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Review

The MRL proteins: Adapting cell adhesion, migration and growth

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ABSTRACT

MIG-10, RIAM and Lamellipodin (Lpd) are the founding members of the MRL family of multi-adaptor molecules. These proteins have common domain structures but display distinct functions in cell migration and adhesion, signaling, and in cell growth. The binding of RIAM with active Rap1 and with talin provides these MRL molecules with important regulatory roles on integrin-mediated cell adhesion and migration. Furthermore, RIAM and Lpd can regulate actin dynamics through their binding to actin regulatory Ena/VASP proteins. Recent data generated with the *Drosophila* MRL ortholog called Pico and with RIAM in melanoma cells indicate that these proteins can also regulate cell growth. As MRL proteins represent a relatively new family, many questions on their structure–function relationships remain unanswered, including regulation of their expression, post-translational modifications, new interactions, involvement in signaling and their knockout mice phenotype.

Introduction

MIG-10 (abnormal cell migration protein 10), RIAM (Rap1-GTP-interacting adaptor molecule) and Lamellipodin (Lpd) are members of the MRL family of adaptor molecules, a group of proteins involved in the regulation of actin dynamics, cell adhesion and migration, as well as in cell growth (Krause et al., 2004; Lafuente et al., 2004). MIG-10 represents the *Caenorhabditis elegans* ortholog of the MRL proteins (Manser and Wood, 1990), whereas the related Pico protein was characterized as the *Drosophila* MRL ortholog (Lyulcheva et al., 2008).

The MRL proteins share an N-terminal coiled-coil region, a central region with Ras-association (RA) and pleckstrin-homology (PH) domains, a proline-rich C-terminus with multiple FPPPP motifs that interact with Ena/VASP homology 1 (EVH1) domains found in the actin regulatory proteins enabled (Ena) and vasodilator-stimulated phosphoprotein (VASP), as well as XPPPP motifs that interact with profilin and SH3-binding motifs (XP-pXP) (Fig. 1A). MRL proteins localize both in the cytoplasm as well as in lamellipodial protrusions (Krause et al., 2004; Lafuente et al., 2004).

The identification of MRL interaction partners has contributed to define their role as regulators of cell adhesion and actin dynamics. Thus, binding of RIAM to Rap1 and talin revealed its contribution to integrin activation and cell adhesion, whereas RIAM and Lpd interaction with Ena/VASP proteins unveiled their regulatory role on the actin cytoskeleton (Krause et al., 2004; Lafuente et al., 2004). As actin dynamics control cell migration, it was expected that MRL proteins might be involved in cell motility. Indeed, the *mig-10* gene is required for the migration of certain neurons during development, and RIAM silencing in tumor cells leads to blockade of cell migration and invasion (Hernandez-Varas et al., 2011). Furthermore, recent data provided evidence for *pico*- and RIAM-dependent cell growth regulation in *Drosophila* and melanoma cells, respectively (Hernandez-Varas et al., 2011; Lyulcheva et al., 2008). In this review we summarize the present knowledge of MRL protein structure and functions.

MRL proteins: structure and interactions

RIAM

RIAM was first termed APBB1IP (amyloid beta (A4) precursor protein-binding, family B, member 1-interacting protein), because it bound to the WW (tryptophan-tryptophan) domain of APBB1 (Ermekova et al., 1997), as well as RARP1 (retinoic acid response proline-rich protein), as it was differentially induced in cells exposed to all-trans-retinoic acid (Inagaki et al., 2003). In a subsequent yeast two-hybrid screening for Rap1-GTP binding proteins in T cells that are involved in Rap1-dependent regulation of integrin-mediated cell adhesion and actin dynamics, Lafuente et al. isolated the *RIAM* gene that defined a new class of multi-adaptor molecules together with Lpd and MIG-10 (Lafuente et al., 2004). Following this report, a screen aimed at identifying novel ligands for Ena/VASP family proteins described the murine ortholog of human RIAM, that was named PREL1 (proline-rich EVH1 ligand 1) and which was

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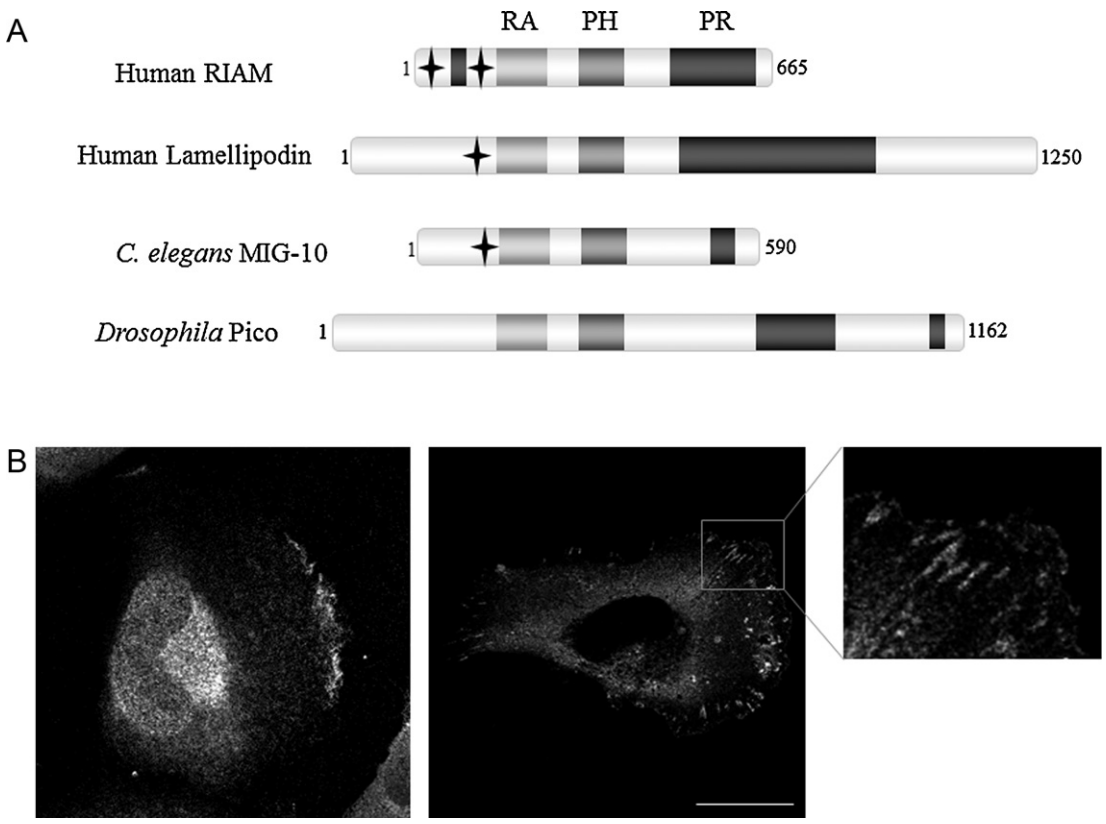

