Lymphocyte activation requires adhesion to antigen-presenting cells. This is a critical event linking innate and adaptive immunity. Lymphocyte adhesion is accomplished through LFA-1, which must be activated by a process referred to as inside-out integrin signaling. Among the few signaling molecules that have been implicated in inside-out integrin activation in hematopoietic cells are the small guanosine triphosphatase (GTPase) Rap1 and its downstream effector Rap1-interacting molecule (RIAM), a multidomain protein that defined the Mig10-RIAM-lamellipodin (MRL) class of adaptor molecules. Through its various domains, RIAM is a critical node of signal integration for activation of T cells, recruits monomeric and polymerized actin to drive actin remodeling and cytoskeletal reorganization, and promotes inside-out integrin signaling in T cells. As a regulator of inside-out integrin activation, RIAM affects multiple functions of innate and adaptive immunity. The effects of RIAM on cytoskeletal reorganization and integrin activation have implications in cell migration and trafficking of cancer cells. We provide an overview of the structure and interactions of RIAM, and we discuss the implications of RIAM functions in innate and adaptive immunity and cancer progression.

**Identification of RIAM**

The human Rap1-interacting molecule (RIAM) was identified in a yeast two-hybrid screen for candidate effectors of the small guanosine triphosphatase (GTPase) Rap1 (1). Before its identification as a Rap1-interacting molecule, RIAM was identified as a binding partner of the amyloid β (A4) precursor protein–binding, family B, member 1 (APBB1; also known as Fe65) and was named amyloid β (A4) precursor protein–binding, family B, member 1 interacting protein (APBB1IP) accordingly (2). This interaction is mediated by the WW (tryptophan-tryptophan) domain of Fe65 interacting with the proline-rich regions of RIAM (2). In an independent study, the gene encoding RIAM was also identified as transcriptionally induced in response to all-trans retinoic acid (ATRA) in the promyeloleukemic HL-60 cell line, and the protein was accordingly named retinoic acid-responsive proline-rich protein 1 (RARP-1) (3). In that system, it was found that forced expression in various cell types suppressed transactivation of activator protein 1 (AP-1) and serum response element (SRE), leading to the conclusion that this protein was functionally involved in cell growth arrest. An independent group also identified RIAM as an interactor of Enabled/vasodilator-stimulated proteins (Ena/VASP) family, which are involved in cell motility and actin polymerization, and named it proline-rich EVH1 ligand 1 (PREL1) (4). This study reported that RIAM colocalized with Ena/VASP proteins at the tips of lamellipodia and at focal adhesions in response to epidermal growth factor (EGF) treatment of fibroblasts. Because this event coincided temporally with Ras activation, the authors suggested that RIAM might link Ras signaling to cytoskeleton remodeling during cell migration and spreading (4). However, direct evidence for such interaction was not identified.

**Structure and Homologs of RIAM**

The open reading frame of RIAM is 1998 base pairs and encodes a protein of 665 amino acids. Structurally, RIAM contains an RA (RalGDS/AF-6 or Ras-association) domain, a PH (pleckstrin homology) domain, and two proline-rich regions. Two putative coiled-coil regions are present at the N terminus (amino acids 62 to 89 and amino acids 149 to 181) (Fig. 1A) (1).

Upon identification as a Rap1-interacting molecule and structural characterization of RIAM, database searches for homologous genes revealed that the proteins with highest homology to RIAM are 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, 5). Furthermore, RIAM is related to proteins CG11940 (AAF49029) in *Drosophila melanogaster* and Mig-10 (P34400) in *Caenorhabditis elegans* (Fig. 1A. Comparison of the domain structures of these proteins indicated that RIAM, Lpd, CG11940, and Mig-10 have a proline-rich region at the C terminus and a highly conserved pattern of 27 amino acids predicted to be a coiled-coil region immediately N-terminal to the RA domain. In addition, comparison of the RA and PH domains in RIAM-related proteins showed regions of these domains to be conserved among the proteins (1). Collectively, these proteins define the MRL (Mig-10/RIAM/Lpd) family (6). Phylogenetic analysis showed that the MRL proteins are conserved during evolution but *Drosophila* and *C. elegans* each only have one gene encoding an MRL family member (1). Mig-10 is the first member of the MRL family and was identified in a screen for mutations associated with neuronal cell migration defects during *C. elegans* embryogenesis. Specifically, the mig-10 gene is required for the long-range anteroposterior migration of the two canal-associated neurons, anterior lateral microtubule cells, and hermaphrodite-specific neurons and for proper development of the excretory canals (7).

Subsequently, mutations in the *Drosophila* MRL ortholog CG11940 were identified, and the gene was named *pico* due to the retarded growth phenotype resulting from *pico* knockdown or loss-of-function mutation (8). Reduction in *pico* expression in *Drosophila* resulted in animals with...
RIAM is proline-rich (12.9%) and contains six putative profilin-binding motifs (XP PPPP) and six putative Ena/VASP homology 1 (EHV1) protein-binding motifs (D/E)(F/L/W/Y)PPPXP(D/E)(D/E) (9). In addition, RIAM contains binding motifs for Src homology 3 (SH3) domain– and WW domain–containing proteins (Fig. 1B) (10). The N-terminal structure of RIAM differs from other MRL family members and contains a short, proline-rich N-terminal region with two putative EVH1-binding sites and one additional coiled-coil domain. RIAM and Lpd share conserved RA and PH domains, whereas the N- and C-terminal regions are more divergent (29.1% amino acid identity in the N-terminal region and 23.2% amino acid identity in the C-terminal region). In addition, the C-terminal region of Lpd is 500 amino acids longer than that of RIAM (1, 5).

Expression and Subcellular Localization of RIAM

Northern blot analysis has shown that RIAM is expressed broadly. Two transcripts of 5.4 and 2.8 kb were detected in hematopoietic tissues in approximately equal amounts, whereas in nonhematopoietic tissues, the larger transcript predominated. The functional importance of the differential expression of the two RIAM transcripts in these tissues has not yet been determined. Nonhematopoietic tissues, in which the RIAM transcript has been identified, include heart, brain, lung, liver, skeletal muscle, kidney, and pancreas. The abundance of the RIAM protein varies among tissues and cell types, but the greatest amount is detected in cells of hematopoietic origin (1, 3, 4).

