RIAM, an Ena/VASP and Profilin Ligand, Interacts with Rap1-GTP and Mediates Rap1-Induced Adhesion

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Summary

The small GTPase Rap1 induces integrin-mediated adhesion and changes in the actin cytoskeleton. The mechanisms that mediate these effects of Rap1 are poorly understood. We have identified RIAM as a Rap1-GTP-interacting adaptor molecule. RIAM defines a family of adaptor molecules that contain a RA-like (Ras association) domain, a PH (pleckstrin homology) domain, and various proline-rich motifs. RIAM also interacts with Profilin and Ena/VASP proteins, molecules that regulate actin dynamics. Overexpression of RIAM induced cell spreading and lamellipodia formation, changes that require actin polymerization. In contrast, RIAM knockdown cells had reduced content of polymerized actin. RIAM overexpression also induced integrin activation and cell adhesion. RIAM knockdown displaced Rap1-GTP from the plasma membrane and abrogated Rap1-induced adhesion. Thus, RIAM links Rap1 to integrin activation and plays a role in regulating actin dynamics.

Introduction

The small GTPase Rap1 (Krev-1), a member of the Ras superfamily, was identified by its ability to revert K-Ras-induced transformation (Kitayama et al., 1989). Recent data indicate that Rap1 does not simply antagonize Ras function. In mammalian cells, Rap1 plays a role in cell adhesion and spreading. Fibroblasts deficient for C3G, a Rap1-GEF, display impaired adhesion and accelerated migration, phenotypes that can be reversed by overexpression of the Rap1-GEFs CalDAG-GEFI or Epac (Ohba et al., 2001). Similarly, a dominant-negative form of C3G blocked HGF-induced Rap1 activation and adhesion (Sakkab et al., 2000), as did overexpression of the Rap1-GAP Spa1 (Tsukamoto et al., 1999). In lymphoid cells,

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Rap1-GTP regulates LFA-1-mediated adhesion upon TCR, CD31, or CD98 ligation (Katagiri et al., 2000; Reedquist et al., 2000; Suga et al., 2001). Rap1 is also involved in chemokine-induced β2 and α4 integrin activation and T cell migration (Shimonaka et al., 2003). However, the mechanism that links Rap1 to integrin activation remains unclear.

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 (ATP) complexes. When bound by Profilin, actin monomer is added only to the barbed ends of F-actin (Pollard and Borisy, 2003). 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 contact site (Krause et al., 2003). They contain an EVH1 domain that interacts with the proline-rich motif (D/E)(F/L/W/Y)PPPPX (D/E)(D/E) (abbreviated as FPPPP) present in proteins such as Zyxin and Vinculin that target Ena/VASP proteins to focal adhesions (Niebuhr et al., 1997) or in Fyb/SLAP that recruits Ena/VASP to the T cell:APC interface (Krause et al., 2000; Renfranz and Beckerle, 2002). They also have proline-rich regions that bind to SH3 domain-containing proteins and to Profilin, and an EVH2 domain that mediates their tetramerization and interacts with G- and F-actin (Krause et al., 2003).

Here, we report the cloning and the structural and functional characterization of RIAM, an interactor of Rap1-GTP. Overexpression of RIAM promoted the active conformation of β1 and β2 integrin and integrin-mediated adhesion. Knockdown of RIAM expression displaced Rap1-GTP from the plasma membrane and reverted Rap1-induced adhesion. RIAM also interacted with Profilin and Ena/VASP proteins. Overexpression of RIAM induced changes in actin cytoskeleton, reflected by increased cell spreading and lamellipodia formation. In contrast, RIAM knockdown cells had reduced polymerized actin content. Therefore, RIAM plays a key role in Rap1-induced adhesion and interacts with modulators of the actin cytoskeleton.

Results

Identification of RIAM as a Rap1-Interacting Protein

To search for Rap1-interacting molecules in T cells, we used the constitutively GTP bound mutant Rap1E63 (Kitayama et al., 1990) as a bait to screen a Jurkat cDNA library using yeast two-hybrid system. We isolated several clones encoding for RGL2, a known interactor of Rap1 (Peterson et al., 1996), along with clones encoding partial cDNAs with homology to mouse PRP48 (AF020313). We named this protein RIAM for Rap1-GTP-interacting adaptor molecule (AY152730). Later, sequences encoding for this protein were deposited (NM_019043). RIAM also has homology to a human clone (LOC54518) that is identical to RIAM residues 1–261 except for its last 27 amino acids (aa), suggesting that it is a splice variant of RIAM.

The open reading frame of RIAM is 1998 bp and encodes a protein of 665 aa. The human RIAM gene locus is
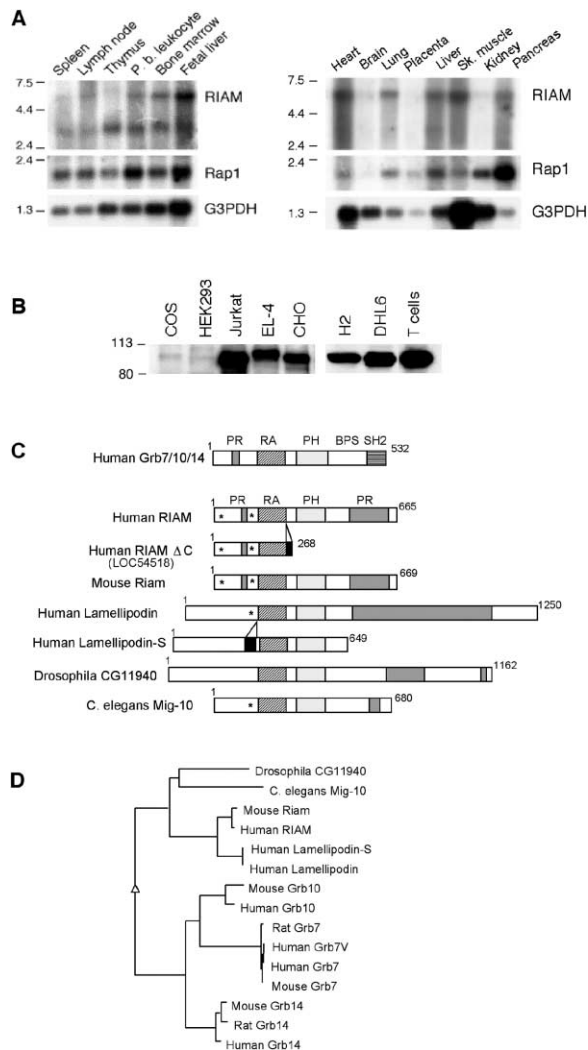
Figure 1. RIAM Is Part of the MRL Protein Family

(A) Human multiple immune tissue and human multiple tissue Northern blots were sequentially hybridized with full-length RIAM, Rap1, and G3PDH cDNAs.
(B) RIAM expression detected by Western blot in different cell lines, in human B cell lymphoma cell lines (H2 and DHL6) and in human primary T cells.
(C) Schematic representation of domain structure of human Grb7/10/14 and MRL family proteins (asterisks represent a coiled-coil region).
(D) Dendrogram of full-length proteins of the Grb7 and MRL families. RA, Ras-association domain; PH, pleckstrin homology domain; PR, proline rich; SH2, Src homology domain; BPS, between PH and SH2 domain.

on chromosome 10p12.1. Northern blot analysis showed that RIAM, like Rap1, is expressed broadly (Figure 1A). Two transcripts of 5.4 and 2.8 Kb were detected in immune tissues. In nonimmune tissues, the larger transcript predominated. We raised rabbit polyclonal antibodies against the C and N termini of RIAM that recognized a 110 kDa band in Western blots of various cell types (Figure 1B). In vitro translation of full-length RIAM cDNA produced a protein of 110 kDa (Figure 2B), confirming that the protein recognized by our antibody is RIAM.

Sequence analysis indicated that RIAM contains a 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 (aa 62–89 and aa 149–181) (Figure 1C).