Fig. 1. Domain composition of MRL proteins and RIAM cell localization. (A) RA, Ras-association domain; PH, pleckstrin-homology domain; PR, proline-rich; the stars represent coiled-coil regions. (B, left) Expression of endogenous RIAM in BLM melanoma cells was detected with anti-RIAM antibodies. (B, right) Shown is RIAM-GFP in BLM transfectants. Bar, 25 μm.

found to localize at lamellipodia tips and focal adhesions (FAs) in response to Ras activation (Jenzora et al., 2005).

The human *RIAM* gene locus resides on chromosome 10p12.1 (Lafuente et al., 2004). Two transcripts of 5.4 and 2.8 kb were reported in immune tissues, whereas only the larger transcript was found in non-immune tissues. The human and murine RIAM open reading frames are 1998 and 2007 bp, respectively, with 665 and 669 amino acids (Fig. 1A) (Jenzora et al., 2005; Lafuente et al., 2004). Although the predicted molecular weights (MW) for human and murine RIAM are 73 and 74 kDa, most anti-RIAM antibodies recognize 100–110 kDa bands in Western blots. Initial database analyses revealed that RIAM presented homology with the growth factor receptor-bound (Grb) adaptor molecules Grb7, Grb10 and Grb14. However, RIAM as well as Lpd and MIG-10, lack the SH2 and BPS domains found on these Grb proteins. While the RA and PH domains of RIAM and Lpd are conserved, their N-terminal and C-terminal regions show considerable divergence. At its N-terminus, RIAM contains an additional proline-rich region with two putative EVH1 binding sites and an extra coiled-coil motif that is not present in other MRL members. The RIAM C-terminus contains a proline-rich motif comprising five profilin-binding sites and five EVH-1 binding motifs.

Both RIAM and Lpd share the capacity to bind talin. A talin-binding region localizes within the first 100 amino acids of the RIAM N-terminus (Fig. 2A and B), a region that lacks the RA, PH and most of Ena/VASP binding motifs (Lee et al., 2009). Mapping studies indicated that the N-terminal 30 residues of RIAM predicted to form amphipathic helices contain a minimal binding site for direct interaction with talin. Contrary to Lpd, RIAM interacts both *in vitro* and *in vivo* with active Rap1. This interaction requires both the RA and PH domains, whereas the RIAM N-terminus was found to enhance this interaction (Lafuente et al., 2004). Moreover, RIAM also bound to active Ras although to a lower extent compared with RIAM-Rap1 interaction. PH domains bind inositol phosphates and help protein localization at cell membranes (Lemmon, 2003). Another difference between RIAM and Lpd resides within the PH domain binding preferences. *In vitro* phospholipid immobilization on membranes revealed that the RIAM PH domain bound with high affinity to phosphatidylinositol (PtdIns) monophosphates PtdIns(3)P and PtdIns(5)P. Instead, the PH domain of Lpd preferentially binds phosphatidylinositol biphosphate [$PI(3,4)P_2$] (Jenzora et al., 2005; Krause et al., 2004). Since phosphoinositides represent early polarizing molecules during cell activation, the differential binding capacities of Lpd and RIAM PH domains may contribute to distinct temporal and spatial localization of these proteins.

RIAM and Lpd have N-terminal and C-terminal domains that contain proline-rich EVH1 binding sites and profilin binding motifs, which mediate their interaction with Ena/VASP proteins and with profilin, respectively (Fig. 2A and B) (Jenzora et al., 2005; Lafuente et al., 2004). RIAM–VASP interaction takes place preferentially with the dephosphorylated form of VASP, as observed in β3 integrin-null animals that showed impaired PKA activation leading to dephosphorylated VASP (Worth et al., 2010). Interestingly, the increase in RIAM–VASP binding led to enhanced talin-β1 association and upregulated FA turnover.