RIAM is constitutively localized to the cytosol and is recruited to sites of actin dynamics upon cell activation. In human Jurkat T cells, human embryonic kidney (HEK) 293 cells, and mouse fibroblasts [both Swiss Albino 3T3 (SW3T3) and NIH 3T3] overexpressing RIAM, the protein is detected in the cytoplasm but also localizes to the plasma membrane and is concentrated at the tips of lamellipodia (1, 4). Studies in B16F1 mouse melanoma cells transfected with green fluorescent protein (GFP)–tagged RIAM showed that, in fixed cells seeded on fibronectin, RIAM was present at the tips of the lamellipodia and in focal adhesions, whereas in live cells moving on laminin, RIAM was targeted mainly to the tips of lamellipodia and, to a lesser extent, to focal adhesions (4). Treatment of live NIH 3T3 mouse fibroblasts expressing GFP-tagged RIAM with phorbol myristate acetate or with microinjection of a constitutively active human Ras mutant (RasV12) also induced recruitment of RIAM to focal adhesions and to the tips of lamellipodia, suggesting that recruitment of RIAM to these locations may depend upon the activation state of the cell.

Upon T cell activation, RIAM translocates to the actin cytoskeleton, as demonstrated by subcellular fractionation experiments and by confocal imaging of fixed cells (11). In human platelets plated on fibrinogen to induce spreading, RIAM localizes to vinculin-rich filopodia and lamellipodial edges (12). In fully spread platelets, RIAM is localized with vinculin and actin in structures that resemble focal adhesion complexes. In mouse fibroblasts, RIAM localizes to the focal adhesions in a manner that depends on interaction with the nonphosphorylated form of VASP (13).
Molecular Interactions of RIAM

Interactions of the RA and PH domains

The small GTPase Rap1 of the Ras oncogene superfamily has been linked to secretion and cell proliferation and migration. RIAM functions downstream of Rap1 in inside-out integrin signaling. In vitro data have suggested that RIAM interacts with guanosine triphosphate (GTP)–bound Rap1, but not with guanosine diphosphate–bound Rap1, and exhibits weak, rather nonspecific binding to other Ras GTPases (1, 14). The RA domain of RIAM has also been shown to interact with Ras by pull-down experiments using a recombinant glutathione S-transferase (GST)–tagged RIAM RA domain and lysates from NIH 3T3 cells transfected with Myc-tagged RasV12. Although the RIAM RA domain binds to both GTP-bound Rap1 and constitutively active Ras in vitro with similar affinities, only Rap1 controls RIAM translocation in intact cells (1, 4).

Interaction of Rap1-GTP with the RA domain of RIAM after T cell receptor (TCR)–mediated cell activation is required for translocation of Rap1-RIAM to the plasma membrane (1, 14). The PH domain of RIAM is also found to be required for Rap1-GTP to interact with RIAM in vitro (1). Although this in vitro finding was initially attributed to the biochemical properties and folding of the small recombinant proteins used to test these interactions, the crystal structure of the RIAM RA-PH revealed that these two domains form a single structural unit that is critical for RIAM function (14). RIAM colocalizes with Rap1-GTP only at the plasma membrane and not in any intracellular membrane compartment, in which Rap1-GTP is present, in contrast to the R-ras–binding domain (RBD) of guanine nucleotide dissociation stimulator for Ral (RalGDS-RBD), which colocalizes with Rap1-GTP in both compartments. Plasma membrane localization of RIAM requires Rap1-GTP but not Ras-GTP.

The affinity of the RIAM PH domain for various members of the phosphatidylinositol (PtdIns) family of lipids was initially investigated by probing lipid-coated membranes with various RIAM domains fused to GST. These studies showed that the PH domain of RIAM has specificity for phosphatidylinositol monophosphates PtdIns(3)P, PtdIns(4)P, and PtdIns(5)P (4, 5). Subsequently, by using various fluorescently labeled phosphatidylinositols (PPIs), recombinant RIAM RA-PH, and fluorescence polarization, it was discovered that RIAM has the highest binding affinity for phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂; also called PIP₂], which is present in the plasma membrane and a substrate for phospholipase C–γ1 (PLC–γ1), making this PIP the most likely physiological target of the RIAM PH domain (14). Analysis of the binding affinities of the RIAM RA and PH domains showed that both the RA and PH domains have relatively low affinity for their specific binding partners Rap1-GTP and PIP(4,5)P₂, respectively. Furthermore, the crystal structure of RIAM RA-PH showed that the RA and PH domains of RIAM form a single structural unit through an extensive RA-PH domain interface, which is further fortified by interactions from residues in the intervening linker region. On the basis of the crystal structures of other GTPase-RA domain complexes (15, 16), Lys₂¹³ in the α1 helix of the RIAM RA domain was predicted to interact with Asp¹⁹³ of Rap1. Mutation of this lysine residue prevented colocalization of RIAM RA-PH–GFP to the plasma membrane. The amino acid sequence in the B₁–B₂ loop region of the PH domain of RIAM conforms to the consensus K-X₉-X(K/R)—X-R sequence, which is associated with PIP binding (17). Mutation of Lys₂¹³, Arg⁴¹⁹, and Lys₂³⁷ within this sequence abrogated recruitment of RIAM RA-PH–GFP to the plasma membrane (14). These results provide evidence that binding of both components of the integrated RA-PH unit to their natural partners and RA-PH structural integration are required for recruitment of RIAM to the plasma membrane. This dual binding is likely required because both the RA and the PH domain bind their partners with relatively low affinity.