RIAM Belongs to the MRL Family of Adaptor Molecules

Database searches revealed that RIAM has similarities with Grb7, Grb10, and Grb14 adaptor molecules. The protein with highest homology to RIAM is Lamellipodin (Lpd) (KIAA1681, AY494951) and Lpd-S (ALS2CR9, BAB69020), characterized in the accompanying article by Krause et al. (2004 [this issue of *Developmental Cell*]). Furthermore, RIAM is related to proteins CG11940 (AAF49029) in *D. melanogaster* and Mig-10 (P34400) in *C. elegans* (Figure 1C).

Comparison of the domain structure of these proteins indicated that they comprise two distinct families (Figure 1C). Grb7-related proteins have a conserved central region containing a RA domain and a PH domain (Manser and Wood, 1990; Wojcik et al., 1999), a Src homology 2 (SH2) domain at their C-terminal region, and a "between PH and SH2" (BPS) domain (Han et al., 2001). In contrast, RIAM, Lpd, CG11940, and Mig-10 lack SH2 and BPS domains and have a proline-rich region at the C terminus. Moreover, all proteins in the latter group contain a highly conserved pattern of 27 aa predicted to be a coiled-coil region immediately N-terminal of the RA domain. In addition, comparison of the RA and PH domains shows differences between the Grb7/10/14- and RIAM-related proteins and conserved regions among the proteins within each group (Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/7/4/585/DC1/). Dendrograms of sequence relatedness of full-length protein (Figure 1D), the RA and PH domains (data not shown), confirmed that Grb7- and RIAM-related proteins are separated into two distinct families. We propose to name the family of RIAM-related adaptor molecules the MRL (Mig-10/RIAM/Lpd) family.

RIAM Interacts with Rap1-GTP In Vitro and In Vivo

We examined the specificity of the RIAM-Rap1 interaction and its dependence on the activation state of Rap1. In a two-hybrid assay, yeast was cotransformed with RIAM with either Rap1WT, the GTP bound mutants Rap1E63 and Rap1V12, or Rap1N17, a mutant that remains in nucleotide-free state. Interaction was observed with Rap1V12, Rap1E63, and Rap1WT but not with Rap1N17 (Figure 2A). As previously reported, when transformed in yeast, Rap1WT is mostly in GTP bound state (McCabe et al., 1992). Only a weak interaction was observed when RIAM was cotransformed with RasV12 (Figure 2A), suggesting that RIAM preferentially interacted with Rap1-GTP compared to Ras-GTP. Since Rap1 and Ras share identical effector domains, sequences outside the Rap1 effector domain may contribute to the preferential binding of RIAM with Rap1. When RIAM was cotransformed with RalA, RalB, Rho, Cdc42, and Rac, interaction was not detected (data not shown, see accompanying article by Krause et al. [2004]).

The RIAM/Rap1 interaction was tested by in vitro protein association assay (Figure 2B). GST-Rap1WT, GST-Rap1E63, and GST coupled to glutahione-sepharose
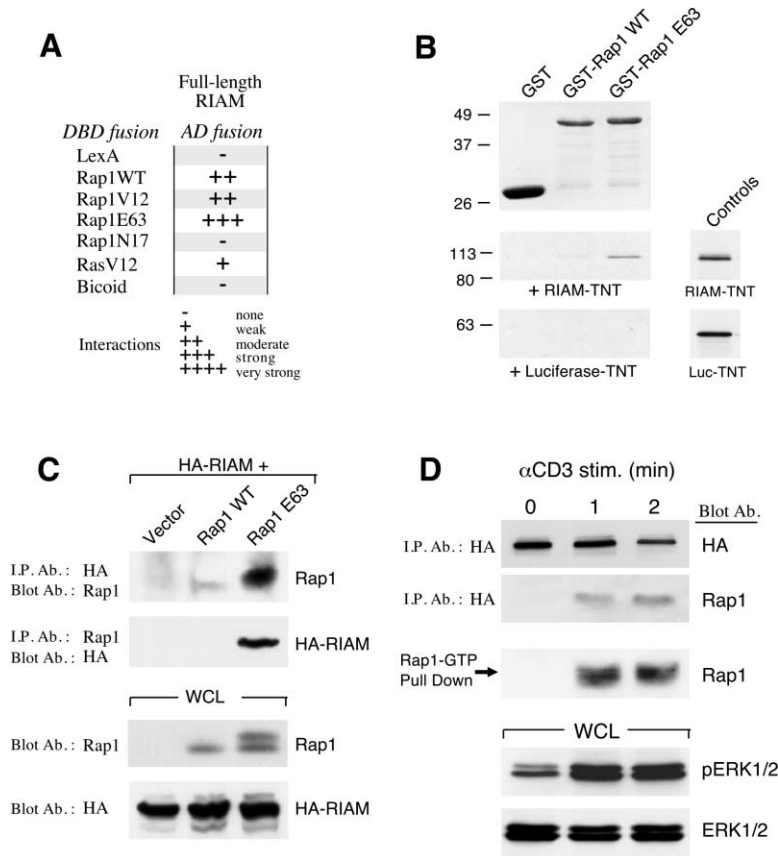
A

Full-length
RIAM

DBD fusion	AD fusion
LexA	-
Rap1WT	++
Rap1V12	++
Rap1E63	+++
Rap1N17	-
RasV12	+
Bicoid	-

Interactions		
-	none	
+	weak	
++	moderate	
+++	strong	
++++	very strong	

B

49 —
37 —
26 —

113 —
80 —
+ RIAM-TNT

Controls
RIAM-TNT

63 —
+ Luciferase-TNT

Luc-TNT

GST   GST-Rap1 WT   GST-Rap1 E63

C

HA-RIAM +

Vector   Rap1 WT   Rap1 E63

I.P. Ab.: HA
Blot Ab.: Rap1   Rap1

I.P. Ab.: Rap1
Blot Ab.: HA   HA-RIAM

WCL

Blot Ab.: Rap1   Rap1

Blot Ab.: HA   HA-RIAM

D

αCD3 stim. (min)

0   1   2   Blot Ab.

I.P. Ab.: HA   HA

I.P. Ab.: HA   Rap1

Rap1-GTP
Pull Down   Rap1

WCL

pERK1/2

ERK1/2

Figure 2. RIAM Interacts with Rap1-GTP

(A) The yeast was transformed with full-length RIAM together with the indicated constructs. LexA and Bicoid were used as negative controls.

(B) Purified fusion proteins and GST control were analyzed by Coomassie staining (top panel). Interactions between the indicated proteins and [^{35}S]methionine-labeled in vitro translated RIAM (middle panel) or Luciferase (lower panel) were examined by SDS-PAGE and exposure on film. In vitro translated RIAM and Luciferase are shown as controls.

(C) COS cells were cotransfected with HA-RIAM and either empty vector, Rap1WT, or Rap1E63. Immunoprecipitations were done with anti-HA followed by immunoblot with Rap1 antiserum (first panel) or with Rap1 antiserum followed by immunoblot with anti-HA mAb (second panel). Equal amounts of whole-cell lysates (WCL) were immunoblotted with anti-Rap1 and anti-HA antibodies (third and fourth panels).

(D) Jurkat cells were transfected with HA-RIAM and stimulated with anti-CD3 mAb. Cell extracts were immunoprecipitated with anti-HA antibody and sequentially immunoblotted with HA mAb and Rap1 antiserum. Rap1 activation was determined by pull-down assays using GST-RalGDS-RBD (third panel). To confirm cell activation, the same samples were immunoblotted with anti-pERK1/2 antibody (fourth panel) and with ERK1/2-specific antiserum (fifth panel).

were incubated with [^{35}S]methionine-labeled RIAM or Luciferase, as negative control. RIAM bound preferentially to Rap1E63, whereas binding to Rap1WT was almost undetectable. Binding to Luciferase was not detected (Figure 2B, lower panel).

