Focal adhesion disassembly is regulated by a RIAM to MEK-1 pathway

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Summary

Cell migration and invasion require regulated turnover of integrin-dependent adhesion complexes. Rap1-GTP-interacting adaptor molecule (RIAM) is an adaptor protein that mediates talin recruitment to the cell membrane, and whose depletion leads to defective melanoma cell migration and invasion. In this study, we investigated the potential involvement of RIAM in focal adhesion (FA) dynamics. RIAM-depleted melanoma and breast carcinoma cells displayed an increased number, size and stability of FAs, which accumulated centrally at the ventral cell surface, a phenotype caused by defective FA disassembly. Impairment in FA disassembly resulting from RIAM knockdown correlated with deficient integrin-dependent mitogen-activated protein kinase (MEK)-Erk1/2 activation and, importantly, overexpression of constitutively active MEK resulted in rescue of FA disassembly and recovery of cell invasion. Furthermore, RIAM-promoted Ras homologue gene family, member A (RhoA) activation following integrin engagement was needed for subsequent Erk1/2 activation. In addition, RhoA overexpression partially rescued the FA phenotype in RIAM-depleted cells, also suggesting a functional role for RhoA downstream of RIAM, but upstream of Erk1/2. RIAM knockdown also led to enhanced phosphorylation of paxillin Tyr118 and Tyr31. However, expression of phosphomimetic and nonphosphorylatable mutants at these paxillin residues indicated that paxillin hyperphosphorylation is a subsequent consequence of the blockade of FA disassembly, but does not cause the FA phenotype. RIAM depletion also weakened the association between FA proteins, suggesting that it has important adaptor roles in the correct assembly of adhesion complexes. Our data suggest that integrin-triggered, RIAM-dependent MEK activation represents a key feedback event required for efficient FA disassembly, which could help explain the role of RIAM in cell migration and invasion.

Key words: Cell migration, Adhesion turnover, Focal adhesion, Integrin, Signalling

Introduction

Following membrane protrusion during cell migration, integrin-dependent adhesion complexes are assembled, subsequently mature and finally disassemble to allow cells to move in a polarised manner (Parsons et al., 2010). Focal adhesions (FAs) are composed of structural (such as talin, paxillin and vinculin) and signalling molecules [including focal adhesion kinase (FAK) and Src]. FAs are dynamic platforms that link the extracellular matrix with the actin cytoskeleton and mediate integrin-dependent outside-in signalling (Carragher and Frame, 2004; Lock et al., 2008; Parsons et al., 2010; Zaidel-Bar et al., 2007a; Zamir and Geiger, 2001). Upon integrin-mediated cell adhesion, FAK is recruited to FAs and undergoes autophosphorylation at Tyr397, allowing the binding of Src kinases, which further phosphorylate FAK at Tyr576/Tyr577. Subsequently, the FAK–Src complex triggers phosphorylation of other FA proteins, including paxillin at Tyr31 and Tyr118 residues (Deakin and Turner, 2008; Mitra et al., 2005), which leads to recruitment and localised MEK-Erk activation (Fincham et al., 2000; Ishibe et al., 2003). As discussed above, disassembly of FAs is crucial for cell migration. Microtubule (MT)-directed FA disassembly and disassembly controlled by the activation of different signalling pathways have previously been reported (Ezratty et al., 2005; Kaverina et al., 1999; Franco et al., 2004; Parsons et al., 2010; Schober et al., 2007; Webb et al., 2004). However, the mechanisms involved in FA turnover remain poorly understood.

Rap1-GTP-interacting adaptor molecule (RIAM) is a member of the Mig-10–RIAM–Lamellipodin (MRL) family of adaptor proteins that contains an N-terminal coiled-coil region that interacts with talin (Coló et al., 2012; Lafuente et al., 2004; Lee et al., 2009). RIAM was first characterised as a homolog (ENA)–vasodilator-stimulated phosphoprotein (VASP) proteins (Han et al., 2006; Jenzora et al., 2005; Lafuente et al., 2009). RIAM contains central Ras association (RA) and pleckstrin homology (PH) domains together conforming Rap1-binding regions, a C-terminal proline-rich domain, as well as several FPPP motifs with the potential of interacting with enabled homolog (ENA)–vasodilator-stimulated phosphoprotein (VASP) proteins (Han et al., 2006; Jenzora et al., 2005; Lafuente et al., 2004; Lee et al., 2009). RIAM was first characterised as a molecule that promotes Rap1-dependent β1 and β2 integrin activation in T lymphocytes (Lafuente et al., 2004). RIAM, but not lamellipodin, interacts with active Rap1 and functions as a scaffold for recruitment of talin to the cell membrane. Furthermore, confocal microscopy studies revealed that RIAM co-localises with talin in lamellipodia and filopodia (Han et al.,
2006; Lee et al., 2009; Watanabe et al., 2008), which might reflect an event involved in the localisation of activated integrins at these cell membrane protrusions. In addition, RIAM–VASP association was found to be increased in integrin β3-null cells, leading to regulated formation of talin–RIAM complexes at FAs (Worth et al., 2010).