In stimulated T cells, RIAM was found to be a substrate for the tyrosine kinases Fyn, Lck and ZAP-70 (Patsoukis et al., 2009). Furthermore, the RIAM C-terminal proline-rich region constitutively interacts with the SH3 domain of phospholipase C-γ1 (PLC-γ1) (Fig. 2A), and this interaction augmented following T cell activation (Patsoukis et al., 2009). Moreover, the region spanning the RA and PH domains of RIAM constitutively interacts with Src kinase-associated phosphoprotein of 55 kDa (SKAP55)

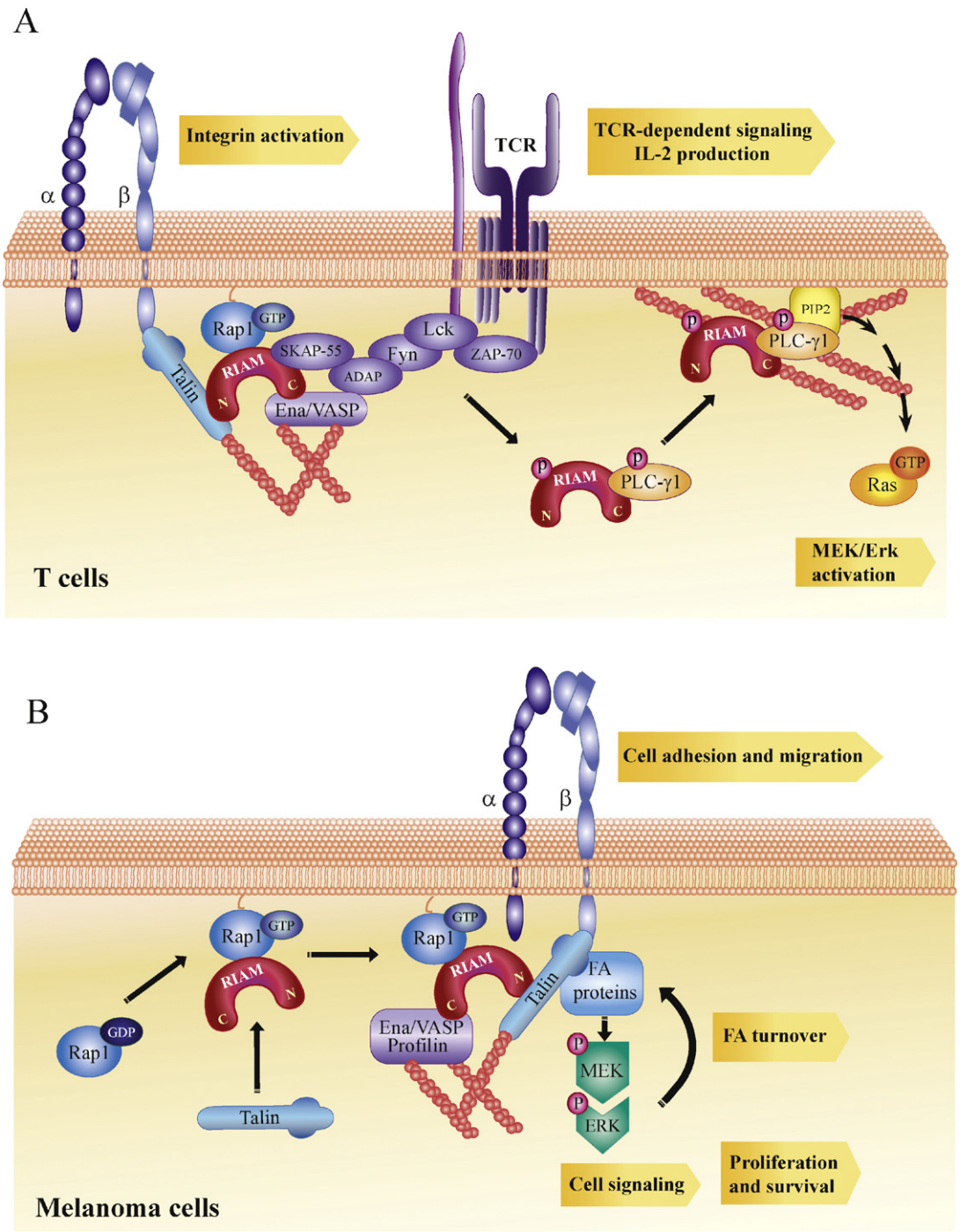

Fig. 2. Models for RIAM functions in T cells and in fibroblasts and melanoma cells. (A, left) Engagement of the TCR leads to integrin activation and increased T cell attachment to integrin ligands. RIAM could connect signaling transmitted by the SKAP-55/ADAP module and Rap1 activation to contribute to integrin activation (Menasche et al., 2007). (A, right) In addition, TCR engagement results in kinase-dependent RIAM tyrosine phosphorylation, PLC-γ1 activation and RIAM/PLC-γ1 binding, which is needed for PLC-γ1 translocation to the actin cytoskeleton and for PLC-γ1 reaction with PtdIns(4,5)P_2 (PIP2) (Patsoukis et al., 2009). (B) In melanoma cells, a RIAM/Rap1-GTP complex recruits talin to the plasma membrane where it binds the β1 integrin subunit, a mechanism modulating cell adhesion. RIAM binding to talin contributes to focal adhesion assembly and stimulates integrin-dependent outside-in activation which regulates cell proliferation and survival (Hernandez-Varas et al., 2011). A feed-back signaling from RIAM-dependent MEK-Erk1/2 activation to focal adhesions triggers the disassembly of these adhesion complexes (Coló et al., unpublished data).

(Fig. 2A), forming a module that associated with adhesion and the degranulation promoting adaptor protein (ADAP) and with active Rap1 (Menasche et al., 2007). In addition, recent data indicated that a RIAM/Mst/Kindlin-3 complex associated with an ADAP/SKAP55 module on chemokine-activated T cells (Kliche et al., 2012).