To examine whether RIAM interacted with Rap1 in vivo, HA-tagged RIAM was expressed in COS cells with empty vector, Rap1WT, or Rap1E63. Immunoprecipitation with anti-HA antibody followed by Rap1 immunoblot and reciprocal immunoprecipitation with Rap1 antiserum followed by anti-HA immunoblot (Figure 2C) showed that RIAM preferentially interacted with Rap1E63. When RIAM was coexpressed with RasV12, only minimal amounts of Ras interacted with RIAM (data not shown). Thus in vivo, RIAM preferentially interacts with Rap1-GTP as compared to Ras-GTP. Interaction of RIAM with Rap1-GTP was also detected upon physiological stimulation. Jurkat T cells transfected with HA-RIAM were stimulated with anti-CD3 mAb (Figure 2D). Rap1 activation was monitored by pull-down assay with GST-RalGDS-RBD followed by Western blot with Rap1 antiserum (Reedquist and Bos, 1998). Immunoprecipitations with anti-HA antibody followed by Rap1 immunoblot indicated that RIAM interacted with endogenous Rap1 after formation of Rap1-GTP (Figure 2D). Thus, RIAM interacts only with Rap1-GTP, suggesting that it is a Rap1-effector.

To determine the region of RIAM that associates with Rap1, we generated truncation mutants and assayed them for interaction with Rap1 by yeast two-hybrid (Supplemental Figure S2). Only RIAM fragments containing

both RA and PH domains interacted with Rap1-GTP (RIAM 150–665, RIAM 150–503, RIAM 150–430). Residues 1–150 enhanced this interaction as RIAM 1–503 or full-length RIAM interacted with Rap1 more robustly than RIAM 150–665. Thus, the RIAM RA-like and PH domains together are necessary and sufficient for interaction with Rap1-GTP, and N-terminal RIAM sequences enhance this interaction.

RIAM Interacts with Ena/VASP Proteins and Profilin

RIAM is proline-rich (12.9%) and contains six putative Profilin binding motifs (XPPPPP) and six putative EVH1 binding motifs (D/E)(F/L/W/Y)PPPPX(D/E)(D/E) (Figure 3A; Niebuhr et al., 1997), suggesting that RIAM may interact with Profilin and EVH1 domain-containing proteins. In addition, RIAM contains binding motifs for SH3 and WW domain-containing proteins (Holt and Koffer, 2001).

Using the yeast two-hybrids, we detected a strong interaction between RIAM and Profilin of similar intensity to that between RIAM and active Rap1 (Figure 3B). We also detected interaction between RIAM and VASP by in vitro association assay. GST and GST-fusion proteins of VASP or VASP mutant (VASPΔ) in which the Profilin binding region of VASP (Gertler et al., 1996) was eliminated were coupled to glutathione-sepharose and incubated with [^{35}S]methionine-labeled RIAM or Luciferase, as control. Both VASP and VASPΔ bound to RIAM (Figure 3C), indicating that RIAM interacts directly with VASP.
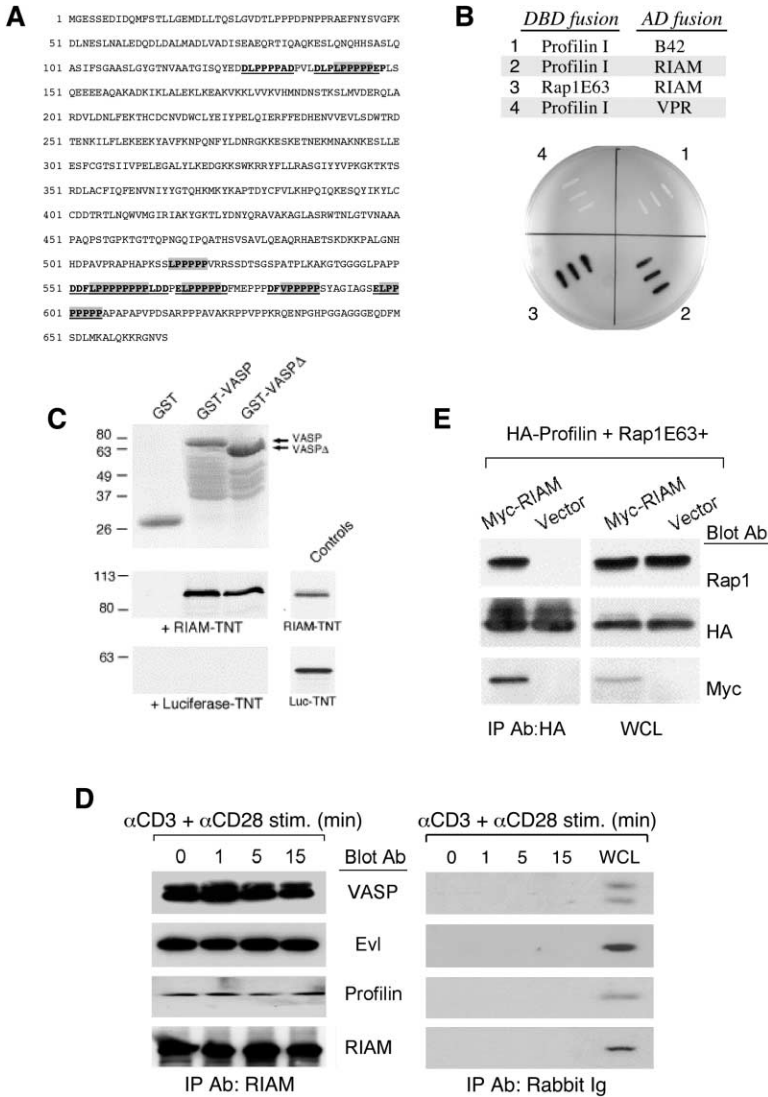
A