The crystal structure of RIAM RA-PH in complex with Rap1-GTP revealed that several side-chain interactions are critical in determining specificity of recognition of RIAM by Rap1-GTP (18). In particular, Lys¹⁸⁸ of Rap1, which is oppositely charged compared with the Glu¹¹¹/Asp³¹ residue in other Ras GTPases, forms a salt bridge with the RIAM residue Glu²³⁷, making it the key specificity determinant of the interaction. Disruption of these interactions results in the reduction of Rap1-RIAM association, leading to a loss of colocalization and cell adhesion, consistent with the finding that an intact Rap1-RIAM module is required for inside-out integrin activation (19).

Interactions of the proline-rich regions

RIAM has an N- and C-terminal proline-rich region, which contains well-defined proline-rich motifs (Fig. 1B). RIAM has six putative profilin-binding motifs (XPXPXP) and six putative EVH1-binding motifs (D/E)(F/L/W/Y)PPPPXXD/E(D/E). In addition, RIAM contains proline-rich motifs for binding of SH3 domain– and WW domain–containing proteins (10). The proline-rich regions of RIAM mediate interaction with the WW domain of Fe65 as determined by communoprecipitation of endogenous proteins (2). WW domain–mediated interactions have also been identified with formin-binding protein 11 (FBP11) and growth arrest–specific protein 7 (Gas7) (20). The proline-rich regions of RIAM also interact with profilin and with the EVH1 domain of Ena/VASP proteins, as determined by yeast two-hybrid assays, in vitro association of recombinant proteins, and communoprecipitation of endogenous proteins in Jurkat T cells (1). The interaction of RIAM with Ena/VASP family proteins was also identified by communoprecipitation of endogenous mammalian Ena (Mena) with GFP-tagged RIAM using NIH 3T3 cells. Interaction with Ena/VASP proteins was also detected by pull-down experiments using GST-tagged EVH1 domains and lysates of NIH 3T3 cells transfected with GFP-tagged N- or C-terminal RIAM constructs (4). RIAM-VASP interaction preferentially occurs with the nonphosphorylated form of VASP, as determined by immunoprecipitations of endogenous proteins in lysates of mouse fibroblasts isolated from wild-type and B₃ integrin–deficient animals, in which VASP was not phosphorylated due to impaired activation of protein kinase A (PKA) (13).

Profilin and Ena/VASP family proteins are important regulators of the actin cytoskeleton. Profilin associates with G-actin and promotes nucleotide exchange to create profilin-actin(adenosine 5′-triphosphate) complexes. When bound to profilin, actin monomers are added only to the barbed ends of F-actin (21). The Ena/VASP family members Mena, VASP, and Evl are recruited to sites of actin cytoskeleton remodeling, such as lamellipodia, filopodia, focal contacts, and the T cell–APC (antigen-presenting cell) contact site (22) but do not have an active role in the formation of T cell–APC conjugates (23). Each contains an EVH1 domain that interacts with the proline-rich motif D/E)(F/L/W/Y)PPPPXXD/E(D/E) (abbreviated as FPPP) present in proteins such as zyxin and vinculin that target Ena/VASP proteins to focal adhesions (9) or in FYN binding protein (FYB-120/130), also known as FYB, ADAP (adhesion- and degranulation-promoting adapter protein), and SLAP-130 (SLP-76–associated phosphoprotein of 130 kDa) [FYb/SLAP(ADAP)], which recruits Ena/VASP to the T cell–APC interface (24, 25). They also have proline-rich regions that bind to SH3 domain–containing proteins and profilin and an EVH2 domain that mediates their tetramerization and interacts with both G- and F-actin (22). Thus, by its interactions with profilin and with Ena/VASP family proteins, RIAM is linked to cytoskeletal modulation.
The proline-rich C-terminal region of RIAM interacts with the SH3 domain of PLC-γ1. This interaction was identified by in vitro association of recombinant RIAM and PLC-γ1 proteins and by pull-down experiments using various GST-tagged domains of PLC-γ1 and lysates from primary human T cells and from Jurkat T cells and, conversely, using GST-tagged domains of RIAM and lysates from the same cell types (11). These findings are intriguing because the RIAM PH domain has specificity for the PLC-γ1 substrate PI(4,5)P2 (14). Functional implications of RIAM–PLC-γ1 interactions are discussed in the section on T cell–specific signaling interactions of RIAM.

Interaction with talin

After Rap1 activation and recruitment of RIAM to the plasma membrane, RIAM recruits talin through an N-terminal talin-binding (TB) sequence of 103 amino acids that is predicted to form amphipathic helices. This interaction was identified by coimmunoprecipitation using lysates of Chinese hamster ovary (CHO) cells stably expressing integrin α1β1, and transiently cotransfected with hemagglutinin (HA)–tagged talin and various GFP-tagged human RIAM constructs of different lengths (26). Subsequently, it was discovered that the N-terminal domain of RIAM has two distinct TB sites (TB1 and TB2), but only TB1 can recruit talin to the plasma membrane (27). RIAM TB1 and TB2 can recognize multiple sites in talin-R (rod) and talin-H (head) F2F3 regions, suggesting that multiple RIAM molecules bind to a single talin molecule. The primary RIAM–integrating sites of talin are located in the F3 and R8 regions of talin. RIAM binding to talin-F3, which is adjacent to talin’s integrin-binding site, also located in talin-F3, competes with the autoinhibitory R9 domain of talin for binding to the talin-F3 domain, thereby promoting the unmasking of the integrin-binding site and allowing it to bind to integrin (28). Intriguingly, the TB region of RIAM may also promote RIAM autoinhibition by blocking the interaction between RIAM and Rap1 (14). Because the TB region is highly negatively charged, it may mask the positively charged PI(4,5)P2-binding surface in the PH domain. Additional aspects of the RIAM-talin interaction are discussed below in the section about the integrin activation machinery.

