



Molecular basis for autoinhibition of RIAM regulated by FAK in integrin activation

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RAP1-interacting adapter molecule (RIAM) mediates RAP1-induced integrin activation. The RAS-association (RA) segment of the RA-PH module of RIAM interacts with GTP-bound RAP1 and phosphoinositol 4,5 bisphosphate but this interaction is inhibited by the N-terminal segment of RIAM. Here we report the structural basis for the autoinhibition of RIAM by an intramolecular interaction between the IN region (aa 27–93) and the RA-PH module. We solved the crystal structure of IN-RA-PH to a resolution of 2.4-Å. The structure reveals that the IN segment associates with the RA segment and thereby suppresses RIAM:RAP1 association. This autoinhibitory configuration of RIAM can be released by phosphorylation at Tyr45 in the IN segment. Specific inhibitors of focal adhesion kinase (FAK) blocked phosphorylation of Tyr45, inhibited stimulated translocation of RIAM to the plasma membrane, and inhibited integrin-mediated cell adhesion in a Tyr45-dependent fashion. Our results reveal an unusual regulatory mechanism in small GTPase signaling by which the effector molecule is autoinhibited for GTPase interaction, and a modality of integrin activation at the level of RIAM through a FAK-mediated feedforward mechanism that involves reversal of autoinhibition by a tyrosine kinase associated with integrin signaling.

RIAM | integrin signaling | focal adhesion kinase | autoinhibition | small GTPase

Adhesion of leukocytes to blood vessels, antigen-presenting cells (APCs), and target cells is critical to a wide range of immune functions and must be tightly regulated (1). Leukocyte adhesiveness is controlled, in large part, by integrins on their surface, including LFA-1 ($\alpha_L\beta_2$), MAC-1 ($\alpha_M\beta_2$), and VLA4 ($\alpha_4\beta_1$) (2). Integrins are type I transmembrane receptors composed of $\alpha\beta$ heterodimers where the extracellular domains interact with extracellular matrix or cognate ligands on cells to establish adhesion. LFA-1 is the principal integrin on lymphocytes and binds to a family of intercellular adhesion molecules (ICAM-1,2,3) on the surface of endothelial cells and APCs (3). Like all integrins, LFA-1 exists in multiple affinity states that correlate with various conformations of the ectodomains. These conformations are, in turn, controlled by the proximity of the α and β cytoplasmic domains, which is regulated by a variety of cytoskeletal proteins including talin (4). The process of increasing adhesion by transmitting conformational information from the cytoplasmic domains to the ectodomains is referred to as inside-out signaling (5). Inside-out signaling follows leukocyte activation downstream of a variety of receptors, including the T cell receptor (TCR) and chemokine receptors (6). The molecular events that mediate inside-out signaling have been the subject of intense investigation.

Among the signaling molecules that have been implicated in inside-out signaling is the small GTPase RAP1. Small GTPases are binary molecular switches that invariably signal through effector molecules that bind to the GTPases only when they are GTP-bound. Two such effectors of RAP1 have been implicated in inside-out signaling to integrins, RAPL and RAP1-interacting adaptor molecule (RIAM), the latter is a hematopoietic-restricted homolog of the more ubiquitously expressed lamellipodin (LPD).

Knockout *RAP1* or *RIAM* diminishes lymphocyte adhesion and lymphocyte homing (7, 8), which recapitulates the immunological phenotype of LFA-1 deficiency in mice (9, 10). Thus, RIAM appears to be essential for inside-out signaling to integrins in lymphocytes. Consistent with this observation, RIAM expression in T cells is recently reported to be necessary for immune-mediated diabetes (11).

RIAM is a multidomain scaffold protein that is regulated by recruitment from the cytosol to the plasma membrane (PM). The RAS-association (RA) and Pleckstrin-homology (PH) regions of RIAM fold into a single structural module (RA-PH) that mediates PM association by functioning as a coincidence detector for GTP-bound RAP1 and phosphoinositol 4,5 bisphosphate [PI(4,5)P₂] (12). The N terminus of RIAM binds talin, suggesting that RIAM may act as a bridge between the small GTPase switch and the cytoskeletal regulator of integrins. Indeed, RAP1, RIAM, and talin were recently found to colocalize at the tip of actin protrusions of migrating cells forming a “sticky finger” (13).