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  1 MGESSEDIDQMFSTLLGEMDLLTQSLGVDTLPPPDPNPPRAEFNYSVGFK
 51 DLNESLNALEDQDLDALMADLVADISEAEQRTIQAQKESLQNQHHSASLQ
101 ASIFSGAASLGYGTNVAATGISQYEDDLPPPPADPVLDLPLPPPPPEPLS
151 QEEEEAQAKADKIKLALEKLKEAKVKKLVVKVHMNDNSTKSLMVDERQLA
201 RDVLDNLFEKTHCDCNVDWCLYEIYPELQIERFFEDHENVVEVLSDWTRD
251 TENKILFLEKEEKYAVFKNPQNFYLDNRGKKESKETNEKMNAKNKESLLE
301 ESFCGTSIIVPELEGALYLKEDGKKSWKRRYFLLRASGIYYVPKGKTKTS
351 RDLACFIQFENVNIYYGTQHKMKYKAPTDYCFVLKHPQIQKESQYIKYLC
401 CDDTRTLNQWVMGIRIAKYGKTLYDNYQRAVAKAGLASRWTNLGTVNAAA
451 PAQPSTGPKTGTTQPNGQIPQATHSVSAVLQEAQRHAETSKDKKPALGNH
501 HDPAVPRAPHAPKSSLPPPPPVRRSSDTSGSPATPLKAKGTGGGGLPAPP
551 DDFLPPPPPPPLDDPELPPPPPDFMEPPPDFVPPPPPSYAGIAGSELPP
601 PPPPPAPAPAPVPDSARPPPPAVAKRPPVPPKRQENPGHPGGAGGGEQDFM
651 SDLMKALQKKRGNVS
```

B

	DBD fusion	AD fusion
1	Profilin I	B42
2	Profilin I	RIAM
3	Rap1E63	RIAM
4	Profilin I	VPR

C

D

E

HA-Profilin + Rap1E63+

Figure 3. RIAM Interacts with Profilin and Ena/VASP Family Proteins

(A) Six putative Profilin binding motifs are shown in gray boxes and six putative EVH1 binding motifs are shown underlined.

(B) The yeast was cotransformed with full-length RIAM and either Profilin or with Rap1E63 as positive control. B42AD and VPR were used as negative controls. Colonies from each transformation were assayed for β-galactosidase activity.

(C) The indicated purified GST-fusion proteins and GST were visualized by Coomassie staining (top panel). Interactions between the indicated proteins and [35S]methionine-labeled in vitro translated RIAM or Luciferase were examined by SDS-PAGE and exposure on film (middle and bottom panel). In vitro translated RIAM and luciferase are shown as controls.

(D) Cell extracts from primary T cells either unstimulated or stimulated with anti-CD3 and anti-CD28 mAbs were immunoprecipitated with RIAM antibody (left panels) or with control IgG (right panels) followed by immunoblot with VASP, Evl, Profilin, and RIAM antibodies.

(E) COS cells were cotransfected with HA-Profilin and Rap1E63 with either Myc-RIAM or empty vector, and immunoprecipitations were done with anti-HA mAb followed by immunoblot with Rap1, HA, and Myc antibodies. Equivalent amounts of whole-cell lysates (WCL) were used as controls for Rap1E63 and HA-Profilin expression.

To determine if RIAM interacts with Profilin and Ena/VASP in vivo, we immunoprecipitated RIAM from primary human T cell extracts. Coimmunoprecipitation of Profilin, VASP, and Evl with endogenous RIAM was detected prior and after stimulation via TCR/CD3 and CD28, indicating that these interactions are constitutive in T cells (Figure 3D).

RIAM Is a Link between Rap1-GTP and Profilin

Rap1-GTP localizes at the T cell:APC interface (Katagiri et al., 2002) and is involved in integrin activation (Katagiri et al., 2000; Reedquist et al., 2000). These events require reorganization of the cytoskeleton. However, the mechanism that regulates docking of Rap1 to the cytoskeleton has not been determined. Our observation that RIAM associates with Ena/VASP and Profilin suggests that RIAM might provide a link between Rap1-GTP and the cytoskeleton. Therefore, we examined whether Rap1-GTP, RIAM, and Profilin may be present in complexes. HA-Profilin and Rap1E63 were expressed in COS cells with either Myc-RIAM or empty vector. Immunoprecipitation with anti-HA antibody followed by immunoblot

with Rap1 antiserum (Figure 3E, first panel) showed that Rap1 was present in a complex with Profilin (Figure 3E, second panel) only when RIAM was expressed, as indicated by immunoblot with anti-Myc antibody (Figure 3E, third panel). These results indicate that RIAM can function as a link between Rap1-GTP and the actin regulator Profilin either by direct RIAM binding to Profilin or by RIAM recruitment of Ena/VASP-Profilin complexes.

RIAM Colocalizes with F-Actin and Induces Cell Spreading

The interaction between RIAM and the regulators of actin polymerization, Profilin and Ena/VASP, prompted us to determine the effect of RIAM on the actin cytoskeleton. HEK293 cells stably transfected with RIAM or empty vector were examined by confocal microscopy. As shown in Figure 4, cells overexpressing RIAM were more spread and had almost double the area of controls (HEK293 RIAM = 1760 ± 310 μm^2, n = 50; HEK293 Vector = 932 ± 342 μm^2, n = 50). Staining with RIAM antibody revealed that RIAM is distributed throughout
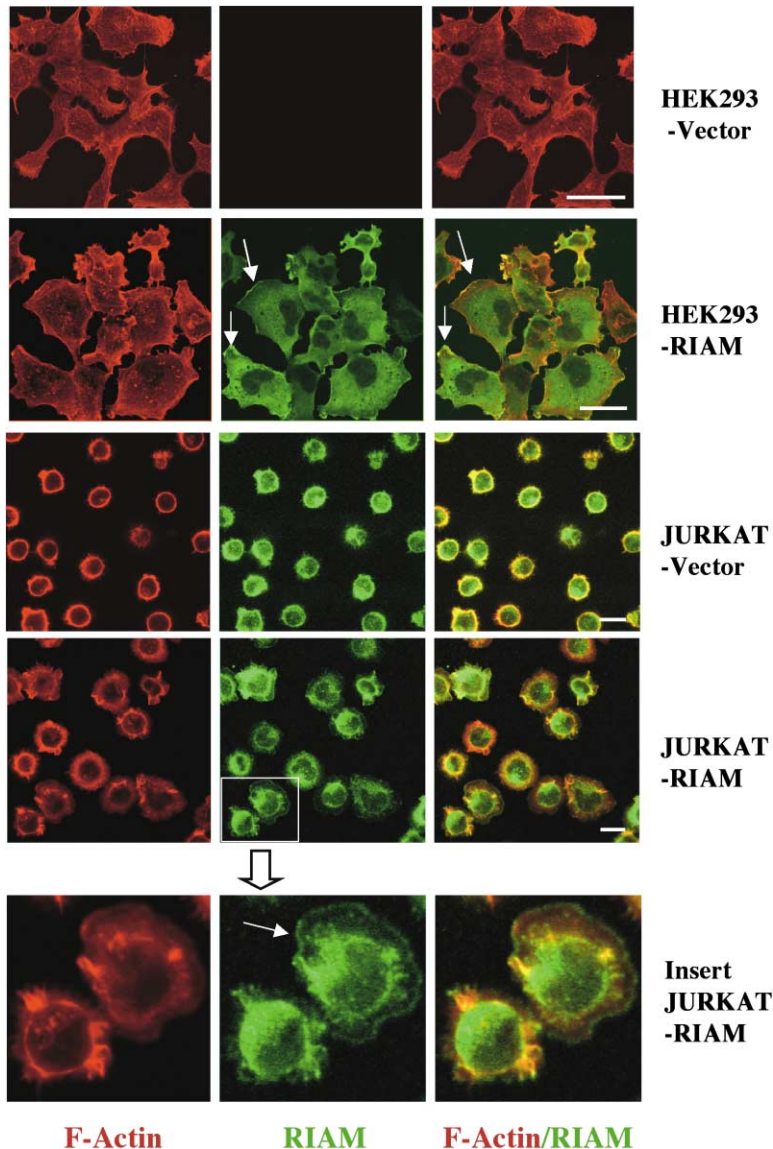
Figure 4. RIAM Induces Cell Spreading and Lamellipodia Formation

HEK293 and Jurkat T cells stably transfected with empty vector or with RIAM were seeded on fibronectin (HEK293) or poly-Lysine (Jurkat). Cells were fixed, stained, and analyzed by confocal microscopy. RIAM was visualized with RIAM-specific antibody (green) and polymerized (F)-actin was detected with Cy5-Phalloidin (red). Points of RIAM localization at the lamella are marked with arrows. Scale bars equal 50 μm for HEK293 cells and 10 μm for Jurkat cells.

the cytoplasm and at the leading edge where it colocalizes with F-actin (Figure 4, arrows). This localization of RIAM is consistent with the reported localization of Ena/VASP proteins at the leading edge (Gertler et al., 1996; Rottner et al., 1999), where they are recruited to the fast growing ends of actin filaments (Bear et al., 2002; also see accompanying article by Krause et al. [2004]).

To determine whether cell spreading was induced by overexpression of RIAM in other cell types, we stably transfected Jurkat T cells with RIAM or empty vector. RIAM-transfected Jurkat cells formed extensive lamellipodia (Figure 4). In these cells, RIAM was detected in the perinuclear region and at the leading edge (Figure 4, bottom row, arrow).

RIAM Induces β1 and β2 Integrin-Mediated Adhesion
Rap1-GTP induces β1 and β2 integrin-mediated adhesion in T cells. To determine if RIAM had a similar effect, stable Jurkat T cell lines transfected with RIAM,