Interactions with T cell signaling proteins

RIAM participates in TCR signaling events. The TCR-proximal Src family kinases Pyn and Lck associate with and induce tyrosine phosphorylation of RIAM (29). RIAM is a critical node of signal integration downstream of the signalosome containing linker for activation of T cells (LAT) and SH2 domain–containing leukocyte protein of 76 kDa (SLP-76) and is critical for activation of PLC-γ1 downstream of the TCR (11). Through its proline-rich C-terminal region, RIAM interacts constitutively with the SH3 domain of PLC-γ1 (Fig. 2). Upon TCR activation, RIAM promotes PLC-γ1 recruitment to the actin cytoskeleton, which is essential for PLC-γ1 activity (30, 31). Hence, knockdown of RIAM in T cells results in impaired PLC-γ1 activity as determined by a diminished induction of inositol triphosphate production and reduced intracellular calcium release. Reduced production of these second messengers by knockdown of RIAM impairs activation of Ras guanyl-releasing protein 1 (RasGRP1) and calcium- and diacylglycerol-regulated guanine nucleotide exchange factors (CaDLG-GEFs), resulting in defective GTP loading of Ras and Rap1 and reduced nuclear translocation of the nuclear factor of activated T cells (NFAT) family of transcription factors. The combined defects in TCR-mediated activation of PLC-γ1 and Rap1-induced activation of integrins when RIAM is knocked down cause a profound reduction of interleukin-2 (IL-2) production in T cells.

The finding that RIAM mediates its effects on PLC-γ1 activation downstream of LAT–SLP-76 signalosome explains why abrogation of either SLP-76 or RIAM results in impaired activation of PLC-γ1 and production of IL-2.

RIAM was also found to interact constitutively with the scaffold protein Src kinase–associated phosphoprotein of 55 kDa (SKAP55), as determined by using GST–RIAM fusion proteins and lysates from Jurkat T cells (32). RIAM binding to SKAP55 did not interfere with RIAM binding to Rap1, but abrogation of the SKAP55–RIAM interaction led to impaired cell adhesion after TCR activation. This study identified the sequence encompassing the RA–PH domains of RIAM as the region through which association with SKAP55 was mediated. Because the RA domain of RIAM also interacts with Rap1 and the PH domain of RIAM also interacts with PI(4,5)P2, it remains unclear whether RIAM can interact with SKAP55, Rap1, and PI(4,5)P2 simultaneously or whether separate intracellular pools of RIAM might be involved in distinct interactions and functions. A summary of the domain-specific interactions of RIAM is shown in Fig. 2.

RIAM Is an Integral Part of the Integrin Activation Machinery

The Rap1–RIAM module is involved in inside-out integrin activation

Almost every cell process involves adhesion to the microenvironment through the extracellular matrix (ECM), a process that is mediated by integrins. Integrins are heterodimeric transmembrane receptors composed of one α and one β subunit. They link the cytoskeleton to the ECM by binding to ECM proteins through their large ectodomains and binding to the actin cytoskeleton microfilaments through their cytoplasmic tails. Integrin signaling promotes cell adhesion, proliferation, and migration and promotes cell survival through cross-talk with receptor tyrosine kinases (33–35). In migrating cells, nascent adhesions form at the leading edge after ECM engagement of integrins and are associated with polymerization of actin (36). Some of the nascent adhesions disassemble within minutes, whereas other persist, grow, and mature into focal complexes and then into focal adhesions. This evolution of adhesion complexes is driven by mechanical force (37). The ability of integrins to bind to ECM proteins is controlled by a distinct “inside-out” signaling mechanism, wherein a receptor-mediated intracellular signal induces a conformational change of the integrin cytoplasmic domain, which is relayed through the transmembrane region to the ectodomain, converting it from a low-affinity to a high-affinity ligand-binding state (38).

The small GTPase Rap1 has a key role in the activation of inside-out signaling that induces β1 and β2 integrin conformational changes, leading to cell adhesion. This function of Rap1 is induced in response to T cell stimulation by CD31 [also known as platelet endothelial cell adhesion molecule–1 (PCAM-1)] with an anti-CD31–specific antibody but also with phorbol ester and, in addition to cell adhesion, mediates T cell migration in response to chemokines (39–41). These in vitro findings regarding the role of Rap1 in promoting integrin activation were validated and confirmed by findings in lymphocytes from Rap1A-deficient mice, which exhibited impaired LFA-1 polarization in vitro, although no measurable defects in lymphocyte function were observed in vivo (42). Conversely, T cells from mice expressing constitutively active, GTP-bound Rap1A have increased integrin activation and integrin-mediated adhesion (43). In contrast to Rap1A, which is ubiquitously expressed, Rap1B (encoded by a different gene) is expressed predominantly in platelets and is involved in the activation of αIIbβ3 integrin (44).
When RIAM was identified as a Rap1 effector molecule, it was investigated whether the Rap1-RIAM interaction might be involved in inside-out integrin activation. Using in vitro cell adhesion models, it was shown that RIAM is a Rap1 effector in inside-out signaling and that RIAM is required for Rap1-induced affinity changes in β, and β integrins in T cells (1). Subsequent studies demonstrated that RIAM is also involved in Rap1-mediated activation of αβ integrin in platelets (45). Platelet aggregation requires incubation with a compound that induces αβ activation, which is mediated by Rap1-GTP and talin. Using a pathway reconstruction CHO cell experimental system, it was determined that RIAM is mandatory for this process because knockdown of RIAM abrogated αβ activation in the presence of Rap1-GTP. In platelets, RIAM localizes to filopodia, lamellipodia, and focal adhesion–like structures. In primary megakaryocytes, RIAM knockdown blocks αβ activation induced by thrombin protease–activated receptors, which is mediated in a manner that depends on Rap1-GTP and talin (45). The molecular and functional properties of RIAM and its selective effect in promoting conformational changes of the integrin β chain distinguish RIAM from the Rap1 adaptor regulator of adhesion and cell polarization enriched in lymphoid tissues (RapL), which promotes LFA-1 activation by interacting with the integrin α chain (46).