Whereas the isolated RA-PH module of RIAM tagged with green fluorescent protein (GFP) readily translocates to the PM upon activation of lymphocytes and can be seen constitutively at the PM in cells expressing activated RAP1-G12V, full-length RIAM-GFP remains in the cytosol (12). Deletion of the N terminus of RIAM significantly enhances colocalization with RAP1-G12V on the PM, suggesting that accessibility of the RA-PH module is autoinhibited by the N terminus. Here we identify an intramolecular interaction between the RA region of the

Significance

Adhesion of leukocytes to blood vessels, antigen-presenting cells, and target cells is critical to a wide range of immune functions. Adhesion is mediated by integrins on the cell surface, activity of which is controlled by a RAP1-dependent “inside-out” signaling pathway. Here we report the structural basis for an unusual regulatory mechanism of RAP1, a Ras-family small GTPase, by which its effector RAP1-interacting adapter molecule (RIAM) is autoinhibited, and can be activated through phosphorylation by focal adhesion kinase (FAK). Interestingly, FAK also mediates the outside-in integrin signaling at focal adhesions. Thus, our findings also suggest a feedforward mechanism that links outside-in to inside-out integrin signaling through FAK.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, [www.wwwpdb.org](http://www wwwpdb.org) (PDB ID code 6E31).

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RA-PH module and a helical sequence near the amino terminus of RIAM that we designate the inhibitory region (IN). Our results provide a structural basis for how RIAM is autoinhibited as a RAP1 effector by an intramolecular interaction, and reveal regulation of this autoinhibition by phosphorylation of RIAM Tyr45 by focal adhesion kinase (FAK).

Results

An Inhibitory Segment (IN) Near the RIAM N Terminus Interacts Directly with the RA-PH Module and Inhibits Translocation of RIAM to the PM. By assaying colocalization on the PM of GFP-tagged RIAM and mCherry-tagged constitutively active RAP1-G12V, we identified an autoinhibitory segment immediately downstream of the talin-binding site (TBS, aa 1–30) (12). To better define the IN segment that could interact with the RA-PH module, we generated a series of constructs containing various functional segments of RIAM for biochemical and crystallographic analyses (Fig. 1A). To define intramolecular interactions, we performed affinity pull-down assays with GST extended with various RIAM N-terminal segments and purified 6xHis-RA-PH. Whereas GST-TBS pulled down no more RA-PH than did GST alone, GST extended with the N terminus of RIAM (GST-NT, residues 1–93) efficiently pulled down the RA-PH module. Importantly, GST extended with aa 27–93 (GST-IN) was equally efficient in pulling down RA-PH (Fig. 1B). Thus, the IN segment of the RIAM N terminus interacts with the RA-PH module, suggesting that this intramolecular interaction could mediate autoinhibition by blocking access to active RAP1 and/or PI(4,5)P₂.

To assess the effect of the interaction of IN and RA-PH on RAP1-dependent membrane association of RIAM, we examined the ability of various GFP-tagged RIAM proteins to colocalize

with constitutively active mCherry-RAP1-G12V in Jurkat T cells. As expected, full-length RIAM (1–670) showed minimal translocation. A truncation missing only the TBS segment (RIAM-ΔTBS) behaved like the full-length protein and exhibited minimal PM-colocalization with RAP1 (Fig. 1C and D). In contrast, removal of the N terminus (NT) or the helical region of the IN segment (RIAM-ΔNT and RIAM-ΔIN) showed robust colocalization. Further removal of the C-terminal segment downstream of the PH domain and N-terminal fragments (RIAM_{94–437}, RIAM_{128–437}, and RIAM_{150–437}) showed a degree of colocalization equivalent to RIAM-ΔIN and RIAM-ΔNT, suggesting that the IN segment is sufficient to inhibit the RAP1-dependent PM translocation of RIAM.

A Crystal Structure of the RIAM RA-PH Module Extended with the IN Segment Reveals the Atomic Basis for an Intramolecular Interaction and Steric Inhibition of RAP1 Binding.