Rap1E63, or empty vector were added in plates coated with increasing concentrations of fibronectin. Cells transfected with RIAM had a 3- to 4-fold increased adhesion compared to controls (Figure 5A). RIAM-induced adhesion was comparable to that induced by Rap1E63 and was increased by PMA (Figure 5A). Adhesion was blocked by an antibody against the β1 chain of VLA-4 (Figure 5B; Nojima et al., 1990), indicating that, similar to Rap1-GTP, RIAM induced β1 integrin-mediated adhesion. To determine whether RIAM induced active conformational changes in β1 integrins, we analyzed activation epitope exposure in cells transfected with empty vector, RIAM, or Rap1E63. Cells were incubated with HUTS4 antibody that specifically binds to β1 integrins in their active conformation (Carman and Springer, 2003; Luque et al., 1996). In basal conditions, RIAM-transfected cells showed increased binding to HUTS4 to levels comparable to cells transfected with Rap1E63 (Figure 5C).
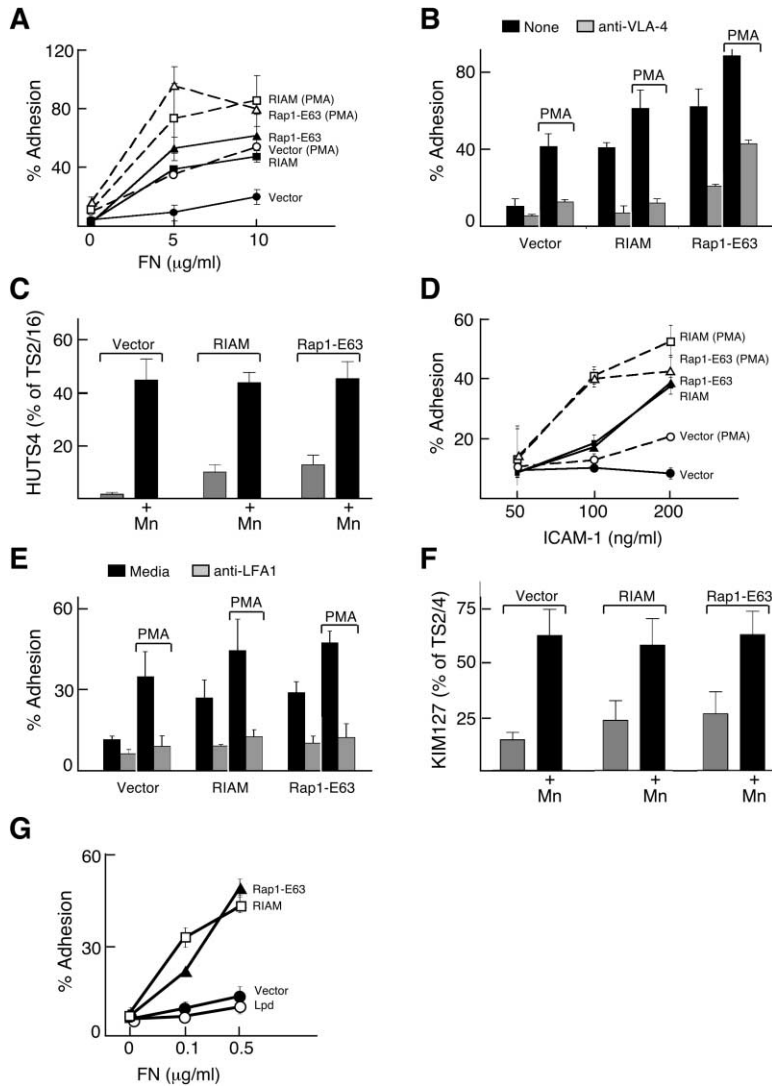
To examine if RIAM also regulates β2 integrin-mediated

Figure 5. RIAM Induces Cell Adhesion Mediated by β1 and β2 Integrins

(A and D) Jurkat cells stably transfected with RIAM (closed square), Rap1E63 (closed triangle), or vector (closed circle) were untreated (solid line) or treated (dashed line and open marker) with PMA, labeled, and seeded on triplicate wells coated with the indicated concentrations of fibronectin (A) or ICAM-1 (D). Bound cells are expressed as percentage of the total seeded cells. Data are means of at least four independent experiments. (The p values for RIAM binding to fibronectin are 0.000328 and 0.028 for cells untreated and treated with PMA, respectively. The p values for RIAM binding to ICAM are 0.00014 and 0.0033 for cells untreated and treated with PMA, respectively.)

(B and E) Jurkat cells stably transfected with RIAM, Rap1E63, or vector were untreated or stimulated with PMA. Cells were plated on 20 μg/ml fibronectin in the presence (gray bars) or the absence (black bars) of anti-VLA-4 antibody (B) or on 200 ng/ml ICAM-1 in the presence (gray bars) or the absence (black bars) of anti-LFA-1 antibody (E). Bound cells are expressed as percentage of the total seeded cells. Data are means of four independent experiments.

(C and F) Jurkat cells stably transfected with RIAM, Rap1E63, or vector were stained with the β1 activation-dependent antibody HUTS4 (C) or with the β2 activation-dependent antibody KIM127 (F) in the presence (black bars) or absence (gray bars) of Mn^{2+} and analyzed by flow cytometry. Signals from HUTS4 or KIM127 were expressed as a percentage of signals from the conformation-independent TS2/16 and TS2/4 antibodies, respectively.

(G) HEK293 cells stably transfected with RIAM (open square), Rap1E63 (closed triangle), Lpd (open circle), or vector alone (closed circle) were labeled and seeded on triplicate wells coated with the indicated concentrations of fibronectin. Bound cells are expressed as percentage of the total seeded cells.

adhesion, RIAM-, Rap1E63-, and vector-transfected Jurkat cells were seeded in ICAM-1-coated plates. RIAM-transfected cells displayed 3- to 4-fold increase of adhesion as compared to controls (Figure 5D). This adhesion was comparable to that induced by Rap1E63 and was increased by PMA. Addition of LFA-1 blocking mAb confirmed that increased adhesion was mediated by LFA-1 (Figure 5E). As with β1 integrins, RIAM exhibited a tendency to promote active conformation of LFA-1 as determined by using KIM127 antibody that specifically binds to β2 integrins in their active conformation (Figure 5F).

To test whether RIAM-induced adhesion requires Ena/VASP proteins, we transfected the Jurkat cell line stably overexpressing RIAM with proline-rich repeats of ActA to block interaction between Ena/VASP and proteins containing EVH1 binding motifs (Krause et al., 2000). RIAM-induced adhesion was unchanged (data not shown), suggesting that RIAM binding of Ena/VASP proteins is not required for RIAM-induced adhesion.

RIAM and its homolog Lpd have RA and PH domains and both interact with Ena/VASP proteins. Therefore, we compared the adhesive properties of HEK293 cells overexpressing RIAM or Lpd. RIAM-transfected cells had increased adhesion on fibronectin to levels comparable to Rap1E63-transfected cells. Surprisingly, Lpd-transfected cells showed no increase in adhesion but rather had a small but reproducible decrease in adhesion compared to controls (Figure 5G).

RIAM Knockdown Reverts Rap1-Induced Cell Adhesion

The ability of RIAM to bind Rap1-GTP, to induce integrin activation, and to increase adhesion prompted us to determine whether RIAM is a downstream effector of Rap1. Rap1E63 Jurkat cell line was stably transfected with a vector containing specific RIAM shRNA sequences (this line is named Rap1E63-RIAM-KD) or with shRNA vector control (this line is named Rap1E63-control-KD). Rap1E63-RIAM-KD cells had 80% reduction in RIAM protein compared with controls as determined by Western blot (Figure 6A). Rap1E63-RIAM-KD, Rap1E63-control-KD, and

A

Blot Ab.

RIAM

GAPDH

(lanes: Rap1-E63, RIAM-KD [Rap1-E63], Control-KD [Rap1-E63])

B Fibronectin

■ Media
□ PMA

% Adhesion

Rap1-E63

Vector, Rap1-E63, RIAM-KD, Control-KD

C ICAM-1

■ Media
□ PMA

% Adhesion

Rap1-E63

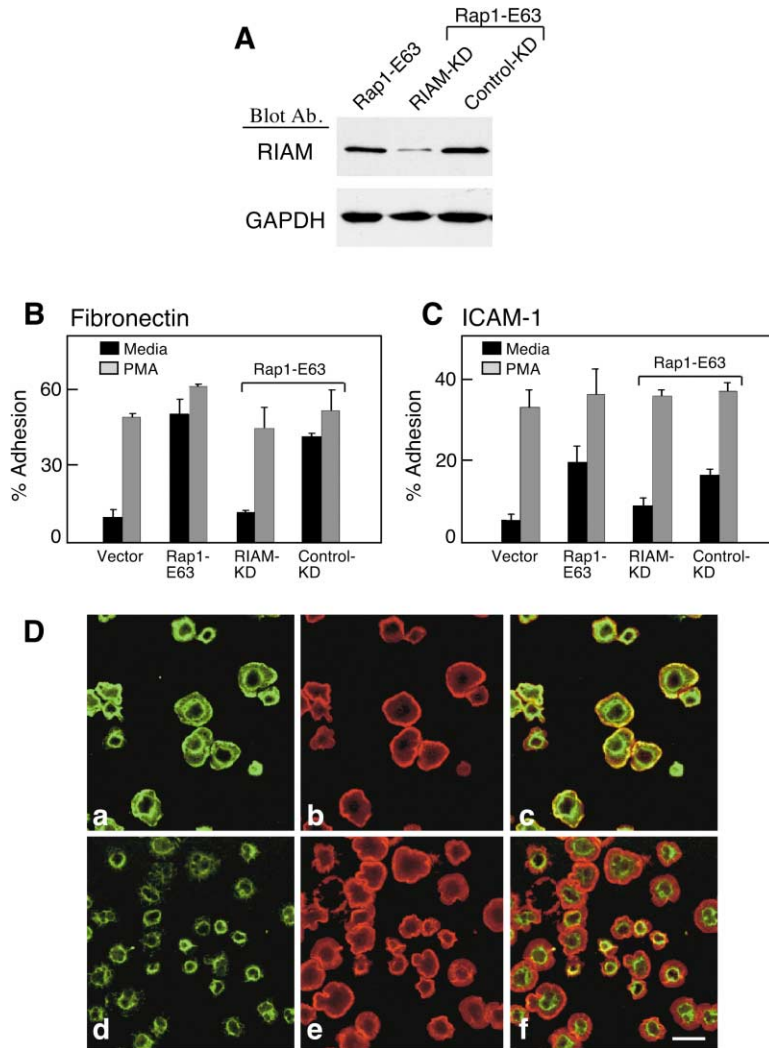Vector, Rap1-E63, RIAM-KD, Control-KD

D

a b c
d e f

Figure 6. RIAM Knockdown Abrogates Rap1-GTP-Mediated Cell Adhesion and Displaces Rap1-GTP from the Plasma Membrane

(A) RIAM expression was analyzed by Western blot in the indicated Jurkat cell lines. The amount of protein in each lane was normalized by expression of GAPDH.

(B and C) The indicated Jurkat cell lines were untreated (black bars) or treated (gray bars) with PMA and seeded in triplicate wells coated with fibronectin (B) or ICAM-1 (C). Bound cells were expressed as percentage of the total seeded cells. Results are representative of four independent experiments.

(D) Rap1E63-control-KD Jurkat cell line (a–c) or Rap1E63-RIAM-KD Jurakt cell line (d–f) were seeded on slides coated with anti-CD3 mAb. Cells were fixed, permeabilized, and incubated with GST-RalGDS-RBD followed by anti-GST antibody to detect localization of active Rap1 (a and d) and with Phalloidin to detect F-actin (b and e) and analyzed by confocal microscopy. Overlapping images are shown in (c) and (f). Scale bars equal 20 μm. Control staining showed no detectable binding (Supplemental Figure S3).