The RIAM-talin module promotes conformational changes required for integrin activation
Talin is a large protein that is organized into an N-terminal head (1 to 433, talin-H; 50 kDa), which contains an F0 domain and a FERM domain (including F1, F2, and F3 subdomains), and a C-terminal rod (482 to 2541, talin-R; 220 kDa), which is made up of 13 consecutive helical bundles followed by an actin binding motif (Fig. 3A). RIAM recruits talin through the specific TBS1 corresponding to RIAM residues 7 to 30 (26). The Rap1-RIAM complex promotes localization of talin to the plasma membrane through the membrane-anchoring capacity of RIAM’s RA (to Rap1) and PH (to PI(4,5)P2) domains (44). Multiple interacting sites between talin-R domains and RIAM have been identified. A second TB site in RIAM (TBS2, residues 50 to 85) was also identified (47). This region can interact with the R2 and R3 domains of talin. However, TBS1, but not TBS2, interacts with talin in the cytoplasm, and the R8 domain of talin, which forms hydrophobic and electrostatic interactions with RIAM, is the strongest binding site for the TBS1 region of RIAM. The interaction of RIAM TBS1 with talin R8 is responsible for recruiting talin to the plasma membrane (27). Furthermore, RIAM TBS1 binds to talin-H region at the F3 subdomain, located in close proximity to the integrin-binding site, which is also located in the F3 subdomain of talin (28). The interaction of RIAM TBS1 with talin-F3 promotes the conformational opening of latent talin, leading to the binding and activation of integrin (Fig. 3B).

The multiple binding sites of RIAM on talin-R may cooperatively strengthen RIAM binding to talin, thus leading to effective RIAM-talin colocalization to the membrane. However, it is the interaction of RIAM TBS1 with the talin-H region that leads to talin activation by sterically preventing the binding of talin-R domain with the talin-H domain, an autoinhibitory interaction that masks the integrin-binding site of talin-H (Fig. 3B) (28). RIAM also binds talin-R through a site in talin-R that partially overlaps the binding site for vinculin, a major focal adhesion adaptor. Structural and functional analysis revealed that vinculin can displace RIAM on talin-R and can function as a switch to promote the maturation of focal adhesions and their turnover. Thus, after integrin activation, RIAM may be displaced from talin by vinculin, which triggers focal adhesion reassembly. The vinculin-talin-integrin and RIAM-talin-integrin modules differ in abundance at various cellular sites, in the mechanisms that regulate their formation, and in their major functions (47).

The MRL-talin-integrin complex drives migratory protrusions
RIAM is abundant at the cell edge and at the lamellipodium, where it promotes protrusion (48). Protrusive activity is likely due to the ability of RIAM to increase actin polymerization, most likely due to its interaction with profilin and Ena/VASP family proteins. Consistent with this hypothesis, overexpression of RIAM in Jurkat T cells and in HEK293 cells induced lamellae formation and cell spreading, whereas knockdown of RIAM resulted in defective actin polymerization and reduced the amount of polymerized actin (1). RIAM seems to interact preferentially...
with the nonphosphorylated form of VASP, which is a substrate of PKA. Impaired activation of PKA in mouse fibroblasts deficient in β3 integrin resulted in loss of PKA-dependent phosphorylation of VASP, enhanced association of nonphosphorylated VASP with RIAM, and enriched formation of the VASP-RIAM complex at focal adhesions, leading to increased binding of talin to β3 integrin. These events resulted in enhanced focal adhesion turnover and migration (13). RIAM colocalizes with talin in lamellipodia and filopodia, and this might play a role in the localization of activated integrins at these membrane protrusions (12, 26, 45). In contrast to RIAM, vinculin is enriched in maturing focal adhesions, which are present on and at the border of lamellipodia, where vinculin reinforces the ability of the adhesion to transmit and bear force (47, 49).

Live cell imaging combined with indirect bimolecular fluorescence complementation indicated that a RIAM–integrin α5β3–talin complex is enriched at the tips of growing actin filaments in lamellipodial and filopodial processes, thus revealing the molecular basis of the formation of the so-called sticky fingers of these protrusions (50). Although RIAM is most abundant at the cell edge and lamellipodium, it is subsequently reduced in mature adhesions due to direct competition with vinculin for binding to talin (48). Vinculin stabilizes adhesions and increases their ability to transmit forces, whereas RIAM promotes lamellipodial protrusion. The transition of integrin-based adhesions from drivers of lamellipodial protrusion that contain RIAM, but not vinculin, to stable focal adhesions that contain vinculin, but not RIAM, delineates a molecular switch in adhesion maturation. Disruption of this complex by mutations that affect the TB site in RIAM results in impaired cell protrusion (48). The protrusive activity stimulated by RIAM is likely mediated by reorganization of the actin cytoskeleton through its binding to both profilin and Ena/VASP proteins (1).

A similar result was also observed when α5β1 integrin was used instead of integrin α5β3, or the formation of Lpd-talin-integrin complexes (50). In this experimental approach, talin-bound Lpd, which is a RIAM paralog present in all species except C. elegans and Drosophila, also formed a complex with α5β1 integrin at the tips of actin-based protrusions. Together, these findings lead to the conclusion that either mammalian MRL protein (RIAM or Lpd) can form a complex with activated integrin and talin (called the MIT complex) at the tips of sticky fingers. In this complex, the N terminus of the MRL protein binds to and recruits talin to the plasma membrane to induce integrin activation. The C-terminal portion of RIAM and other MRL proteins stimulate progressive actin polymerization, in part, by recruiting Ena/VASP, thereby propelling the movement of the “sticky fingers” ends of lamellipodia and filopodia.

**Involvement of RIAM on Cancer Cell Adhesion, Invasion, Migration, and Growth**

Rap1 is among the most closely related proteins to members of the Ras oncogene superfamily (51) but serves functions that are distinct from those of H-, K-, and N-Ras. Rap1 has been linked to cellular proliferation and migration (52, 53), and aberrant Rap1 activity can lead to integrin hyperactivity that is linked to tumor development and metastasis in several cancer types (54–59). Sustained activation of Rap1 in mouse hematopoietic stem cells causes expansion of hematopoietic progenitors, followed by a myeloproliferative disorder mimicking chronic myeloid leukemia (58). Notably, both a constitutively active Rap1 mutant and Rap1 that has been activated in response to EGF1-mediated stimulation localizes predominantly in the nucleus in epithelial cells (60). It was determined using a high-throughput proteomic approach that several gene products encoding cytoskeletal regulator proteins, signaling molecules, transcription factors, viability regulators, and protein transporters are induced by Rap1-GTP, the active form of Rap1. Notably, under these conditions, fused in sarcoma–translocated in sarcoma (FUS-TLS), which is involved in the regulation of adhesion by the formation of spreading initiation centers (61), is highly induced by Rap1-GTP (60).