Pull-down experiments with fusion GST proteins followed by mass spectrometry have revealed that RIAM may associate with novel additional partners in T cells. Thus, GST-fused WW domains from formin-binding protein 11 (FBP11) and growth arrest-specific protein 7 (Gas7) bound to RIAM from Jurkat T cell lysates (Ingham et al., 2005). Moreover, RIAM interacts with GST-SH3 domains of the Pombe Cdc15 homology family members PACSIN1, PACSIN2 and PACSIN3, three proteins involved in regulation of cytoskeleton organization and endocytosis (Linkermann et al., 2009).

Lamellipodin

Lamellipodin, also known as Ras-associated and Pleckstrin homology domains containing protein 1 (RAPH1), was identified in a database search for proteins containing proline-rich motifs (FPPPP) predicted to bind Ena/VASP proteins (Krause et al., 2004). Lamellipodin was named after its subcellular localization, as it was enriched at the tips of lamellipodia and filopodia, where it co-localized with Ena/VASP proteins. Its cDNA sequence presents considerable homology to the *C. elegans* MIG-10 and to *D. melanogaster* Pico.

The human Lpd locus localizes in the chromosome 2q33. Database analyses reveal that besides the main Lpd isoform, there are nine additional splice isoforms (RMO, RMO1a, RMO1b, RMO1c, RMO1ab/Lpd-S, RMO1ac, RMO1bc, RMO1abc and RMO-RAPH1). These isoforms are shorter versions of Lpd with a length ranging from 592 to 649 amino acids, and some of them bear an insertion of 53 amino acids near the RA domain. Northern blot analyses using an N-terminal Lpd probe revealed the presence of at least 4 transcripts of 9, 5, 2 and 2.2 kb. EST and gene prediction programs suggested that the 9 and 5 kb transcripts represent Lpd mRNAs with different 3′UTR regions, while the 2.2 and 2 kb transcripts represented splice variants lacking the Lpd's C-terminus (Krause et al., 2004). The shorter variant RMO1 lacks the proline-rich motifs present in Lpd, bearing instead a serine-rich region. Another isoform, ALS2CR9, also called RMO1ab or Lpd-S, was first cloned as one of the genes localized in the genomic region associated to amyotrophic lateral sclerosis 2 (ASL2) (Hadano et al., 2001). However, mutations in this isoform are not related with this disease.

The main Lpd isoform is a 1250 amino acid protein with a MW of 200 kDa (Fig. 1A), with a highly charged N-terminus that contains a putative coiled-coil motif, but it is devoid of the proline-rich motif present in RIAM. At the C-terminus, Lpd diverges more from RIAM in sequence, with only 23.3% amino acid identity, as well as in length, with an extra-long 500 aa sequence extension. This region contains eight potential SH3 binding sites, three profilin interacting motifs and six EVH-1 domain binding regions. Individually, all the EVH-1 binding sites are involved in the interaction with Ena/VASP proteins (Krause et al., 2004). At present it is not known whether Lpd binds directly to profilin. A recent report demonstrated that srGAP3, a member of the Slit-Robo GAP family with specificity for Rac1, interacts through its SH3 domain with Lpd and co-localizes with it at the lamella (Endris et al., 2011).

Similarly to RIAM, an amphipathic helical motif in Lpd located in its N-terminal 1–355 region can bind talin (Lee et al., 2009). At the central region, Lpd contains the RA and the PH domains which are highly conserved in MRL proteins. The binding properties of the RA domain in Lpd have not been clearly established. Using pull-down assays, a short variant of Lpd was reported to bind active K-Ras, N-Ras, H-Ras or R-Ras (Rodriguez-Viciana et al., 2004). Krause and co-workers, however, could not find an interaction between Lpd and classical GTPases of the Ras and Rho family (Krause et al., 2004). Later it was reported that Rac could interact with Lpd and with MIG-10 (Quinn et al., 2006), a finding confirmed by Endris and co-workers (Endris et al., 2011). Nevertheless, there are no reports indicating that Lpd binds active Rap1 as it does RIAM, suggesting that both proteins may work under different GTPase signaling pathways.

Tyrosine phosphorylation has also been reported for Lpd. Following PDGF and netrin-1 stimulation of fibroblasts and primary neurons, Lpd is phosphorylated by the c-Abl kinase at residues Y426 and Y456 in the PH domain, and at Y1226 in the C-terminus (Michael et al., 2010). The c-Abl/Lpd interaction is mediated via the SH2 domain of c-Abl and tyrosine phosphorylated residues in Lpd.

MIG-10 and Pico

The lamellipodin/RIAM ortholog *mig-10* was identified in a screen for defective neuronal cell migration during *C. elegans* embryogenesis (Manser and Wood, 1990). The phenotype of mutations in *mig-10* indicated its requirement for long-range antero-posterior migration of embryonic neurons CAN (canal-associated neurons), ALM (anterior lateral microtubule cells) and HSN (hermaphrodite-specific neurons), as well as for efficient development of excretory canals. Defects in migratory neurons were the result of recessive *mig-10* alleles. Presently, four recessive alleles of the gene have been identified, all of them characterized by truncation of cell migration. *Mig-10 (ct41)* shows the strongest defects in excretory canal truncation due to an amber stop codon that effectively destroys protein function (Manser et al., 1997). Notably, disrupted embryonic cell migration responses shown by *mig-10* mutants resemble those of mutants for *unc-34*, the *C. elegans* Ena/VASP ortholog (Forrester and Garriga, 1997).