We recently reported that RIAM depletion in human melanoma cells leads to impairment in persistent cell migration.
directionality, causing deficient cell invasion (Hernández-Varas et al., 2011). Furthermore, melanoma cells depleted of RIAM display significant inhibition of lung metastasis in a mouse xenograft model. The defective invasion of RIAM-knockdown cells was found to correlate with deficient association between β1 and talin and with inhibition of β1 integrin-dependent activation of the Erk1/2 mitogen-activated protein (MAP) kinases. Given that RIAM binds talin and controls integrin-dependent outside-in signalling and cell invasion and migration, it could be involved in adhesion turnover. Here, we investigated the role of RIAM in the dynamics of FAs and show that melanoma cells depleted of RIAM have increased numbers and stability of FAs, which is the result of defective FA disassembly. Alteration of FA turnover in these cells was associated with deficient MAP kinase kinase (MEK) activation and, importantly, upregulation of constitutively active forms of MEK rescued FA disassembly and cell invasion. Therefore, FA disassembly in melanoma cells is controlled by RIAM-dependent MEK activation, which might explain the role of RIAM in melanoma cell invasion.

**Results**

RIAM-depleted cells display altered number, distribution and stability of FAs

To investigate FA distribution in RIAM-knockdown cells, paxillin staining was analysed in BLM melanoma cells stably interfered for RIAM expression (D11 and F8-Luc transfectants), as well as in cells transiently transfected with siRNA for RIAM (Hernández-Varas et al., 2011). Interestingly, whereas mock cells displayed a predominantly peripheral distribution of paxillin-containing FAs either on fibronectin or type I collagen, adhesions accumulated centrally on the ventral cell surface in RIAM-depleted D11 cells (Fig. 1A,B; see also supplementary material Fig. S1A). RIAM knocking down led to higher number and stability of adhesions, and to a larger cell area covered by adhesions compared with mock cells (Fig. 1C, left; supplementary material Fig. S1B). Furthermore, D11 cells displayed FAs located on average more distantly from the cell periphery than in mock cells (Fig. 1C, right). Alterations in FA distribution in D11 cells were directly the result of RIAM depletion, because expression of His-tagged RIAM in these cells led to rescue of the typical pattern of peripheral FA distribution shown by mock cells (Fig. 1D).

Along with the altered distribution and number of paxillin-stained FAs, RIAM depletion also led to enrichment of β1 integrin, talin, vinculin and p130Cas in centrally localised adhesions (Fig. 1E,F). In addition, whereas F-actin fibres in mock cells were predominantly elongated, fibres in D11 cells were reflected the increased number and size of FAs across the cell surface, showing radial distribution and enhanced thickness (supplementary material Fig. S1C).

Alterations in adhesion complex distribution were not only seen in BLM-knockdown cells, but RIAM depletion in MV3 melanoma cells (Hernández-Varas et al., 2011) also led to formation of adhesion complexes that covered the central area (supplementary material Fig. S1D). FA distribution was also affected in RIAM-depleted breast carcinoma MDA-MB-468 cells, as detected by the accumulation of paxillin- and vinculin-containing FAs at the central part of the ventral cell surface, and F-actin fibres were also thicker than in cells transfected with control siRNA (supplementary material Fig. S1E,F). Together, these data indicate that RIAM knockdown affects the number, distribution and stability of FAs.