We next sought to understand the structural basis for the interaction between the IN segment and the RA-PH module. Attempts at cocrystallizing the isolated IN fragment with the RA-PH module failed as a consequence of poor protein solubility. We therefore tested a series of constructs that keep the IN segment with the RA-PH module in a single polypeptide. We determined the crystal structure of a protein consisting of IN (aa 27–93) followed by the coiled-coil (CC) segment and the RA-PH module (aa 150–437), designated IN-RA-PH (Fig. 1A). The structure was determined to a resolution of 2.4-Å (Table 1). In the crystal structure of the IN-RA-PH, the RA-PH module is identical to our previous structure of RA-PH with the CC extension (PDB code 3TCA), with an rmsd of 0.29 Å (residues 178–437, all atoms). The RIAM IN segment is well ordered from residues 44–73 and forms two short α-helices. The α1 helix consists of residues 51–59 and the α2 helix

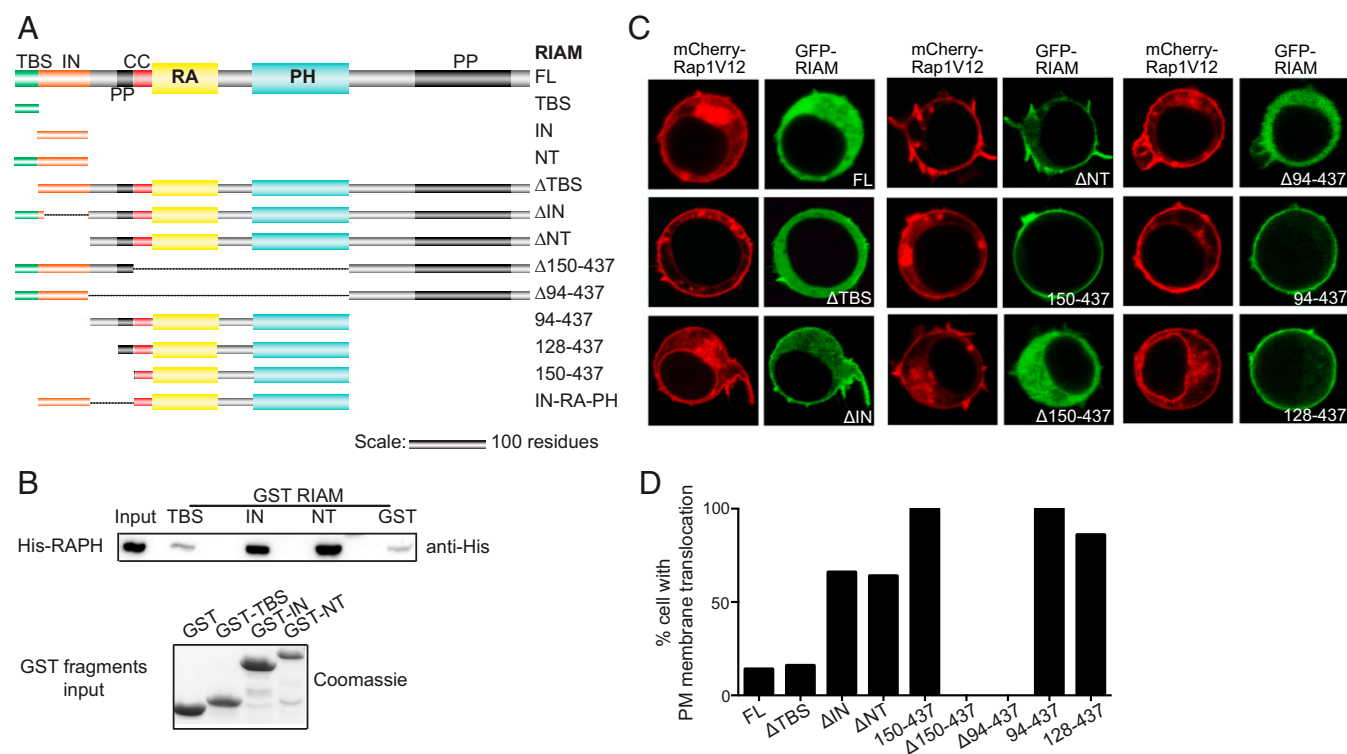


Fig. 1. Identification of an autoinhibitory region in RIAM. (A) Schematic representation of the domain organization of RIAM. Full-length RIAM (FL) possesses residues 1–670. Talin-binding site (TBS) is colored in green; IN segment is in orange; CC segment is in red; poly-proline (PP) segment is in black; the Ras-association domain (RA) is in yellow; and the Pleckstrin-homology domain (PH) is in cyan. TBS includes residues 1–30; IN includes residues 27–93; NT includes residues 1–93; ΔTBS: RIAM lacking residues 1–30; ΔIN: RIAM lacking residues 50–85; ΔNT: RIAM lacking residues 1–93; IN-RA-PH represents the protein construct used in the crystallographic study, including residues 27–437 with residues 94–149 deleted. (B) In vitro pull-down of His-tagged RIAM RA-PH by various GST-tagged RIAM fragments. (C) Coexpression of the GFP-tagged RIAM constructs with mCherry-RAP1-G12V in Jurkat T cells. (D) Quantification of the PM colocalization of the indicated GFP-RIAM with mCherry-RAP1-G12V.

Table 1. Data collection and refinement statistics

	RIAM IN-RA-PH (6E31)
Data collection	
Space group	C2
<i>a</i> , <i>b</i> , <i>c</i> , Å	136.5, 84.0, 45.2
α , β , γ , °	90.0, 91.1, 90.0
Resolution, Å	50.00–2.40
Completeness, %	98.0 (97.7)
<i>R</i> _{sym} , %	4.9 (41.0)
<i>I</i> / σ , <i>I</i>	27.3 (2.0)
Unique reflections	19178
Redundancy	3.4 (3.2)
Refinement	
Resolution, Å	50.00–2.40
<i>R</i> _{work} / <i>R</i> _{free} , %	21.1/24.0 (33.7/35.6)
RMSD bonds, Å	0.007
RMSD angle, °	1.073
Protein atoms	2422
Solvent atoms	24
Residues omitted	36–43, 74–156, 279–293
Average <i>b</i> -factors, Å ²	