Cloning of the gene revealed that *mig-10* can potentially generate two protein isoforms that differ in their N-terminal regions, MIG-10A (667 aa) and MIG-10B (650 aa), but that share RA and PH domains and a proline-rich region (Fig. 1A) (Manser et al., 1997). The Ras Association-Pleckstrin Homology (RAPH) domain of MIG-10 interacts with activated CED-10, the *C. elegans* Rac1 ortholog (Quinn et al., 2008). Furthermore, MIG-10 binding to Unc-34 causes MIG-10 co-localization with actin, leading to the formation of lamellipodia (Quinn et al., 2006).

The *Drosophila* MRL ortholog *pico* (CG11940) encodes the only member of the MRL family in *Drosophila*. Two transcripts generated from alternative transcription star sites were identified: *pico* and *pico-L* (Lyulcheva et al., 2008). *Pico-L* encodes an 1159 amino acid protein that was identical to the protein encoded by *pico*, except for the presence of additional 128 N-terminal amino acids. The protein Pico contains RA and PH domains, as well as proline-rich Ena/VASP binding sites characteristic of the MRL members (Fig. 1A). Lyulcheva and co-workers reported that the Pico RA-PH region was capable of binding constitutively active forms of Ras and Rap1 in a yeast two hybrid system, suggesting that Pico may constitute a Ras or Rap1 GTPase effector. Furthermore, Pico interacts with the EVH1 domain of *Drosophila* Ena and influences actin dynamics.

Expression and subcellular distribution

RIAM is expressed in immune (thymus, spleen, lymph nodes and bone marrow) as well as non-immune tissues (lung, heart, liver, brain, kidney, skeletal muscle and pancreas) (Inagaki et al., 2003; Lafuente et al., 2004). RIAM protein has been detected in primary human T cells, as well as in several human T and B cell lines, in platelets, megakaryocytes, bone marrow macrophages, dendritic cells and fibroblasts (Han et al., 2006; Inagaki et al., 2003; Jenzora et al., 2005; Lafuente et al., 2004; Patsoukis et al., 2009). In addition, RIAM is expressed in melanoma cell lines and in melanoma cells metastasizing regional lymph nodes, as well as on stromal components of the tumors (Hernandez-Varas et al., 2011). Additionally, we found that RIAM is expressed on the MDA-MB-468 breast carcinoma cell line (Coló et al., unpublished data).

The Lpd isoform RMO1 is ubiquitously expressed with highest levels in liver, brain, heart, ovary and developing embryo, and absent in peripheral leukocytes. Furthermore, this isoform shows reduced expression in metastatic osteosarcoma, suggesting an inhibitory role in tumor progression (Eppert et al., 2005).

At the cellular level, RIAM localizes both in the cytoplasm as well as in lamellipodia at the leading edge of the cell membrane, where it co-localizes with F-actin and with Ena/VASP proteins at lamellipodia tips (Jenzora et al., 2005; Lafuente et al., 2004). In the human melanoma cell line BLM plated on fibronectin, RIAM localizes in the cytoplasm, but also concentrates at the edges of cell

protrusions in migrating cells (Fig. 1B). In metastatic melanoma tissue, RIAM distribution on melanoma cells was found to be heterogeneous, and thus some cells presented a cytoplasmatic localization, whereas in other cells RIAM was predominantly at the cell membrane (Hernandez-Varas et al., 2011).

The presence of RIAM at focal adhesions seems to depend on their maturation stage. Thus, looking at talin-containing FAs, Han et al. found that some early FAs contained RIAM at initial cell spreading phases, whereas RIAM was absent on mature FAs and fibrilar adhesions (Han et al., 2006). This could explain the fact that in migrating B16-F1 cells, transfected RIAM-GFP distributed prominently in lamellipodial protrusions but less in FAs (Jenzora et al., 2005). In platelets, RIAM was found at vinculin-rich filopodia and at lamellipodial edges, and in fully spread platelets, RIAM colocalized with vinculin and actin in FA-like structures (Watanabe et al., 2008). Finally, in Jurkat T cell transfectants, GFP-RIAM mostly localized in the cytosol but also to the T cell-antigen presenting cell contact site, where it co-localized with SKAP-55 and ZAP-70 (Menasche et al., 2007; Patsoukis et al., 2009).

Cellular Lpd is located at the plasma membrane, probably by interactions between its PH domain and the $PI(3,4)P_2$ present in the inner left of the membrane. When cells are activated via receptors for EGF, PFGF or Netrin-1, Lpd is recruited at the tips of filopodia and lamellipodia where it co-localizes with Ena/VASP proteins. However, this localization is independent of Ena/VASP, as cell treatment with cytochalasin D, an inhibitor of actin polymerization, displaced these proteins from the leading edge without affecting Lpd localization (Krause et al., 2004). Different from RIAM and other proteins containing EVH1 domain binding motifs, Lpd does not co-localize with Ena/VASP proteins at focal adhesions (Krause et al., 2004). The exclusion of endogenous Lpd from FAs in fibroblasts may reflect non-overlapping functions for Lpd and RIAM. In other cell types, however, forced expression of Lpd results in its recruitment to FAs where it co-localizes with SrGAP3, which is highly expressed in the developing brain (Endris et al., 2011).

MRL protein functions

MRL proteins are involved in integrin activation and cell adhesion, they regulate actin dynamics and cell migration, and they modulate signaling pathways that ultimately control cell growth. Table 1 shows updated information of the main functional properties of these proteins.