We previously showed that RIAM depletion only moderately affects β1 integrin activation in BLM cells, and does not significantly alter binding of soluble fibronectin, as assessed by flow cytometry (Hernández-Varas et al., 2011). Furthermore, incubation of mock and D11 cells with Mn2+, a potent integrin activator, led to similar increased levels of β1 integrin activation and binding of soluble fibronectin (Hernández-Varas et al., 2011). Cell treatment with Mn2+ resulted in significant increase in paxillin-containing peripheral FA number in mock cells, whereas Mn2+ did not correct the altered FA distribution of RIAM-depleted cells and did not further enhance their FA number (Fig. 1G). These results indicate that the altered pattern of FA localisation in RIAM-knockdown cells is independent of the degree of β1 integrin activation.

**RIAM-depleted cells have defective FA disassembly**

Given that the increase in size and number of FAs observed in RIAM-knockdown cells was connected to enhanced adhesion stability, we hypothesised that a defect in adhesion disassembly causes this phenotype. As microtubules contacting adhesions promote FA disassembly (Kaverina et al., 1999; Small et al., 2002), we disrupted MTs with nocodazole to freeze FA disassembly, and followed adhesion turnover after removal of this inhibitor, using established protocols for analysing FA disassembly (Ezratty et al., 2005). As expected, nocodazole blocked MT polymerisation in both mock and D11 cells, and MTs started to gradually regrow following removal of the inhibitor (supplementary material Fig. S2). Blockade of MT polymerisation by nocodazole caused an inhibition of FA disassembly that led to accumulation of adhesion complexes in mock and D11 cells, as detected by paxillin immunostaining (Fig. 2A). Upon MT regrowth, FAs disassembled in mock cells and were clearly visible again on the cell periphery by 200 minutes following nocodazole wash-out. By contrast, most FAs in D11 cells were resistant to disassembly upon nocodazole
release and remained stable throughout the wash-out period (Fig. 2A). Remarkably, fluorescence recovery after photobleaching (FRAP) experiments targeting peripheral FAs revealed that whereas bleached areas in mock cells were rapidly recovered by neighbouring paxillin-DsRed2 molecules, showing recovery half-times of 28 seconds, rescue was strongly inhibited in D11 cells (Fig. 2B; see also supplementary material Movies 1, 2). Importantly, re-expression of RIAM-His in D11 cells led to recovery half-times resembling those of mock cells, indicating the rescue of adhesion dynamics. Together, these results show that FA disassembly is defective in RIAM-depleted melanoma cells.

**Associations between FA proteins are affected by RIAM knockdown**

RIAM-depleted cells display impaired talin-β1 integrin binding (Hernández-Varas et al., 2011), prompting us to analyse whether associations between other FA proteins might also be altered. The FAT domain of FAK binds talin and paxillin and promotes FAK localisation in FAs (Mitra et al., 2005). Co-immunoprecipitation experiments showed that FAK associated constitutively with talin and paxillin in mock cells (Fig. 3A,C). Interestingly, both anti-FAK and anti-talin immunoprecipitating antibodies co-precipitated substantially less amounts of talin and FAK, respectively, in D11 than in mock cells (Fig. 3A, top and middle panels). Furthermore, even when FAK and paxillin were recruited to FAs and displayed co-localisation (Fig. 3B), a decrease in association between these proteins was observed in RIAM-knockdown cells (Fig. 3A, top and bottom panels; Fig. 3C). The Erk1/2 MAP kinase binds to and phosphorylates paxillin (Ishibe et al., 2003; Ku and Meier, 2000; Liu et al., 2002), a process contributing to FA turnover (Ishibe et al., 2003). Co-immunoprecipitation analyses revealed constitutive Erk1/2-paxillin association in mock cells, and deficient paxillin-Erk1/2 and Erk1/2-FAK associations were observed in RIAM-depleted cells (Fig. 3D). On the contrary, complex formation between talin and phosphatidyl-inositol phosphate kinase I-gamma (Barsukov et al., 2003; Di Paolo et al., 2002; Ling et al., 2002) was not affected by RIAM knockdown (Fig. 3E), suggesting that altered assembly between FA proteins is selective. The alterations in protein association were independent of the cell lysis conditions, because when we used NP-40-containing lysis instead of buffers containing digitonin, a clear decrease in FAK co-immunoprecipitation with talin, paxillin and Erk1/2 was again observed (Fig. 3F). Furthermore, deficient FAK–talin and FAK–
paxillin associations were also detected in RIAM-knockdown F8-Luc cells compared with control-Luc counterparts (Fig. 3G). Control experiments indicated that defective protein associations in RIAM-depleted cells were not the result of cellular material selectively left behind following SDS-directed lysis in plates containing RIAM-knockdown cells, as assessed by immunoblotting and confocal microscopy (not shown). Therefore, the data indicate that, although FAs are assembled upon RIAM depletion, associations between their component proteins are less stable.