As adhesion receptors, integrins transduce signals in a bidirectional manner (62). Integrin signaling can be triggered by an inside-out pathway,
which requires the recruitment of active Rap1, RIAM, and talin to the plasma membrane (1, 26). Conversely, during outside-in signaling, integrin binds to the ECM, forms highly organized clusters, and initiates downstream signaling cascades in the cytoplasm (37). As mentioned above, in migrating cells, nascent adhesions form at the leading edge after ECM engagement of integrins, and these are associated with polymerization actin (36). A proportion of these nascent adhesions grow and mature into focal adhesions that have a central role in driving cancer cell migration and invasion. It has been determined that RIAM depletion in human melanoma cells leads to impairment in persistent cell migration directionality, thus reducing melanoma cell invasion (63). Furthermore, RIAM-depleted melanomas display fewer lung metastatic lesions in a mouse xenograft model. The compromised invasiveness of RIAM knockdown cells correlated with deficient association between β1 integrin and talin and with inhibition of β1 integrin–dependent activation of the extracellular signal–regulated kinase 1 (ERK1) and ERK2 mitogen-activated protein (MAP) kinases. In an independent study, RIAM-depleted melanoma cells were found to have increased numbers and stability of focal adhesions, which was interpreted as resulting from defective focal adhesion disassembly (64). Alteration of focal adhesion turnover in these cells was associated with deficient activation of MAP kinase kinase (MEK), and overexpression of constitutively active forms of MEK rescued focal adhesion disassembly and cell invasion.

Additional studies support the intriguing hypothesis that the Rap1-RIAM module might play an important role in cancer growth by mediating the recruitment of cancer-promoting myeloid lineage cells to the tumor microenvironment. Recruitment of myeloid lineage cells into the tumor microenvironment promotes angiogenesis, immunosuppression, and metastasis (65). A key mechanism involved in the recruitment of CD11b+Gr1hi monocytic lineage cells and CD11b+Gr1hi granulocytic lineage cells by tumor-derived chemokines is increased numbers and stability of focal adhesions, which is associated with defective activation of MAP kinase kinase (MEK), and overexpression of constitutively active forms of MEK rescued focal adhesion disassembly and cell invasion.

RIAM Has an Active Role in Innate Immunity

RIAM is also a regulator of leukocyte recruitment (adhesion, transmigration) and pathogen clearance through complement-mediated phagocytosis, revealing an important role of RIAM in activation and modulation of innate immune responses.

Complement-mediated phagocytosis

Opsonization is the process by which antigens are molecularly tagged to enhance their phagocytosis by immune cells. The β2 integrin phagocytic complement receptors CR3 and CR4 are classically involved in the recognition and internalization of particles bound to iC3b, a proteolytically inactive cleavage fragment of complement component 3b (C3b) (Fig. 4A). These complement receptors play an important role in eliminating complement-opsonized pathogens and apoptotic particles and contribute to cell homeostasis during tissue remodeling (69). Their role as important modulators of the host inflammatory response is highlighted by the fact that they are exploited by pathogenic bacteria and viruses to potentiate host cell invasion, hijacking signaling downstream of the receptors to reduce inflammatory cytokine production (70, 71). In a similar fashion, activation of CR3 in dendritic cells during apoptotic cell clearance promotes intracellular tolerogenic signals (72). Recent reports describe a role of RIAM in the regulation of CR3 activity.

The CR3 receptor is present mainly in myeloid cells (monocytes, macrophages, neutrophils, and dendritic cells) and in a certain subset of natural killer (NK) cells. It is implicated in phagocytosis, cell-mediated killing, and chemotaxis through its interaction with structurally unrelated ligands [iC3b, intercellular adhesion molecule-1 (ICAM-1), fibrinogen, coagulation factor X, denatured proteins, and various pathogen-derived molecules] (73). CR3 activity is tightly regulated, requiring inside-out integrin signaling downstream of various receptors, such as FcγR, TRLA, fMLP receptor, cytokine receptors, and CD44 (74, 75). Activation of these receptors culminates in the binding of talin to the short cytoplasmic tail of the β chain of integrin αMβ2 (also called macrophage-antigen or Mac-1). Ligand binding further stabilizes the high-affinity conformation of integrin αMβ2, which entails separation of the integrin cytoplasmic tails, allowing for the recruitment of intracellular effector proteins (76). Outside-in signaling is then initiated, promoting actin cytoskeleton remodeling to ensure particle internalization, cell proliferation, and survival, or phagocytosis-induced apoptosis (77, 78).

The increase in complement-dependent phagocytosis after inside-out activation of integrin αMβ2 by CR3 depends on Rap1 activity. In macrophages, expression of the constitutively active mutant Rap1V12 triggered complement-mediated phagocytosis in the absence of agonist stimulation, whereas expression of the dominant-negative mutant Rap1N17 blocked lipopolysaccharide (LPS)–induced phagocytosis (79). Rap1 activation correlates with talin recruitment to integrin αMβ2 during phagocytosis (80), although there is no evidence of a direct interaction between Rap1-GTP and talin. Different Rap1 effectors were under consideration as potential candidates to promote talin recruitment to phagocytic cups, among them RapL, afadin adherens junction formation factor 6 (AF-6), Krev interaction trapped protein 1 [Krit-1; also known as cerebral cavernous malformation-1 (CCM1)], and RIAM (81). RIAM has been shown to promote the acquisition of the high-affinity state of αMβ2 as demonstrated by a reduction in the binding of an activation reporter monoclonal antibody (CBRM1/5) to the αM subunit in human promyelocytic cell lines HL-60 and THP-1, in which RIAM was knocked down by RNA interference. Whereas in control cells LPS or N-formyl-Met-Leu-Phe (fMLP) chemotactic peptide stimulation resulted in increased CBRM1/5 binding, in RIAM knockdown (KD) cells CBRM1/5 binding was abrogated, which points to RIAM as essential for full αMβ2 activation (82).