Rap1E63 cell lines were used in adhesion assays. To determine baseline adhesion, the vector control Jurkat cell line used in Figure 5 was also included. Compared to Rap1E63, Rap1E63-RIAM-KD cells displayed reduced adhesion on fibronectin to levels similar to vector-transfected Jurkat cells (Figure 6B). In contrast, Rap1E63-control-KD and Rap1E63 cells showed comparable adhesion to fibronectin. Similarly, Rap1E63-RIAM-KD cells had reduced adhesion to ICAM-1 as compared to Rap1E63 cells (Figure 6C). Thus, RIAM is required for Rap1-induced adhesion. This observation places RIAM downstream of Rap1-GTP.

**RIAM Is Required for the Recruitment
of Rap1-GTP to the Plasma Membrane**
Rap1-GTP localizes at the plasma membrane, and this localization is required for Rap1 to mediate cell adhesion (Bivona et al., 2004). Based on our observation that Rap1E63-RIAM-KD Jurkat T cells displayed reduced adhesion, we examined whether distribution of Rap1-GTP was affected by depletion of RIAM. We used GST-RalGDS-RBD, which interacts with Rap1-GTP (Reedquist and Bos, 1998) and has been used to determine

intracellular localization of Rap1-GTP (Bivona et al., 2004). Rap1E63-RIAM-KD and Rap1E63-control-KD Jurkat cells were seeded on anti-CD3-coated slides, fixed, and probed with GST-RalGDS-RBD. In Rap1E63-control-KD cells (Figure 6D, panels a–c), Rap1-GTP was localized in the perinuclear region and at the plasma membrane (panel a), consistent with previous reports (Bivona et al., 2004), where it colocalized with F-actin (panel c). Strikingly, in Rap1E63-RIAM-KD Jurkat cells (Figure 6D, panels d–f), Rap1-GTP was displaced from the plasma membrane and was detected only in the perinuclear region (panel d). In these cells, no colocalization of Rap1-GTP with F-actin was detected (panel f). These results indicate that RIAM is required for localization of Rap1-GTP at the plasma membrane.

**RIAM Is Required to Maintain the Cellular
Content of F-Actin**
Our studies showed that RIAM-overexpressing cells display extensive lamellipodia. The physical force for lamellipodia formation is provided by polymerization of actin underneath the plasma membrane. RIAM interacts with Profilin and Ena/VASP proteins, important regulators of

A

B

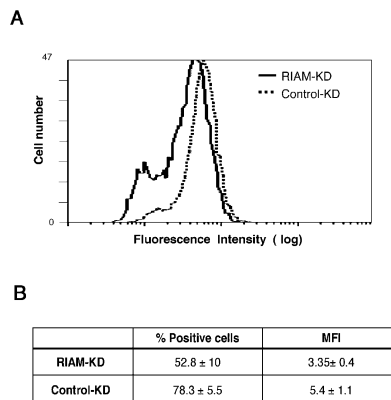|	% Positive cells	MFI
RIAM-KD	52.8 ± 10	3.35± 0.4
Control-KD	78.3 ± 5.5	5.4 ± 1.1

Figure 7. RIAM Is Required to Maintain the Cellular Content of F-Actin

(A) Stable RIAM-KD and Control-KD Jurkat T cell lines were stained with Rhodamine-Phalloidin and examined by FACS analysis for F-actin content. Results of one representative experiment are shown.

(B) Summarized results of five independent experiments are shown as percentage of positive cells and as mean fluorescense intensity (MFI) of Rhodamine-Phalloidin binding (p values for % of positive cells and MIF are 0.045 and 0.032, respectively).

actin polymerization. For these reasons, we examined whether diminished expression of RIAM might affect the content of polymerized F-actin. We used our vector control Jurkat cell line stably transfected with RIAM shRNA (RIAM-KD) or with control shRNA (control-KD). In RIAM-KD cells, expression of RIAM was reduced by 80% (Supplemental Figure S4). F-actin content was detected by staining with Phalloidin. Quantification by flow cytometry showed that RIAM depletion resulted in significant reduction in F-actin content, as RIAM-KD cells had 38.8% ± 9% downshift in the mean fluorescence intensity of Phalloidin binding compared to control-KD cells (Figure 7). However, the total actin content was comparable in RIAM-KD and control-KD cells (data not shown), suggesting that RIAM regulates the ratio of F-actin: G-actin content.

Discussion

Rap1 is a small GTPase with a critical role in cell adhesion and spreading (Bos et al., 2001). Recent studies have shown that TCR, CD98, and CD31 mediate activation of LFA-1 integrin in T cells, in a Rap1-GTP-dependent manner. We have identified RIAM, an adaptor protein that may link Rap1-GTP to integrin activation in T cells. First, RIAM and Rap1 associated upon TCR ligation in Jurkat T cells. Second, localization of Rap1-GTP at the plasma membrane that correlates with the ability of Rap1 to induce integrin-mediated adhesion (Bivona et al., 2004) was abrogated in RIAM knockdown cells. Consistently, knocking down RIAM eliminated adhesion mediated by Rap1-GTP. Third, overexpression of RIAM promoted the active conformation of integrins and enhanced cell adhesion. Overexpression of RIAM enhanced adhesion even in the presence of Spa1, a Rap1 GAP that inactivates Rap1, indicating that RIAM functions downstream of Rap1 (data not shown).

Our data showed that in T cells, RIAM and active Rap1 induced a comparable increase in cell adhesion and RIAM expression is required for Rap1E63-mediated adhesion. Interestingly, in HEK293 cells, in which expression of either RIAM or Rap1 could induce adhesion, we detected only minimal expression of RIAM protein. It is possible that these low levels of RIAM in HEK293 cells are sufficient to mediate Rap1-induced adhesion. It is also possible that Rap1 may induce adhesion by mechanisms that do not require RIAM. Consistent with the latter possibility is our recent observation that Rap1E63-transfected HEK293 cells have increased number of focal adhesions but a low change in active conformation of β1 integrins (E.M.L., C.V.C., A.A.F.L.v.P., M.K., A.B., and V.A.B., unpublished data). Thus, in certain cell types, Rap1 may mediate increase in adhesion by regulating integrin redistribution rather than integrin affinity.