Integrin activation

RIAM contributes to integrin activation and promotes cell adhesion (see model on Fig. 2A and B). A well-known regulator of integrin activation in inside-out signaling is Rap1 (Bos, 2005). Following the finding that RIAM interacts with GTP-bound active Rap1, it was demonstrated that RIAM was required for Rap1-GTP recruitment to the plasma membrane and for Rap1-induced adhesion dependent on β1 and β2 integrins in Jurkat T cells, indicating that RIAM is a Rap1 downstream effector in the signaling leading to integrin activation (Lafuente et al., 2004). As noted above, RIAM and Lpd contain binding sites for talin, a cytoskeleton protein whose interaction with the cytoplasmic region of β integrin subunits constitutes a final step in integrin activation (Tadokoro et al., 2003). The connection between Rap1, RIAM and talin in integrin activation came from studies using the integrin αIIbβ3 as a model, which showed that talin can be detected in Rap1-RIAM complexes in platelets and fibroblasts, and this correlated with a RIAM requirement for β3 integrin activation (Han et al., 2006). It was later demonstrated that talin recruitment and activation of β3 that is mediated by active Rap1 needs RIAM (Watanabe et al., 2008), and that both talin and Rap1 binding sites on RIAM are required for integrin activation (Lee et al., 2009). Notably, in primary murine megakaryocytes silenced for RIAM expression, agonist-induced αIIbβ3 activation was inhibited, therefore identifying RIAM as a required distal element of thrombin-mediated and talin-driven αIIbβ3 activation (Watanabe et al., 2008).

T cell receptor (TCR)-mediated signals lead to integrin activation (Smith-Garvin et al., 2009). Both ADAP and SKAP-55 re-localize Rap1 and RIAM to the plasma membrane after TCR stimulation, and thus RIAM connects the ADAP/SKAP-55 module to Rap1 to facilitate TCR-mediated integrin activation (Menasche et al., 2007) (Fig. 2A). Furthermore, the SKAP-55-RIAM complex was shown to be essential for efficient conjugate formation between T cells and antigen presenting cells. More recent work has demonstrated that loss of the ADAP/SKAP55 module in T cells impairs chemokine CCL21-mediated affinity and avidity regulation of the LFA-1 integrin (Kliche et al., 2012). The same study identified two independent pools of the ADAP/SKAP55-module in CCR7-activated cells; one contains RIAM, Mst1, talin, kindlin-3 and Rap1, which associates with the β-chain of LFA-1, and the other includes RAPL, Mst1 and Rap1, which binds to the α-chain of this integrin.

Similarly to RIAM, Lpd can activate the αIIbβ3 integrin in a αIIbβ3-CHO cell system, but independently of Rap1 activation (Watanabe et al., 2008). Interestingly, the Lpd talin-binding site fused to a Ras membrane-targeting sequence was sufficient for the recruitment of talin to αIIbβ3 and for activation of this integrin (Lee et al., 2009), raising the possibility that Lpd might contribute to integrin activation via Ras proteins. Yet, Lpd overexpression in HEK293 cells leads to reduced adhesion to fibronectin (Lafuente et al., 2004). Although data so far suggest that Lpd can activate integrins, it remains to be determined whether this activation results in a subsequent increase in cell adhesion, and if endogenous Lpd does activate integrins. In addition, further work is needed to determine the differential contributions of Lpd and RIAM to integrin activation.

Although the above data indicate that RIAM contributes to integrin activation, this may well be cell-type specific. Thus, RIAM depletion in melanoma cells caused only minor reductions in β1 integrin activation that was associated with modest decreases in soluble binding of fibronectin (FN) and with partial inhibition in cell adhesion to fibronectin and collagen I, even if talin-β1 association was diminished in these cells (Hernandez-Varas et al., 2011).

Actin dynamics

All MRL proteins contain Ena/VASP binding sites on their sequences, suggesting that they might recruit Ena/VASP proteins to the leading edge and therefore regulate the organization of the actin cytoskeleton. Indeed, RIAM and Lpd co-localize with Ena/VASP proteins at lamellipodia tips and influence actin dynamics (Jenzora et al., 2005; Lafuente et al., 2004). Thus, RIAM overexpression in HEK293 and Jurkat cells promotes cell spreading and lamellipodia formation, two processes that depend on actin reorganization, whereas RIAM silencing causes a reduction in F-actin content, suggesting that RIAM regulates the F-actin:G-actin ratio (Lafuente et al., 2004). In Lpd-overexpressing cells, an increase in lamella protrusion velocity was observed, which constitutes a similar phenotype to the one observed upon Ena/VASP overexpression. On the contrary, Lpd knockdown impaired lamella formation. Indeed, electron microscopy studies showed that the F-actin content and the branching density of actin filaments at the lamellipodium are impaired in Lpd-silenced cells (Krause et al., 2004), which may explain the severe defects in lamella formation caused by Lpd depletion, since local actin polymerization is the driving force promoting membrane protrusions and lamella formation.

Table 1
Main properties of MRL proteins.