Main chain atoms	83.0
Side chain atoms	80.8
Solvent	62.9
Favored/allowed regions	97%/100%

Parentheses denoted highest resolution bin, 2.46–2.40 Å.

includes residues 61–73. Interestingly, although the CC segment of RIAM is disordered in the structure of RA-PH with the CC extension (12), this segment is well ordered in the IN-RA-PH structure. The helical configuration of the CC segment is supported by the IN segment through side chain interactions including a Asp65:Lys167 salt bridge (Fig. 2*A*), suggesting that the CC segment of RIAM, like that of LPD (PDB 4GMV) (14), maintains stable secondary structure in the full-length RIAM.

The IN segment makes extensive interactions with the RA domain at the RAP1-binding site (Fig. 2*B*). Superposition of the

IN-RA-PH structure with the RAP1:RIAM complex structure (PDB code 4KVG) reveals that the IN segment overlaps with the bound RAP1 molecule, demonstrating steric hindrance on the association of RAP1 and RIAM (15). The canonical interaction between the small GTPases in the RAS superfamily and their effectors is defined by a salt bridge interaction formed by an aspartate in the switch I region of the GTPase with a lysine or arginine from the corresponding effector (15). In the RAP1:RIAM complex structure, this interaction is mediated by Asp33 of RAP1 and Lys213 of RIAM. Notably, in IN-RA-PH, Lys213 forms salt bridges with Glu60 and Asp63 of the IN segment (Fig. 2*B* and *SI Appendix*, Fig. S1). The interaction between the IN segment and the RA domain is further strengthened by a hydrophobic cluster formed by a group of leucine residues (Leu52, Leu56, Leu59, Leu64, and Leu67) from the IN segment and at least two hydrophobic residues (Val182 and Met196) from the RA domain (Fig. 2*C*). The stable conformation of the IN and CC segments and aforementioned side-chain interactions in this autoinhibited structure of RIAM is supported by well-defined electron density (Fig. 2*D*). Thus, the crystal structure reveals a mechanism for autoinhibition in atomic detail.

Interestingly, the IN segment only contains one tyrosine residue, Tyr45, which also makes a close contact with the RAP1-binding site of the RA segment (Fig. 2*D* and *E*). Inspection of this structure suggests that the phosphorylation of Tyr45 would disrupt the autoinhibitory interaction and thereby promote GTPase binding. Indeed, RIAM has been shown to be a substrate for several nonreceptor tyrosine kinases (16), and mass spectrometric analysis has revealed that Tyr45 is among the residues that are frequently found to be endogenously phosphorylated (17).