RIAM depletion leads to increased paxillin tyrosine phosphorylation at FAs

Tight regulation of tyrosine phosphorylation of FA proteins is needed for adhesion turnover and for cell migration (Parsons et al., 2010). Anti-pTyr antibodies labelled peripheral FAs on both mock and D11 cells, but staining of centrally localised adhesions was particularly evident in RIAM-knockdown cells (supplementary material Fig. S3A), similar to the pattern observed in paxillin-stained adhesions. Paxillin and FAK are tyrosine phosphorylated following cell adhesion (Burridge et al., 1992; Deakin and Turner, 2008), and in the case of paxillin, Tyr118 and Tyr31 are well-defined phosphorylated residues. Notably, phosphorylation at these Tyr paxillin residues was higher in D11 cells than in mock transfectants upon adhesion either onto fibronectin or collagen I (Fig. 4A). Confocal analyses showed that anti-phosphorylated paxillin Tyr118 antibodies stained peripheral FAs in mock cells, whereas staining was primarily located throughout the central cell surface of RIAM-depleted cells, co-localising with paxillin staining (Fig. 4B, left). Furthermore, consistent with the biochemical data, a higher
RIAM controls focal adhesion turnover

Fig. 4. See next page for legend.
relative rate of labelling intensity per adhesion between phosphorylated paxillin Tyr118 and total paxillin in D11 transfectants than in mock cells was obtained from the confocal image analyses (Fig. 4B, right). Increased paxillin Tyr118 phosphorylation was also found in BLM cells transiently interfered for RIAM expression (supplementary material Fig. S3B, left), and they also displayed accumulation of phosphorylated paxillin Tyr118 centrally localised at their ventral cell surface (supplementary material Fig. S3B, right). Enhanced phosphorylation was specifically caused by RIAM depletion, as RIAM-His expression reversed phosphorylation at paxillin Tyr118 in D11 cells to mock cell phosphorylation levels, leading to rescue of predominant peripheral localisation of paxillin-containing adhesions (Fig. 4C). Interestingly, immunoblotting analyses with nocodazole-treated cells indicated that blockade of FA disassembly in RIAM-depleted transfectants was linked to maintenance of higher phosphorylated paxillin Tyr118 and Tyr31 levels throughout the nocodazole wash-out step. By contrast, mock cells displayed increased phosphorylation at these paxillin residues in the presence of this agent, but phosphorylation decreased to untreated cell levels following its removal (Fig. 4D). These data suggest that hyperphosphorylated paxillin in RIAM-knockdown cells cannot be released from the abnormal adhesion complexes during MT repolymerisation.

In addition to paxillin, RIAM-depleted cells had higher total phosphorylation at FAK Tyr576 than mock counterparts (Fig. 4E), whereas phosphorylation at FAK Tyr397 and Tyr925 was not significantly altered (supplementary material Fig. S3C). Immunofluorescence analyses of phosphorylated FAK Tyr576 and Tyr397 revealed a central distribution pattern in D11 cells, whereas mock transfectants displayed labelling primarily on the cell periphery (Fig. 4F, top; supplementary material Fig. S3D). Increased phosphorylation at FAK Tyr576 was reversed to levels similar to those of mock cells following RIAM-His expression, and it correlated with rescue of peripheral localisation of paxillin-containing adhesions (supplementary material Fig. S3E). However, although D11 cells present an overall cellular increase in phosphorylated FAK Tyr576, quantitative analyses from confocal images indicated that their FAs do not contain a higher fraction of phosphorylated FAK Tyr576 per adhesion than FAs in mock cells, as shown by the phosphorylated FAK Tyr576 to total FAK ratio (Fig. 4F, bottom). Thus, the increased overall FAK phosphorylation merely reflects the increase in adhesion number. Therefore, these results suggest that RIAM depletion leads to selective increase in the content of phospho-paxillin Tyr118 within FAs, whereas phosphorylated FAK Tyr576 does not increase. Higher phosphorylation at paxillin Tyr118 and FAK Tyr576 was not only seen in RIAM-depleted BLM cells, but also in MV3 cells knocked down for RIAM (supplementary material Fig. S3F). Moreover, RIAM knockdown in MDA-MB-468 cells also caused upregulation of phosphorylation at paxillin Tyr118 associated with accumulation of centrally localised FAs (supplementary material Fig. S3G).