In accordance with the defective acquisition of the αMβ2 high-affinity state, this study showed that RIAM knockdown reduced complement-dependent phagocytosis in response to LPS or fMLP treatment as compared...
Fig. 4. RIAM in innate immune responses. (A) RIAM enhances pathogen clearance through complement-mediated phagocytosis and ROS production. (B) RIAM is required for neutrophil migration, adhesion, extravasation, and polarity in response to chemokines. (C) RIAM is involved in activation of integrin αMβ2 (Mac-1), which is a critical step in the cooperative actions of neutrophils and platelets in producing neutrophil extracellular traps (NETs). (D) RIAM is involved in talin recruitment to the integrin cytoplasmic tail and Pyk2 activation, which are critical events for NK cell cytotoxic function.
to control cells (82). Moreover, the reduction of $\alpha_M\beta_2$ activation and impaired phagocytosis in RIAM KD cells was observed even when Rap1 was activated with 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (8-CPT-CAMP), a cAMP analog that specifically activates the Rap1–guanine nucleotide exchange factor exchange nucleotide protein directly activated by CAMP (EPAC). Similar results were obtained when RIAM was knocked down in human peripheral blood monocyte–derived macrophages, confirming that $\alpha_M\beta_2$ activation and complement-dependent phagocytosis induced by Rap1 were mediated by RIAM. As pointed out above, talin plays a crucial role in stimulating $\alpha_M\beta_2$ activity by binding to the cytoplasmic tail of the $\beta_2$ integrin subunit and inducing integrin activation. Communoprecipitation experiments done in neutrophil-like differentiated HL-60 cells during complement-dependent phagocytosis demonstrated reduced talin recruitment to $\beta_2$ integrin when RIAM was knocked down. Confocal microscopy studies also indicated that talin localization to phagocytic cups containing the complement receptor was impaired in RIAM KD HL-60 cells (82). Together, these findings point toward a role of RIAM in linking Rap1 activation to talin recruitment to $\alpha_M\beta_2$ integrin, thereby promoting its activation by CR3 and complement-dependent phagocytosis (Fig. 4A).

The role of RIAM in promoting complement-dependent phagocytosis was assessed in an independent study using RIAM-null polymorphonuclear leukocytes (PMNs) and serum-opsonized bacteria. RIAM−/− PMNs presented reduced bacterial uptake compared to wild-type PMNs. Analysis of the respiratory burst in response to tumor necrosis factor–α (TNF-α) revealed a reduction in free reactive oxygen species (ROS) production in RIAM−/− PMNs, pointing to a role of RIAM in $\beta_2$ integrin–mediated outside-in signaling (83). These results are in accordance with data obtained from RIAM KD human promyelocytic cells and indicate an essential role of RIAM in $\beta_2$ integrin–mediated phagocytosis (82).

### Adhesion, extravasation, and motility of innate immune cells

Integrin $\alpha_M\beta_2$ activation and ICAM-mediated adhesion of leukocytes to the endothelium are required for transendothelial migration and extravasation (Fig. 4B). Adhesion and spreading in response to binding ICAM-1, but not to binding vascular cell adhesion molecule–1 (VCAM-1), are reduced in RIAM-null PMNs in vitro, and reduced leukocyte adhesion and extravasation in response to chemokines were observed in vivo. RIAM-null mice exhibit a marked leukocytosis due to an increased number of neutrophils in the peripheral blood (83), a hallmark of leukocyte adhesion deficiency (LAD) syndromes associated with defects in leukocyte extravasation (84). A role of RIAM in neutrophil polarity has also been identified. Specifically, RIAM knockdown in neutrophil-like PLB-985 cells resulted in the loss of a clearly defined leading edge on two-dimensional (2D) surfaces and impaired directionality (49) toward fMLP, decreased migration and chemotaxis velocity, and reduction of protrusion formation in 3D Matrigel (82). These findings contrast with those from migration analyses of cells in which Kindlin-3, an integrin coactivator that interacts with talin, was knocked down. In Kindlin-3 KD cells, polarization was normal, yet cell motility and directionality were impaired because of a reduction in F-actin content at the leading edge (85). On the basis of these observations, Yamahashi et al. (85) speculated that Kindlin-3 works in parallel with RIAM, because Kindlin-3 acts through inside-out signaling to facilitate directionality, whereas RIAM enables outside-in signaling to induce leading edge formation.

### The Role of RIAM in Hematopoietic Cells In Vivo

The role of RIAM in promoting agonist-mediated activation of integrin $\alpha_{Ib}\beta_2$ has been well documented by in vitro reconstitutive studies. Moreover, RIAM knockdown blocks agonist-mediated $\alpha_{Ib}\beta_2$ activation in primary mouse megakaryocytes (12). Despite the compelling in vitro findings, indicating an important role of the Rap1–RIAM–talin module in $\alpha_{Ib}\beta_2$ integrin activation, genetic deletion of RIAM in mice did not affect platelet developmental, homeostasis, or platelet-related integrin functions (83, 86, 87). RIAM protein abundance is low in platelets, whereas Rap1, talin1, and integrins are highly abundant in these cells. Together, these findings indicate that RIAM-independent mechanisms exist for Rap1 to mediate its effects on platelet integrin function under physiologic conditions. Moreover, Rap1-independent mechanisms of integrin activation have been identified in platelets (88), which might have a compensatory role in the absence of a functional Rap1–RIAM pathway.

The selective role of RIAM on integrin function in vivo is also supported by other findings in RIAM knockout mice, which display only a partial defect in the function of $\beta_2$ integrin but a more pronounced impairment of $\beta_3$ integrin activation. As a consequence, RIAM-deficient mice exhibit leukocytosis due to impaired leukocyte-endothelium adhesion and extravasation (83). Consistent with the indispensable role of RIAM in trafficking of B and T lymphocytes to secondary lymphoid organs, RIAM knockout mice display impaired humoral responses to T-dependent antigen (87).