Tyr45, Glu60, and Asp63 of the IN Segment Are Essential for the Intramolecular Interaction Between IN and RA, and for Inhibiting RIAM Binding to RAP1 and Suppressing T Cell Adhesion.

To validate the intramolecular interactions between the IN segment and the RA domain observed in the crystal structure, we mutated Glu60 and Asp63; the two residues interact with Lys213 and block RAP1 binding, and examined the effect of the mutations on the RIAM IN:RA-PH interaction. Substitution of alanine for the two acidic residues (E60A/D63A) in GST-IN completely abolished its binding to RA-PH. Substitution of Tyr45 with

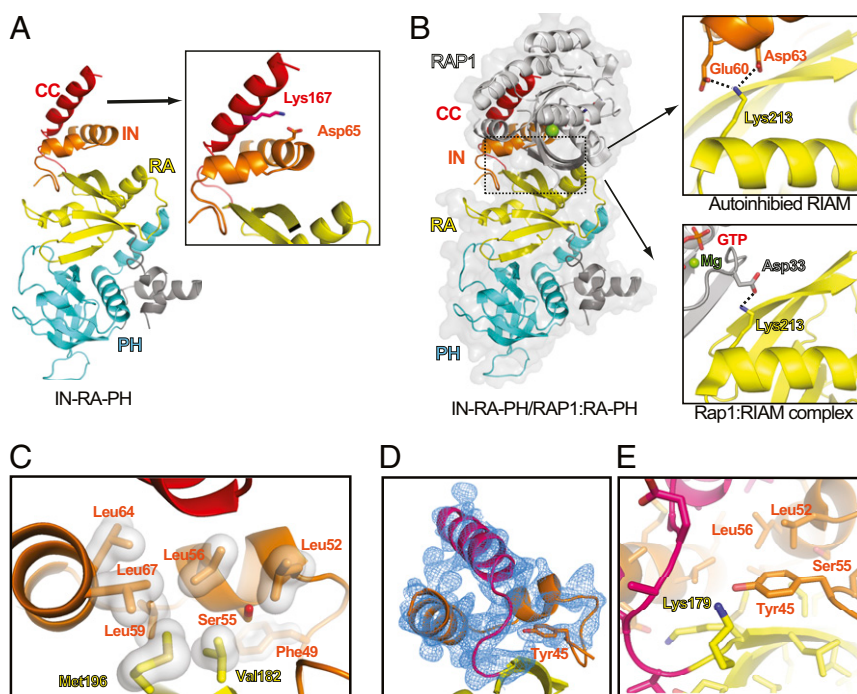


Fig. 2. Crystal structure of RIAM in an autoinhibited configuration. (A) Ribbon diagram of RIAM in an autoinhibited configuration represented by the crystal structure of RIAM IN-RA-PH, with the IN segment in orange, the CC segment in red, the RA domain in yellow, the PH domain in cyan, and the linker between RA and PH in gray. The salt-bridge interaction supporting the cc helix is illustrated in the *Right*. (B, *Left*) Superposition of the autoinhibited RIAM structure with the RAP1:RIAM complex structure. The RAP1:RIAM complex structure is shown as a surface representation. RAP1 is colored light gray. The RA-PH module in the RAP1:RIAM complex is omitted. (B, *Upper Right*) Side-chain interactions formed by Lys213 from the RA domain and Glu60/Asp63 from the IN segment. (B, *Lower Right*) The canonical Ras:effector interaction mediated by Lys213 of RIAM and Asp33 of RAP1. (C) Surface representation of the hydrophobic cluster formed by the residues from the IN segment (orange) and the RA domain (yellow). (D) *2Fo-Fc* electron density map of the IN and CC segments. Side chain of Tyr45 is shown in stick representation. (E) Residues in the proximity of Tyr45 in the autoinhibitory state of RIAM are shown in stick representation.