As deficient adhesion turnover in RIAM-depleted cells correlates with upregulated phosphorylation of paxillin at Tyr118 and Tyr31, we investigated if expression of phosphomimetic paxillin mutated at these residues could recapitulate the altered FA phenotype of these cells. We expressed the phosphomimetic paxillin mutant YFP-Y118E/Y31E (Y2E), as well as the YFP-Y118F/Y31F (Y2F) nonphosphorylatable paxillin mutant (Zaidel-Bar et al., 2007b) in mock and D11 cells (Fig. 4G, left), and analysed the distribution of paxillin-containing FAs. Both Y2E and Y2F paxillin forms were recruited to peripheral FAs in mock cells, and the distribution of the phosphomimetic Y2E paxillin did not recapitulate the altered FA pattern observed in D11 cells (Fig. 4G, middle and right; supplementary material Fig. S4). In addition, the nonphosphorylatable Y2F paxillin did not rescue the altered FA phenotype of RIAM-depleted cells. These results indicate that increased paxillin tyrosine phosphorylation in RIAM-knockdown cells by itself does not represent a mechanism responsible for altered adhesion turnover, but rather is likely a subsequent consequence of the deficient adhesion turnover.

**Defective MEK1/2 kinase activation in RIAM-depleted cells accounts for their impaired FA disassembly**

Melanoma cells knocked down for RIAM have deficient B1 integrin-mediated Erk1/2 activation (Hernández-Varas et al., 2011). This inhibition appears not to be exclusive of melanoma cells, as RIAM depletion in MDA-MB-468 cells also leads to decreased Erk1/2 activation (supplementary material Fig. S3G). Nearly all melanoma cell lines have alterations in Ras-Raf-Erk1/2 signalling resulting from activating mutations (Flaherty et al., 2012; Gray-Schopfer et al., 2007; Ibrahim and Haluska, 2009). As BLM and MV3 melanoma cells carry mutations at N-Ras, and MDA-MB-468 cells have elevated Ras activation levels (Eckert et al., 2004), we tested the melanoma cell line A375, which displays the B-Raf V600E but not N-Ras mutations, for Erk1/2 activation and FA turnover following RIAM knocking down. RIAM-depleted A375 cells plated on FN showed defective Erk1/
Fig. 5. See next page for legend.
2 activation, which was associated with accumulation of FAs at the ventral cell surface, suggestive of impaired FA turnover (supplementary material Fig. S5). These data strongly suggest that the defect in both Erk1/2 activation and FA turnover in RIAM-knockdown melanoma cells is independent of whether activating mutations are carried either by N-Ras or B-Raf.

Notably, paxillin disassembly from FAs in FAK-deficient fibroblasts depends on Erk activity (Webb et al., 2004). Inhibition of MEK1 with UO126 caused a blockade of Erk1/2 activation and an accumulation of paxillin-containing FAs in the central area of mock cells, similarly to RIAM-depleted D11 cells (supplementary material Fig. S6). These results raised the possibility that MEK activation might be affected following RIAM depletion. Immunoblotting analyses aimed to detect active MEK revealed that D11 cells have deficient MEK activation compared to mock counterparts (Fig. 5A). Importantly, overexpression in RIAM-depleted cells of constitutively active forms of MEK1 (MEK CA) led to recovery of the typical peripheral FAs of mock cells (Fig. 5B,C), suggestive of rescue of FA turnover. Furthermore, quantitative analyses from confocal microscopy images showed that D11-MEK CA transfectants had a number of paxillin adhesions similar to those from mock cells, and they also displayed FAs primarily at peripheral locations (Fig. 5D). As with cell treatment with UO126, expression of a dominant negative form of MEK1 (MEK DN) in mock cells gave rise to increased number of paxillin adhesions that were enriched in central areas of the ventral cell surface (Fig. 5C,D).