RIAM interacts with Profilin and with Ena/VASP family proteins, both known to regulate actin dynamics. Since Ena/VASP proteins also bind Profilin, it is possible that RIAM may interact with Profilin directly, indirectly through Ena/VASP, or both. Ena/VASP proteins are involved in spreading of platelets, macrophage phagocytosis, formation and locomotion of lamellipodia, actin reorganization in T cells, and filopodia formation in neuronal growth cones and *Dictyostelium* (Coppolino et al., 2001; Han et al., 2002; Krause et al., 2000; Laurent et al., 1999; Lebrand et al., 2004; Reinhard et al., 1992). Both RIAM and Lpd localize at the leading edge and may recruit Ena/VASP proteins to this site (see accompanying article by Krause et al. [2004]). Thus, RIAM may regulate the actin cytoskeleton through recruitment of Profilin and Ena/VASP. In fact, we have observed that overexpression of RIAM induces cell spreading and lamellipodia formation, processes that require actin cytoskeleton reorganization. Conversely, RIAM knockdown cells have a lower content of F-actin, indicating that RIAM together with its interacting partners Profilin and Ena/VASP proteins may play a role in actin polymerization. Strikingly, Lpd also regulates actin polymerization, as Lpd knockdown cells had a dramatic reduction in F-actin content (accompanying article by Krause et al. [2004]).

Ena/VASP proteins directly regulate actin dynamics (Krause et al., 2003). When all Ena/VASP proteins were delocalized in Jurkat T cells, the reorganization of the actin cytoskeleton at the contact site with anti-CD3-coated latex beads was impaired (Krause et al., 2000). Fyb/SLAP also associates with Ena/VASP proteins (Krause et al., 2000). Interestingly, T cells from Fyb/SLAP-deficient mice do not have detectable differences in the content of polymerized actin as compared to control cells (Peterson et al., 2001). Our present results suggest that RIAM may substitute for Fyb/SLAP, explaining the absence of a dramatic phenotype in Fyb/SLAP-deficient cells.

Disruption of the interaction between RIAM and Ena/VASP by expression of ActA repeats had no effect on the ability of RIAM to mediate adhesion. Therefore, the effect of RIAM on integrin activation is likely Ena/VASP independent. Similarly, despite its overall similarity to RIAM and shared ability to bind Ena/VASP, Lpd overexpression could not induce adhesion. Together, these observations suggest that the ability of RIAM to mediate

Rap1-induced adhesion may be a RIAM-specific, Ena/VASP-independent attribute.

Recently RAPL, another Rap1 effector, was described. RAPL binds to Rap1-GTP and modulates LFA-1 distribution and ligand binding. It has been proposed that RAPL may interact with αL integrin subunit, thereby destabilizing the formation of αLβ2 heterodimer and upregulating ligand binding affinity (Katagiri et al., 2003). Our present data showed that RIAM can induce the active conformation of β1 and β2 integrins. Further work will be required to determine how RIAM induces integrin conformational changes.

We have identified RIAM as a partner of Rap1-GTP. In the accompanying article, Krause et al. (2004) have identified Lpd as a binding partner of Ena/VASP proteins. RIAM and Lpd, along with their *C. elegans* and the *Drosophila* orthologs Mig-10 and CG11940, form a gene family that we named the MRL family. Members of the MRL family share a common domain structure with the presence of a RA domain, a PH domain, and proline-rich motifs. We have shown that the RA domain of RIAM binds preferentially to Rap1. In the accompanying article, Krause et al. showed that the PH domain of Lpd is specific for the phosphoinositide PI(3,4)P_2. The PH domain of RIAM is highly related to the PH domain of Lpd, suggesting that it may have a similar specificity. Since generation of PI(3,4)P_2 is one of the consequences of TCR ligation, recruitment of RIAM via its PH domain to the inner membrane leaflet may take place during T cell activation. TCR ligation induces the recruitment of Rap1-GTP to the T cell:APC contact site (Katagiri et al., 2002). At this location, interaction between Rap1-GTP and RIAM can take place, resulting in integrin activation and enhanced cell adhesion. Our studies showed that RIAM has a critical role in regulating membrane localization of Rap1-GTP, since constitutively Rap1-GTP was displaced from the plasma membrane in RIAM knockdown cells. Although the precise mechanism via which RIAM affects localization of Rap1-GTP remains to be determined, it is possible that via its interaction with phosphoinositides, RIAM may stabilize Rap1-GTP at the plasma membrane. In agreement with this hypothesis, we have observed that RIAM PH domain is required to mediate RIAM/Rap1 interaction. To our knowledge, RIAM is the first Rap1-interacting protein shown to contain a PH domain. Thus, RIAM, an effector of Rap1, controls Rap1 localization at the plasma membrane, mediates Rap1-induced adhesion, and has an additional role in regulating actin dynamics.

Experimental Procedures

Yeast Expression Plasmids and Interaction Trap
pLexA and pJG4-5 (pB42AD) were used as "bait" and "prey" vectors, respectively. The yeast strain EGY191 (Invitrogen) was transformed sequentially with the plasmid pLexARap1E63 and a Jurkat cDNA library in pJG4-5 (Clontech). Interaction trap was done as described (Golemis et al., 1994; Gyuris et al., 1993). Rap1WT, Rap1V12, Rap1N17, Rap1E63 cDNA were cloned in-frame with the DBD of LexA. RIAM deletion mutants amplified by PCR from cDNA clones were inserted in pJG4-5. The yeast strain EGY191 and the plasmids pLexA, pLexAc-H-RasV12, and pRFHM-1 (pLexABicoid) pJG4-5-VPR were kindly provided by Dr. P. Silver (Dana-Farber Cancer Institute).

Isolation of Full-Length RIAM
We isolated full-length clones by ClonCapture cDNA selection kit (Clontech) using a Jurkat cDNA library and RIAM (nt 540–841) biotinylated PCR fragment as probe.

Northern Blot Analysis
Human Multiple Tissue Northern blots (Clontech) were incubated 30 min at 42°C in prehybridization solution (Molecular Research Center, Inc.) and hybridized overnight at 42°C with multiprime randomly labeled [α-^{32}P]dATP probes (Megaprime Labelkit, Amersham).

Mammalian Expression Plasmids
The ORF of Rap1WT, Rap1E63, and c-H-RasV12 (kindly provided by Dr. H. Kitayama, University of Tokyo, Japan) were subcloned into pAXEF-1 (Anumanthan et al., 1998). Myc-tagged RIAM was inserted in pAXEF or pcDNA1.1 (Invitrogen) vectors. pSRαHA (kindly provided by Dr. C. Rudd, DFCI) was used to express RIAM and Profilin as HA-tagged fusions. Profilin was PCR amplified from pGADProfilin I (kindly provided by Dr. L. van Aelst, Cold Spring Harbor). To create plasmid-expressing RIAM shRNA, specific oligonucleotides were cloned into pLL3.7 vector (Rubinson et al., 2003). As controls, vector expressing shRNA for mouse Lpd or empty vector were used. The sequences of the shRNA oligos can be obtained from the authors upon request.