either a phosphomimetic glutamic acid or a phenylalanine also diminished the interaction with RA-PH (Fig. 3A). The reduced association with RA-PH of the Y45E suggests that phosphorylation at Tyr45 is the means of physiologic regulation. Diminished binding of the Y45F substitution suggests that the hydroxyl group of the tyrosine is also crucial for stabilization of the structure. These mutations were also examined for their impact on the interaction of RIAM and RAP1 in cells by coimmunoprecipitation of GFP-RIAM and HA-RAP1-G12V. The isolated RA-PH module interacted with RAP1 much more efficiently than the full-length RIAM (FL-RIAM), supporting autoinhibition. In contrast, FL-RIAM bearing the E60A/D63A double mutation was efficiently pulled down by HA-RAP1-G12V (Fig. 3B). Y45E and Y45F mutations also enhanced the interaction, although to a lesser degree. These data suggest that all three residues of the IN segment, Tyr45, E60, and D63, are essential for the autoinhibited configuration consistent with the crystal structure.

We next examined the effect of these mutations on the RAP1-dependent PM translocation. We transfected Jurkat T cells with GFP-RIAM (wild type or with designated mutations) and mCherry-RAP1-G12V and monitored their colocalization on the PM by live cell fluorescence imaging. Both E60A/D63A and Y45E enhanced the colocalization of RIAM and RAP1 at the PM (Fig. 3C). However, Y45F exhibited little effect, suggesting that the phosphomimetic residue disrupts the autoinhibition more efficiently than does removal of the polar hydroxyl group. Thus, multiple modes of analysis implicate phosphorylation of Tyr45 in the regulation of membrane translocation of RIAM.

The model of autoinhibition of RIAM suggested by our data predicts that ectopic expression of the isolated IN segment in cells might inhibit RIAM function by blocking interaction with RAP1. RIAM regulates T cell adhesion by controlling the adhesive activity of LFA-1. We therefore examined the effect of the IN segment on LFA-1-mediated T cells adhesion to ICAM-1. Transduction of the Jurkat T cells with GFP alone had no effect on adhesion. In contrast, transduction of the cells with GFP tagged IN fragment inhibited cell adhesion by 50% (Fig. 3D).

Importantly, when cells were transduced with GFP-IN bearing Y45E or E60A/D63A mutations, the inhibitory effect of the GFP-IN on cell adhesion was diminished. This result revealed that the isolated IN peptide has the ability to inhibit T cell adhesion, and this inhibition requires Tyr45, Glu60, and Asp63. Together these data strongly support a model whereby RIAM is autoinhibited by an intramolecular association of the IN segment with the RA-PH module. The phosphomimetic mutation Y45E exhibits profound effect on RIAM autoinhibition, suggesting a regulatory mechanism through Tyr45 phosphorylation.

Phosphorylation of Tyr45 upon TCR Activation Is Regulated by Focal Adhesion Kinase (FAK). To determine if Tyr45 can be phosphorylated upon TCR stimulation, we incubated purified GST-IN fragments with cell lysates of unstimulated or anti-CD3 antibodies stimulated Jurkat T cells, and examined the phosphorylation level of GST-IN fragments. Tyrosine phosphorylation of the GST-IN was significantly increased in response to T cell stimulation. Importantly, when Tyr45, the only tyrosine in the IN fragment, is substituted by a glutamic acid or a phenylalanine, tyrosine phosphorylation is completely abolished (Fig. 4A). To identify the kinase responsible for Tyr45 phosphorylation, we examined a series of tyrosine kinase inhibitors for their effect on the phosphorylation of the IN segment based on the computational prediction of cognate protein kinase by Group-based Prediction System (GPS) (18). Two FAK inhibitors, PF431396 and PF572271, significantly diminished tyrosine phosphorylation of the IN segment (Fig. 4B). This result suggests that FAK is one kinase responsible for phosphorylation of the IN segment of RIAM.

To support a physiologic role of FAK in RIAM signaling, we examined the effect of the FAK inhibitors on the RAP1-dependent PM translocation of RIAM in Jurkat T cells. Upon stimulation of the TCRs in Jurkat T cells, GFP-RIAM translocates to the PM in about 60% of cells coexpressing RAP1-G12V, indicating TCR activation releases RIAM autoinhibition, leading to the PM translocation of RIAM. In the cells pretreated with the FAK inhibitors, the PM translocation of RIAM was significantly suppressed, indicating that RIAM remained in the autoinhibited state (Fig. 4C).