Fig. 5. Defective MEK-Erk1/2 kinase activation in RIAM-depleted cells accounts for their impaired FA disassembly. (A) Cells were plated onto fibronectin or collagen I and tested by immunoblotting with antibodies against phospho-MEK or total MEK. Protein loading was controlled with anti-vinculin antibodies. (B) Mock or D11 cells were transfected with MEK CA, MEK DN or control vectors, and transfectants were subjected to immunoblotting using antibodies for the indicated proteins. (C) Transfectants were analysed by confocal microscopy with anti-paxillin antibodies (top), and the images were quantitatively analysed for FA phenotype (bottom). (D) Quantitative analyses displaying different parameters of FAs from the confocal images shown in C (n = 4 experiments, at least 20 cells were analysed per experiment and per condition; shown is a representative result). (E) Cells were incubated with or without UO126 (left), or transfected with control or MEK CA vectors (right), and subsequently tested by immunoblotting using antibodies for the indicated proteins. (F) Mock and D11 cells were transfected with YFP-Y2E or YFP-Y2F vectors, and transfectants were analysed by immunoblotting with antibodies to phospho-Erk1/2 or total Erk1/2. (G) Mock and D11 cells were transfected with control, MEK CA or MEK DN vectors, and the transfectants were incubated with nocodazole (10 μM) and subsequently analysed by confocal microscopy with anti-paxillin antibodies following wash-out of the inhibitor. (H) Quantitative determination of the number of adhesions per cell from confocal images of the same transfectants as in G that had been either left untreated, incubated with nocodazole, or exposed to this inhibitor followed by its removal.

Interestingly, mock cell incubation with UO126 led to upregulated phosphorylation at paxillin Tyr118 to levels close to those of D11 cells (Fig. 5E, left), and importantly, expression of MEK CA in these cells resulted in rescue of Erk1/2 activation and in reversal of paxillin Tyr118 phosphorylation, achieving levels similar to mock cells transfected with empty vector (Fig. 5E, right). Furthermore, D11-MEK1 CA transfectants displayed a decrease in FAK phosphorylation at Tyr576. As UO126-treated mock cells show blockade of adhesion turnover and increased Tyr118 paxillin phosphorylation, together with rescue of both adhesion turnover and paxillin phosphorylation in D11-MEK1 CA transfectants, these data strengthen the above results pointing that paxillin hyperphosphorylation in RIAM-knockdown cells is a follow-up consequence of blockade of adhesion disassembly because of inhibition of MEK-Erk1/2 activation. This model is further supported by the fact that expression of phosphomimetic Y2E paxillin in mock cells does not result in decreased Erk1/2 activation, and that D11 cells expressing the nonphosphorylatable Y2F paxillin do not display rescue of Erk1/2 activation (Fig. 5F).

To assess the role of RIAM-mediated MEK-Erk1/2 activation in FA disassembly, mock and D11 cells transfected with MEK CA or MEK DN forms were incubated with nocodazole, and transfectant FA disassembly was monitored by confocal microscopy following removal of this inhibitor. Notably, MEK CA overexpression in RIAM-depleted cells led to recovery of peripheral FA distribution after nocodazole wash-out, similarly to RIAM-positive cells (Fig. 5G). By contrast, accumulation of centrally located adhesions and, therefore, the phenotype shown by RIAM-depleted cells, was displayed by RIAM-positive mock cells upon MEK1 DN overexpression. Quantitative analyses from confocal images of mock-control transfectants revealed that nocodazole treatment caused a near duplication in the number of adhesions per cell, as well as in the percentage of cell area covered by FAs, which returned to basal condition numbers after wash-out of the inhibitor (Fig. 5H; supplementary material Fig. S7). The D11-control transfectants displayed increased adhesion number and enhanced cell area covered by FAs than mock-control counterparts even upon removal of nocodazole, but MEK CA overexpression in D11 cells lowered these values to levels close to those of control cells (Fig. 5H; supplementary material Fig. S7). In addition, MEK DN expression caused an enhancement in the number of adhesions and cell area covered by adhesions in mock and D11 cells under untreated, nocodazole or washout conditions. Therefore, these data indicate that MEK CA overrides the blockade of FA disassembly displayed by RIAM-depleted cells. Interestingly, whereas Erk1/2 activation after nocodazole wash-out was restored in RIAM-positive cells, Erk1/2 reactivation was deficient in RIAM-knockdown counterparts, coincident with their defective FA disassembly (Fig. 5I).

RIAM knockdown leads to impaired cell invasion (Hernández-Varas et al., 2011). Given that adhesion turnover is required for cell invasion, we tested whether restoring MEK1 signalling rescues invasiveness. Notably, expression of MEK1 CA in D11 cells significantly recovered invasion across matrigel, whereas DN MEK1 transfectants showed blockade of invasion (Fig. 5J). Control experiments indicated that expression of MEK DN and CA forms did not alter transfectant survival along the times used in the invasion assays (not shown). Altogether, these results indicate that deficient MEK-Erk1/2 activation because of RIAM
depletion inhibits the disassembly of FAs, and suggest that promotion of FA turnover in D11-MEK CA transfectants is an important mechanism involved in their recovered invasiveness.

