⁴²⁷ is necessary for membrane localization [20,21]. Src family kinase activation is one of the earliest steps in the outside-in signaling [61] and RIAM itself is involved in outside-in activation [63]. Currently, there are still no reports delineating the phosphorylation status of RIAM under various experimental conditions and cell types. It is also unclear how this modification might affect RIAM subcellular localization and its interactions. Further studies toward this direction will reveal new aspects of RIAM function and might shed light to current uncertainties and inconsistencies in findings generated in different experimental models.

Concluding remarks

RIAM was discovered as a molecule bound to APBB1 and subsequently, in a separate screen, was identified as an interacting partner of Rap1-GTP in T cells and an indispensable mediator of inside-out integrin activation. RIAM has a selective impact on β_2 integrin activation, and its ablation has significant implications on β_2 integrin function but less so on β_1 and β_3 integrins. The Rap1/RIAM module has a relevant role on T cell function not only due to integrin activation but also due to its central role as a hub linking TCR-proximal and distal events, and regulating the generation of second messengers that have a decisive fate in T cell responses. RIAM interacts with Ena/VASP family members and profilin, thereby being linked with actin polymerization and cytoskeletal remodeling.

In fact, the RIAM-VASP protein module work in concert to coordinate β_2 integrin-induced actin cytoskeletal dynamics. Due to these interactions, RIAM is a significant element coupling receptor activation and actin reorganization during complement-mediated phagocytosis. It is increasingly becoming apparent that RIAM might mediate specific functional outcomes, guided by the cell type as well as by the microenvironmental context. As these novel aspects of RIAM function start to unravel, more work is required to determine how the Rap1/RIAM module is able to engage in specific signaling mechanisms and result in selective outcomes in distinct cell types.

Conflicts of interest

VAB has patents on the PD-1 pathway licensed by Bristol-Myers Squibb, Roche, Merck, EMD-Serono, Boehringer Ingelheim, AstraZeneca, Novartis, and Dako. The authors declare no other competing interests.

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