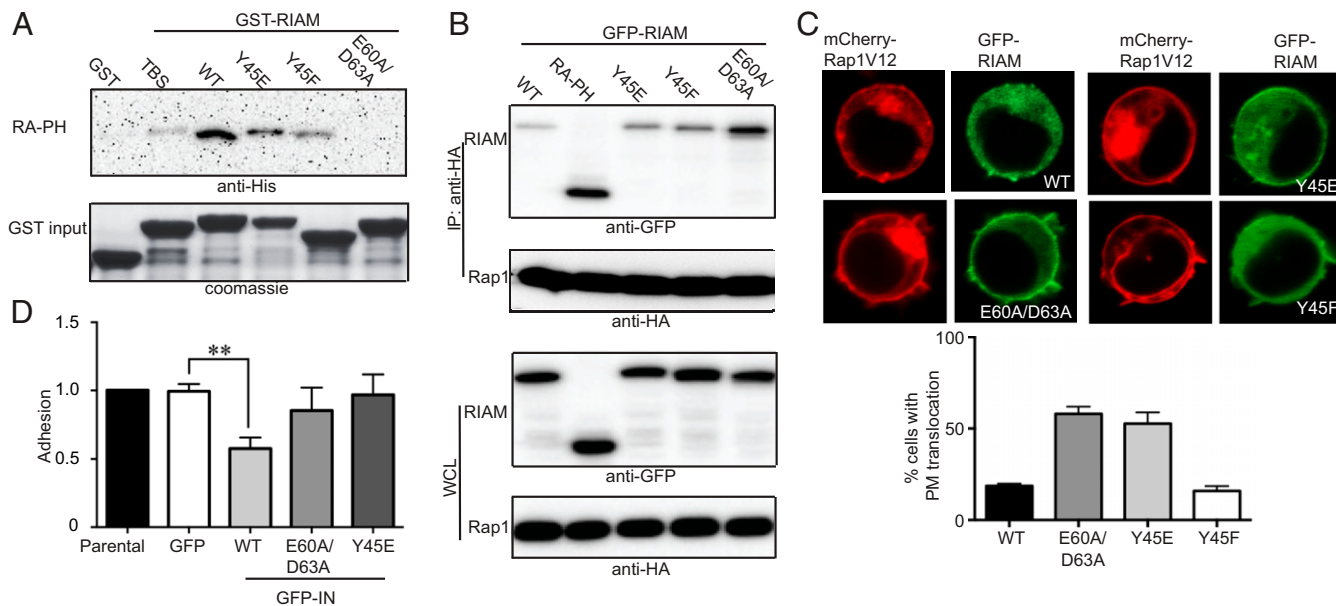


Fig. 3. The IN of RIAM inhibits translocation to the PM and blocks T cell adhesion. (A) In vitro pull-down of His-tagged RA-PH by GST-tagged TBS, IN, and various mutations of IN. Input samples were examined by Coomassie staining. (B) Coimmunoprecipitation of HA-RAP1 and full-length RIAM, or various RIAM mutants. (C, Upper) Coexpression of the GFP-tagged point mutations of RIAM with mCherry-RAP1-G12V in Jurkat T cells. (C, Lower) Quantification of the PM colocalization of the indicated GFP-RIAM with mCherry-RAP1-G12V. Data shown are mean \pm SD, $n = 3$. (D) Jurkat T cells were transfected with indicated constructs. Twenty-four hours later, adhesion assay was performed as described and adherence was determined by analyzing GFP-positive cells using flow cytometry. All results were normalized to untransfected Jurkat cells. Data shown are mean \pm SD, $n = 4$ (** $P < 0.001$).

phosphate would be predicted to disrupt the interaction. Importantly, our RIAM PM translocation and Jurkat adhesion data support the model predicted from the crystal structure.

RIAM is highly expressed in T cells and plays an essential role in T cell adhesion and trafficking (7, 8). Consistent with our observations is the report that pTyr45 is present in the endogenous phospho-peptides derived from RIAM in activated T cells (17). Interestingly, in addition to Tyr45, endogenous phosphorylation of Ser55 in the IN segment has been reported (17). Our data suggested that a phosphomimetic mutation S55E also releases RIAM autoinhibition (*SI Appendix, Fig. S2*). This raises the possibility that, like phosphorylation of Tyr45 by FAK, reversible phosphorylation of Ser55 by a serine/threonine kinase may also contribute to the regulation of autoinhibition. Nevertheless, we did not detect significant changes in pSer55 levels caused by Jurkat cell stimulation, suggesting that pTyr45 is the physiologically relevant modification in activated T cells.