Because the mandatory role of RIAM for $\beta_2$ integrin activation in vivo has been established, there is ample reason to speculate that RIAM is involved in functions of innate immune cells for which $\beta_2$ integrin activation is indispensable. Neutrophils cooperate with platelets to produce NETs, structures composed of histones and DNA that trap extracellular pathogens (Fig. 4C). The formation of these structures is mediated by neutrophil $\alpha_M\beta_2$ integrin and is tightly connected to ROS production (89). RIAM KD cells have reduced integrin activation, and RIAM−/− neutrophils have reduced phagocytosis and ROS production (82, 83). Together, these findings are highly suggestive that NET production could depend on RIAM.

RIAM−/− macrophages exhibit defective autophosphorylation of Pyk2 at Y402 in response to adhesion on ICAM-1 without major changes in phosphorylation of focal adhesion kinase (FAK) or Src. Pyk2 activity after $\beta_2$ integrin activation has also been correlated with neutrophil degranulation responses and efficient clearance of *Staphylococcus aureus* infection (90). Degranulation and ROS production in eosinophils stimulated by granulocyte macrophage colony-stimulating factor (GM-CSF) and platelet activating factor (PAF) are known to be mediated by integrin $\alpha_M\beta_2$ (91) and correlate with greater abundance of active eosinophil $\alpha_M\beta_2$ integrins, as observed in lung eosinophils from asthmatic patients (92).

In NK cells, LFA-1 engagement with ICAM-1–coated beads was found to be necessary and sufficient to produce granule polarization when cells were treated with IL-2 (93). LFA-1–mediated adhesion of NK cells to insect cells expressing human ICAM-1 resulted in target cell killing. Cytotoxicity was diminished when the insect cells coexpressed ICAM-1 and HLA-Cw4, a ligand for killing-inhibitory receptors on NK cells, indicating that $\beta_2$ integrin induces early NK activation signals that can be modulated by these inhibitory receptors (93). LFA-1 activation in NK cells also resulted in Pyk2 autophosphorylation and talin enrichment at the site of contact between the NK cell and the target cell (Fig. 4D) (94). Moreover, granule polarization and cytotoxicity require Pyk2 activation (95). Given that Pyk2 activation is abrogated in neutrophils from RIAM-null mice (83) and that recruitment of talin to the
integrin cytoplasmic tail requires RIAM (27, 50), RIAM could play a role in the activation of NK cell cytotoxic functions.

Thus, multiple lines of evidence converge onto the idea that RIAM is essential for effective innate immune responses. First, RIAM orchestrates the migration of PMNs and macrophages to sites of infection due to its role in regulating adhesion, polarization, directional migration, and extravasation. Second, RIAM plays a critical role in pathogen clearance by promoting complement-dependent phagocytosis and subsequent ROS production. Finally, degradation of PMNs, eosinophils, and NK cells requires $\beta_2$ integrin engagement, which, in turn, is dependent on RIAM function. Therefore, an important role of RIAM in the regulation of innate immune host defense can be inferred.

Concluding Remarks, Challenges, and Future Directions

RIAM was identified as an adaptor defining the MRL family of proteins, a binding partner of APBB1, a protein induced in response to retinoic acid in promyelocytic cells, an Ena/VASP interactor, and a Rap1-interacting molecule and is now a well-established regulator of multiple functions in immune cells. RIAM is involved in reorganization and modulation of the actin cytoskeleton, activation of integrins, cell adhesion, and migration. In vivo, RIAM seems to have an indispensable role in $\beta_2$ integrin activation and function but only a minor impact on activation of $\beta_1$ and $\beta_3$ integrins. RIAM is involved in the regulation of innate and adaptive immune responses and in the invasion, migration, and metastasis of cancer cells. RIAM promotes both inside-out and outside-in integrin signaling. It is also involved in stabilizing PLC-$\gamma$1 in close proximity to the cytoskeleton and at the plasma membrane and interacts specifically with the PLC-$\gamma$1 substrate PI(4,5)P2. These properties of RIAM make it an attractive therapeutic target to selectively modulate inflammatory cell processes mediated by $\beta_2$ integrin and block cancer cell migration.

The selective effect of RIAM on $\beta_2$ integrin activation in vivo indicates that RIAM-independent Rap1-mediated activation of the talin-integrin module promotes $\beta_1$ and $\beta_3$ integrin activation in vivo. Although detailed studies have determined how RIAM-talin interaction leads to talin conformational changes and integrin activation as well as re-localization of the RIAM-talin-integrin complex to the leading edge of cell protrusions during cell migration, it is clear that potent alternative mechanisms for integrin activation are also present. It will be important to identify such mechanisms and dissect their contribution in a cell- and tissue-specific manner. For example, how is talin recruited to the plasma membrane in the absence of RIAM? How is conformational opening of latent talin induced, leading to the integrin binding and activation in RIAM-deficient cells, which retain in vivo activation of $\beta_1$ integrins almost intact? One challenge is to determine and quantify the contribution of RIAM-dependent and RIAM-independent pathways downstream of Rap1 in the function of innate immune cells in the context of infection, inflammation, and cancer. An additional challenge is to identify how the multiple molecules and pathways involved in integrin activation converge to regulate cell adhesion and transendothelial migration.

In the context of cancer, RIAM might have a role in regulating not only cancer cell migration and metastasis but also the properties of the tumor microenvironment by controlling recruitment of tumor-promoting myeloid cells, which confer resistance to cancer immunotherapy. The selective effects of RIAM on $\beta_2$ integrin might provide a novel therapeutic target for selective modulation of immune cells in certain microenvironments while leaving platelet function intact. Such therapeutic approaches might be highly beneficial in autoimmune diseases, inflammation, and cancer.

REFERENCES AND NOTES


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The adaptor molecule RIAM integrates signaling events critical for integrin-mediated control of immune function and cancer progression

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