	RIAM	Lamellipodin	MIG-10	Pico
Main interactions	Binds active Rap1, talin and Ena/VASP proteins The PH domain has specificity for PtdIns(3)P and PtdIns(5)P	Binds active Ras members, talin and Ena/VASP proteins PH domain preferentially binds $PI(3,4)P_2$	Binds active CED-10, the *C. elegans* Rac1 ortholog Binds Ena/VASP proteins	Binds active Ras and Rap1, and Ena/VASP proteins
Post-translational modifications	Substrate for tyrosine kinases Fyn, Lck and ZAP-70	Phosphorylated by the c-Abl kinase	ND	ND
Functions	TCR-mediated integrin activation and regulation of PLC-γ localization Cancer cell invasion Focal adhesion dynamics	Regulation of axon morphogenesis and podocyte morphology	Promotes axonal guidance and growth cones	Required for tissue and organismal growth and viability
Actin dynamics	+	+	+	+
Lamellipodia formation	+	+	ND	ND
Integrin activation	+	+	ND	ND
Cell migration	+	+	+	ND
Cell growth	+	+	ND	+

Lpd also regulates axon morphogenesis in primary hippocampal neurons in vertebrates. In these cells, Lpd depletion reduced the length of the main axon and all axonal branches, a phenotype that was rescued by Lpd overexpression. This effect was found to be mediated by Lpd in cooperation with c-Abl, and it is dependent on Ena/VASP correct localization at the leading edge (Michael et al., 2010). Notably, a transient Lpd phosphorylation was detected upon Netrin-1-promoted activation of primary hippocampal neurons, increasing its interaction with Mena and the rate of lamellipodia formation. Similarly, Lpd is phosphorylated by c-Abl downstream of PDGF in fibroblasts, regulating dorsal ruffle formation by recruitment of Ena/VASP proteins (Michael et al., 2010). Also, in primary fibroblasts the interaction between Lpd and srGAP3 negatively regulates lamellipodial dynamics, and in the neuronal-like cell line N1E-115, srGAP3 and Lpd had opposite effects in the regulation of neurite-like projections (Endris et al., 2011). Lpd, srGAP3 and Rac1 seems to work together regulating actin dynamics by an as yet uncharacterized mechanism. Thus, a model has been proposed by which srGAP3 targeting to the membrane allows its interaction with Lpd, thus releasing the binding between Rac1 and Lpd, which leads to inhibition of membrane protrusion associated with a reduction of the local amount of Rac1-GTP.

Two independent studies proposed an interesting mechanism to modulate Lpd function and lamellipodia dynamics, consisting in the regulation of $[PI(3,4)P_2]$ levels at the leading edge. Venkatareddy and co-workers implicated Lpd in the regulation of podocyte morphology by its ability to regulate lamellipodial dynamics. Lpd was found to work downstream of the receptor Nephrin, in a complex with filamin and SHIP2 at the leading edge. In this location, the activity of PI3K and SHIP2 triggered an increase in the local amount of the phospholipid $[PI(3,4)P_2]$, regulating Lpd recruitment via its PH domain (Venkatareddy et al., 2011). In a similar model, profilin-1 regulated the motility of MDA-MB-231 breast cancer cells by inhibiting the recruitment of Lpd at the leading edge. In this case, profilin-1 bound $[PI(3,4)P_2]$, dampening its accessibility for interaction with the Lpd's PH domain (Bae et al., 2010).

To establish neural circuits between neurons, these cells initiate asymmetric axon outgrowth in response to extracellular guidance cues. At the tips of the growing axons sit the growth cones, which are structures that asymmetrically accumulate F-actin and microtubules where an axon projects forward. Unc-6 and its receptor Unc-40/DCC are conserved regulators of growth cone guidance, and they work together during axon formation to initiate, maintain and orient asymmetric neuronal growth. Studies in *C. elegans* have shown that as the axon forms, Unc-6, Unc-40, as well as the PI3K and PTEN *C. elegans* orthologs AGE-1 and DAF-18, respectively, drive MIG-10 ventrally in HSN neurons to promote asymmetric axon growth (Adler et al., 2006). Furthermore, these results indicated that asymmetrically localized $[PI(3,4)P_2]$ recruits MIG-10 to become itself asymmetrically distributed.

Connected with these data, genetic evidence demonstrated that MIG-10 functions downstream of Unc-6 and of the repulsion guidance cue SLT-1 to direct the ventral migration of the AVM (anterior ventral microtubule) and PVM (posterior ventral microtubule) axons in *C. elegans* (Quinn et al., 2006). These studies showed that overexpression of MIG-10 in the absence of Unc-6 and SLT-1 causes a multipolar phenotype with undirected outgrowths, but that addition of either guidance cue led to neurons becoming monopolar. Therefore, MIG-10 mediates guidance of AVM and PVM axons in response to extracellular Unc-6 and SLT-1. Furthermore, during neuronal development, *mig-10* and the Ena/VASP ortholog *unc-34* cooperate to direct axons to Unc-6 and away from SLT-1 cues (Chang et al., 2006). It was shown that while single mutants of either *mig-10* or *unc-34* displayed mild phenotypes, double mutants arrest early in development with severe axon guidance defects. Moreover, it was demonstrated that the number of filopodia that depend on *unc-34* actions was increased by *mig-10* in axons guided to Unc-6, and that *mig-10* activity in developing axons was stimulated by *age-1*. Collectively, these data indicate that *mig-10* and *unc-34* organize intracellular responses to axon guidance cues, and that *mig-10* and *age-1* lipid signaling promote axon outgrowth.

Genetic analyses revealed that *mig-10* and the Rac ortholog *ced-10* also function together to orient axon growth (Quinn et al., 2008). Importantly, the asymmetric MIG-10 distribution mediated by *ced-10* function in response to Unc-6 correlated with asymmetric accumulation of F-actin and microtubules. Therefore, MIG-10 would act downstream of Rac during axon guidance, and the data suggest that MIG-10/CED-10 interaction triggers local cytoskeletal assembly and polarizes Unc-6-dependent outgrowth-promoting activity. Together with the above observations, the overall picture shows that MIG-10 links Rac and PI3K to proteins that promote actin-based protrusive activity (Quinn and Wadsworth, 2008).

Finally, recent results showed that the muscle branching gene *madd-2* promotes attractive axon guidance to Unc-6 and assists Unc-6- and Unc-40-dependent ventral recruitment of MIG-10 in nascent axons (Hao et al., 2010).