Our results suggest that FAK is among the kinases that can regulate RIAM through phosphorylation of Tyr45. FAK is known to regulate cell adhesion and migration and to be concentrated with integrins at focal adhesions (5). Indeed, FAK has been shown to play an essential role in the outside-in signaling at focal adhesions upon binding of integrins to the extracellular matrix (22). RIAM is a key regulator of inside-out signaling to integrins (3). This suggests that FAK may mediate crosstalk between outside-in and inside-out integrin signaling to integrins. In support of this model FAK has been shown to interact directly with talin at nascent adhesions (23), and this interaction is diminished in RIAM-deficient cells (24). FAK has also been implicated in the strengthening of adhesion in response to mechanical stress (25). Our results suggest that FAK may operate in signaling in both directions through integrins and in so doing mediate a feedforward pathway for amplification of integrin activation.

The regulation of lymphocyte adhesion must be carefully regulated. Too little adhesion would adversely affect host defense and too much could contribute to autoimmunity. Accordingly, inside-out signaling to integrins must have multiple levels of control. Several of these appear to reside at the level of RIAM. Its RA-PH module functions as an AND gate integrating activation of RAP1 and production of PI(4,5)P₂ (12). The results reported here reveal an additional mode of regulation through a reversible intramolecular interaction. It appears that FAK > RIAM > talin may represent the core pathway for a feedforward regulation of integrins and it is interesting to note that each element of this cascade is regulated by the relief of autoinhibition.

Materials and Methods

Plasmid Construction and Protein Purification. The RIAM N-terminal region and RA-PH module were subcloned into modified pET28a expression vector with a His₆-tag and a tobacco etch virus protease cleavage site or pGEX-5X-1 expression vector with a GST-tag. Gene deletion and point mutations were constructed using a site-directed mutagenesis method. Plasmids were transformed into *Escherichia coli* BL21(DE3) for protein expression. Protein samples were extracted from the supernatants of the cell lysates using HisTrap FF or GSTrap FF columns (GE Healthcare). For crystallization, the His-tag was removed by TEV protease. The untagged protein was then further purified using a RESOURCE Q and Superdex 75 column (GE Healthcare).

X-Ray Crystallography. Purified RIAM IN-RA-PH protein (residues 27–437 with a deletion of residues 94–149) was concentrated to 8.9 mg/mL and stored in 20 mM Tris pH 8.0, 100 mM NaCl, and 2 mM DTT. The crystals were grown in 0.1 M magnesium acetate and 6% (wt/vol) PEG 3350 at 4 °C for 10 d by hanging-drop vapor diffusion method. Data collection and refinement statistics are listed in Table 1. The atomic coordinates and structure factors have been deposited to Protein Data Bank with accession number 6E31.

Cell Culture and Confocal Microscopy. Jurkat T cells (ATCC) were maintained in 5% CO₂ at 37 °C in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin. Plasmids were introduced into Jurkat cells by nucleofection (Lonza) according to the manufacturer's instructions, and cells were imaged 24 h later. Cells were starved by 0.5% FBS for two 2 h before anti-CD3 antibodies (2.5 μg/mL) was used for stimulation. In some experiments, FAK inhibitor, PM431396 and PM572271 (400 nM), were used to pretreat cells for 1 h. For cell imaging, Jurkat cells were transfected in suspension. Twenty-four hours later, the transfected cells were plated on poly-lysine-coated 35-mm MatTek dishes before imaging. Cells were imaged in RPMI 1640 media at 37 °C with confocal microscope (Zeiss LSM 800) equipped with a Plan-Apochromat 63 × 1.40 oil objective. Images were processed with Photoshop, and PM translocation of GFP-labeled proteins was quantified by counting 100 cells in each condition.

Methods describing structure determination, GST pull-down, co-immunoprecipitation, phosphorylation assay, and cell adhesion assay are described in detail in *SI Appendix, Supplemental Materials and Methods*.

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