**RhoA partially rescues Erk1/2 activation and peripheral FA distribution in RIAM-knockdown cells**

The GTPase RhoA controls actin stress fibre formation and regulates FA turnover (Parsons et al., 2010). RhoA activation stimulated by the chemokine CXCL12 is defective in RIAM-depleted melanoma cells, and expression of constitutively active RhoA (Rho CA) rescued RhoA activation and cell invasion (Hernández-Varas et al., 2011). As RhoA is needed for downstream Erk1/2 activation in cells plated on FN (Clark et al., 1998; Renshaw et al., 1996), we tested if expression of RhoA CA could reverse the deficient Erk1/2 activation as well as the altered FA distribution shown by RIAM-knockdown cells. RhoA activation was continuously deficient in D11 cells plated on FN compared to its activation in mock cells (Fig. 6A). Overexpression of both RhoA CA and RhoA wt resulted in partial rescue of Erk1/2 activation in RIAM-knockdown cells (Fig. 6B). Notably, these transfectants displayed a reversal of their FA phenotype towards a predominant peripheral FA distribution similar to mock cells, although recovery was not complete (Fig. 6C). Therefore, these data indicate that RIAM-mediated, integrin-dependent RhoA activation is connected to the subsequent stimulation of Erk1/2-triggered FA disassembly in melanoma cells.

**Src kinase and PTP-PEST actions during upregulation of tyrosine phosphorylation in RIAM-knockdown cells**

Deregulated function of Src kinases or tyrosine phosphatases might contribute to the increased paxillin and FAK phosphorylation because of RIAM depletion. To analyse whether Src kinase activity could mediate the enhancement of paxillin phosphorylation in RIAM-depleted cells, we pre-incubated them with the PP2 Src kinase inhibitor or with the inactive PP3 analogue, and analysed the levels of phosphorylated paxillin Tyr118. Immunoblotting analyses revealed that PP2 reversed paxillin phosphorylation to levels shown by PP2-treated mock cells (Fig. 7A). The protein tyrosine phosphatase PTP-PEST (also called PTPN12) is recruited to FAs following direct binding to the LIM3 and LIM4 domains of paxillin (Côté et al., 1999), and it controls paxillin tyrosine phosphorylation (Shen et al., 2000). Furthermore, PTP-PEST−/− cells display increased number of FAs and enhanced paxillin and FAK tyrosine phosphorylation (Angers-Loustau et al., 1999), thus resembling the de-regulated phosphorylation seen in RIAM-depleted melanoma cells. Interestingly, D11 cells overexpressing PTP-PEST displayed reduced phosphorylation at paxillin Tyr118 and FAK Tyr576 to levels similar to mock counterparts (Fig. 7B). In addition, Erk1/2 activation was restored in D11/PTP-PEST transfectants (Fig. 7C), which correlated with recovery of peripheral FA distribution (Fig. 7D). A closer look at the confocal images using anti-phosphorylated paxillin Tyr118 antibodies showed that adhesions in both mock and D11 cells overexpressing this phosphatase had lower intensity than untransfected cells, likely reflecting decreased labelling because of PTP-PEST activity (supplementary material Fig. S8).

Rescue of peripheral FA distribution in D11/PTP-PEST transfectants was associated with a partial but significant recovery of transfectant invasion (Fig. 7E), as well as to rescue of cell migration in wound healing assays (supplementary material Fig. S9). Overexpression of PTP-PEST in mock cells caused a blockade of cell invasion, which might reflect an exaggerated de-phosphorylation of FA proteins to levels incompatible with efficient invasion. A similar defective cell migratory response was also previously observed in cells overexpressing the PTP1B tyrosine phosphatase (Liu et al., 1998). Together, the data suggest that continuous Src kinase activity and/or deficient PTP-PEST tyrosine phosphatase actions...
on paxillin and FAK in RIAM-depleted cells represent consequences of the defective adhesion disassembly, accumulating these FA proteins in a hyperphosphorylated state.