Antibody Production
RIAM (aa 480–665) was cloned into pGEX-6P1 vector (Amersham Pharmacia Biotech). GST-RIAM fusion protein was immobilized on glutathione agarose, digested with Prescission protease (Amersham Pharmacia Biotech), and used to raise the polyclonal rabbit antiserum #4612 (Covance). Purified antibody was used for RIAM immunoprecipitation and immunofluorescence. For Western blot, we used an affinity-purified polyclonal antibody directed against the RIAM peptide $_{79}$AEQRTIQAQKSLQNQHH$_{96}$ (Biosource #5541.1).

In Vitro Protein Interaction Assays
PCR fragments from Rap1 WT, Rap1 E63, VASP, and VASPΔ were inserted into pGEX vectors (Pharmacia Pharmacia Biotech), expressed in *E. coli* DH10B, and purified on glutathione-sepharose (Amersham Pharmacia Biotech). 10 μg of GST and GST fusion proteins were incubated for 1 hr at 4°C with 50 μl of glutathione-sepharose in GST-buffer (1% NP-40, 10% glycerol, 50 mM Tris-Cl [pH 7.5], 200 mM NaCl, and 2 mM MgCl$_2$). 10 μl of [^{35}S]methionine-labeled RIAM or Luciferase synthesized in vitro (TNT T7 System Promega) were suspended in GST buffer and incubated for 1 hr at 4°C with the indicated GST-fusion proteins coupled on glutathione-sepharose. The samples were analyzed on 10% SDS-PAGE and visualized by autoradiography. 1 μl of [^{35}S]methionine-labeled products were analyzed along with the pull-down assays as control.

Stable and Transient Transfections and T Cell Activation
COS, HEK293, and Jurkat cells were cultured routinely. Stable transfections were done by electroporation. Transient transfections in COS cells were done using DEAE-dextran. Stable cell lines expressing shRNAs were generated either by electroporation of the linearized plasmids or by infection with lentivirus according to described protocol (Rubinson et al., 2003). Cells were sorted for GFP expression. Primary T cells were isolated from leukapheresis products of healthy volunteer donors using the RosetteSept Antibody cocktail (Stem Cell Technology). T cells were left untreated or were incubated with 1 μg/ml anti-CD3 mAb and 1 μg/ml anti-CD28 mAb (CLB; Research Diagnostics) for 30 min on ice followed by crosslinking with rabbit-anti-mouse Ig (DAKO; 20 μg/ml) for the indicated time periods.

GST Pull-Down, Immunoprecipitation, and Western Blot
GST-RalGDS-RBD fusion proteins were purified on glutathione-sepharose (Amersham Pharmacia Biotech). 50 μg of fusion protein coupled to beads were incubated with Jurkat cell lysates in GST-buffer. Eluted proteins were immunoblotted with Rap1 antibody. For immunoprecipitations, cell lysates (1 mg) were incubated overnight at 4°C with the indicated antibodies: RIAM polyclonal antibody #4612, Rap1 antiserum (Santa Cruz Biotechnology) precoupled to

Sepharose beads (GammaBind Plus); anti-HA (rat mAb, 3F10 agarose matrix, Roche); and Ras (mouse mAb agarose slurry, Santa Cruz Biotechnology). Proteins were analyzed by immunodetection with antibodies against RIAM #5541, Rap1 (Santa Cruz), Ras (mAb, BD/Transduction Laboratories), c-Myc (mAb 9E10, Santa-Cruz), and HA (mAb 12CA5, Boehringer). Stripping and reblotting of the blots was done as described (Boussiotis et al., 1997).

Confocal Microscopy

HEK293 cells were seeded onto fibronectin-coated coverslips, and Jurkat cells were settled on poly-L-lysine coated slides. Cells were fixed with 4% (w/v) paraformaldehyde in PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 120 mM sucrose [pH 7.3]) and permeabilized with 0.1% (v/v) Triton-x-100 in TBS. Samples were incubated with RIAM polyclonal antibody followed by Texas red-conjugated donkey anti-rabbit IgG. Cy5-Phalloidin or Rhodamine-Phalloidin (Molecular Probes) was used for F-actin staining. For detection of active Rap1, Jurkat cells were settled onto poly-L-lysine slides coated with OKT3 antibody (10 μg/ml), fixed, permeabilized, and incubated with purified GST-RalGDS-RBD or GST followed by mAb against GST and Cy-5-conjugated donkey anti-mouse mAb (Jackson Immunolabs). Samples were analyzed by Zeiss LSM 510 confocal microscope. Images were processed for presentation using Adobe Photoshop.

Cell Adhesion

96-well flat bottom plates (Falcon, BD Biosciences) were coated with human fibronectin (Sigma) or 2% BSA (Sigma) as negative control. For ICAM-1 adhesion, plates were incubated with goat-anti-human IgG (Jackson ImmunoResearch), followed by human recombinant ICAM-1 (Jackson ImmunoResearch). Cells were labeled with 0.5 μM BCECF (Molecular Probes, Eugene, OR), washed, and resuspended in culture media. When indicated, cells where preincubated 30 min at 37°C with media supplemented with 100 ng/ml PMA, anti-VLA-4, or anti-LFA-1 antibodies. Cells were added to triplicates (HEK293 25,000 c/well; Jurkat 100,000 c/well) and incubated 30 min at 37°C. Input was determined using a fluorescence plate reader (Perspective Biosystem). Unbound cells were removed by washing three times with warm 0.2% BSA-PBS. Adherence was calculated as emission of bound cells divided by emission of total cells seeded per well.

Integrin Activation Epitope Exposure Assays

Cells were incubated with HBS (20 mM HEPES [pH 7.2], 150 mM NaCl) containing either 1 mM $MgCl_2$ and 1 mM $CaCl_2$ or 1 mM $MnCl_2$ and either control (X63), β1 expression (TS2/16), LFA-1 expression (TS2/4), β1 activation-dependent (HUTS4), or β2 activation-dependent (KIM127) mAbs. Samples were stained with goat anti-mouse-FITC, washed, resuspended in HBS with 1 mM cations, and analyzed by flow cytometry. Signals from the activation-dependent antibodies HUTS4 or KIM127 were expressed as percentage of signals from activation-independent antibodies TS2/16 and TS2/4, respectively.

Assessment of Cellular Content of F-Actin

Assessment of F-actin content was done as described (Peterson et al., 2001). Cells were fixed and permeabilized with Cytofix/cytoperm buffer (Pharmingen), stained with Rhodamine-Phalloidin, and analyzed by flow cytometry.

Acknowledgments

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