Cell migration

Actin dynamics and cell migration are intimately interconnected processes (Parsons et al., 2010). RIAM regulates cell migration directionality and it is required for actin-dependent

melanoma cell migration and invasion (Hernandez-Varas et al., 2011). This study also revealed that defective invasion of RIAM-silenced melanoma cells correlates with deficient activation of a Vav2-RhoA-ROCK-MLC pathway, suggesting that their impaired invasion is likely based on altered cell contractility and cell polarization.

Cell migration and invasion require regulated formation and disassembly of integrin-dependent adhesion complexes. We recently showed that RIAM regulates focal adhesion dynamics (Coló et al., unpublished data) (Fig. 2B). Hence, RIAM-silenced melanoma and breast carcinoma cells display an increased number, size and stability of FAs, which accumulated centrally located at the ventral cell surface, and which was caused by defective FA disassembly. The impairment in disassembly of FAs due to RIAM silencing correlated with deficient MEK activation, and importantly, overexpression of constitutively active MEK led to rescue of FA disassembly and in recovery of cell invasion. These results indicate that integrin-triggered, RIAM-dependent MEK activation may represent a key feed-back event required for efficient FA disassembly, which may contribute to explain the role of RIAM in cell migration and invasion (Coló et al., unpublished data).

T cell activation responses

The dynamics of the actin cytoskeleton plays key roles in T cell signaling following binding of TCR to antigens (Dustin and Cooper, 2000; Smith-Garvin et al., 2009). Thus, actin filaments provide directional signaling and their importance in TCR-dependent responses is highlighted by the fact that actin destabilization with cytochalasin D blocks IL-2 transcription (Fischer et al., 1998). PLC-γ1 is recruited to the actin cytoskeleton upon TCR activation. Once activated, PLC-γ1 hydrolyzes phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] leading to generation of inositol 1,4,5-trisphosphate (IP_3), which promotes the release of Ca^{2+} from intracellular stores, and diacylglycerol (DAG), a protein kinase C (PKC) activator (Rhee, 2001). Recent data demonstrated that RIAM knock-down in T cells results in deficient activation of PLC-γ1, as translocation of pPLC-γ1 from the cytosol to the actin network was impaired (Patsoukis et al., 2009). As RIAM interacts with PLC-γ1, the results strongly suggest that RIAM might represent a docking site for localizing PLC-γ1 in close proximity to [PtdIns(4,5)P_2]-containing membranes (Fig. 2A).

The involvement of RIAM in the regulation of PLC-γ1 activation suggests that it could mediate Ras and Rap1 stimulation by controlling the activation of their guanine nucleotide exchange factors (GEFs). Indeed, RIAM silencing resulted in impaired translocation to membrane fractions of RasGRP1 and CalDAG-GEFI, which are GEFs for Ras and Rap1, respectively, as well as in the inhibition of Ras and Rap1 activation (Patsoukis et al., 2009). Therefore, RIAM regulation of PLC-γ1 localization plays central roles in TCR signaling and in the transcription of target genes.

Cell growth

The reduction in β1-integrin dependent adhesion in melanoma cells silenced for RIAM expression was seen to correlate with decreased activation of Erk1/2 MAP kinase and phosphatidylinositol 3-kinase (PI3K) (Hernandez-Varas et al., 2011), two central molecules controlling cell growth and cell survival. Notably, these cells exhibited inhibition of anchorage-independent growth, as well as impaired tumor growth and delayed metastasis in SCID mice. These data suggest that defective activation of Erk1/2 and PI3K in RIAM-silenced cells could account for inhibition of melanoma cell growth, and that RIAM might subsequently contribute to the dissemination of melanoma cells.

Similarly to the *Drosophila* MRL ortholog *pico*, Lpd has been implicated in mammalian cell proliferation through regulation of SRF (Lyulcheva et al., 2008), a transcription factor that responds to the cellular G-actin:F-actin ratio. This study showed that Lpd triggers an increase in the local amount of F-actin, releasing the cofactor MAL from its binding to G-actin and allowing its transport to the nucleus where it could work as a cofactor for SRF. The ability of Lpd to regulate SRF activity has been recently implicated in directing pyramidal neurons to select a radial migration pathway along glia rather than a tangential migration mode (Pinheiro et al., 2011).

Studies in *pico* mutant larvae revealed a dramatic reduction in size and early death, suggesting that *pico* is required for tissue and organismal growth and for viability (Lyulcheva et al., 2008). As mentioned above, *pico* activates SRF (Lyulcheva et al., 2008), and genetic analyses revealed that Mal/SRF levels are important for *pico*-mediated regulation of tissue growth. However, a recent report using *pico* RNAi or *pico* overexpression has challenged this finding (Thompson, 2010). Thus, this study concluded that Mal/SRF is not a general regulator of tissue or organismal growth in *Drosophila*. Further studies seem to be needed to clarify this point.

Concluding remarks

Due to their multi-domain composition, MRL proteins have the potential to regulate physiological and pathological processes involving actin reorganization, cell migration and cell growth. Yet, many questions are open to be experimentally addressed, perhaps due to the recent discovery of these proteins. Studies on their transcriptional regulation and post-translational modifications will be important to better know their spatio-temporal functions. The recent reports showing novel MRL protein interactors open the door to potentially new functional roles for these proteins. In addition, further work is needed to ascertain the implication of RIAM and Lpd in tumor cell invasion. Importantly, generation and characterization of RIAM and Lpd knock-out mice will provide more definitive answers on their involvement on development, immune function and pathologies.

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