**Discussion**

Cell migration requires regulated turnover of integrin-dependent adhesion complexes, a process involving adaptor and signalling molecules, actin polymerisation and actomyosin contraction (Parsons et al., 2010). Melanoma cells depleted for RIAM expression have defective cell polarisation and migration directionality, and deficient cell invasion (Hernández-Varas et al., 2011). Here we show that RIAM-knockdown melanoma and breast carcinoma cells have increased FA number, area and stability, suggestive of impaired adhesion turnover. Indeed, experiments with nocodazole and FRAP analyses indicated that RIAM depletion specifically blocked FA disassembly, leading to accumulation of adhesions covering the entire cell surface.

Impaired FA disassembly in RIAM-knockdown melanoma cells correlated with inhibition of MEK activation and importantly, expression of constitutively active MEK recovered both their FA disassembly and cell invasion. Furthermore, RIAM-positive mock cells expressing dominant negative MEK accumulated FAs in the central cell surface caused by impaired adhesion disassembly, and showed inhibition of cell invasion, altogether closely resembling the RIAM-knockdown phenotype. Therefore, our present and earlier results (Hernández-Varas et al., 2011) indicate that RIAM is required for integrin-dependent MEK-Erk1/2 activation, which represents a key feedback event for efficient disassembly of integrin-mediated FA clusters (Fig. 8), which in turn is crucial for cell migration and invasion. RIAM-mediated MEK activation is not only dependent on integrin engagement, as reported here, but it also takes place in TCR-activated T cells (Patsoukis et al., 2009).

Moreover, our results are consistent with the important role of Erk1/2 in paxillin disassembly from FAs that was observed using FAK-null fibroblasts (Webb et al., 2004). Chemokine-promoted RhoA activation is impaired in RIAM-knockdown melanoma cells, and expression of RhoA active forms rescues RhoA activation and cell invasion (Hernández-Varas et al., 2011). The integrin-dependent Rho GTPase activation shows functional connections with activation of the MEK-Erk1/2 pathway. Thus, MEK-Erk1/2 activation regulates Rho GTPase activation (Chen et al., 2003; Ehrenreiter et al., 2005; Klein et al., 2008; Pritchard et al., 2004; Pullikuth et al., 2005), but these GTPases are also capable of controlling downstream Erk1/2 activation following cell adhesion to fibronectin (Clark et al., 1998; Renshaw et al., 1996; Swant et al., 2005; Tang et al., 1999). Our present data indicate that RIAM-depleted melanoma cells have deficient RhoA activation upon adhesion to FN, and that overexpression of RhoA CA or RhoA wt partially rescued Erk1/2 activation. Notably, this rescue was associated with reversal of the centrally-located FAs in RIAM-depleted cells towards a predominant FA distribution at the cell periphery. These results suggest that integrin-dependent, RIAM-mediated RhoA activation is an important step for optimal subsequent Erk1/2 activation, which is a key trigger of FA disassembly. The spatiotemporal and molecular mechanisms involved in Rho-mediated Erk1/2 activation following RIAM engagement have not been yet studied. Earlier works on the Rho/MEK-Erk1/2 cross-cascade relationships reported that active Rho GTPases, including active RhoA, synergised with PAK-1/Raf-1 to induce MEK-dependent Erk1/2 activation (Coles and Shaw, 2002; Frost et al., 1997). Furthermore, active Rac and Rho can activate membrane-localised Raf, which later leads to Erk1/2 activation (Li et al., 2001). Therefore, it is possible that RIAM-promoted RhoA activation following integrin β1-dependent adhesion could contribute to localise MEK-Erk1/2 activation for posterior triggering of FA disassembly.

Erk1/2 is indeed recruited to FAs following integrin engagement and Src kinase activity (Fincham et al., 2000), and it was shown that MEK-paxillin association mediates localised Erk1/2 activation in adhesion complexes (Ishibe et al., 2003). Subsequently, Erk1/2-dependent paxillin and FAK phosphorylation regulates associations between these three FA proteins, a process controlling adhesion turnover (Ishibe et al., 2004; Ishibe et al., 2003; Liu et al., 2002;
Mitra et al., 2005; Parsons et al., 2010). Thus, Erk1/2 binding to paxillin leads to phosphorylation at paxillin Ser83 and induction of paxillin-FAK association. Interestingly, even if FAs were formed and more stable in RIAM-knockdown melanoma cells, co-immunoprecipitation assays revealed that associations between talin and FAK, and among FAK, paxillin and Erk1/2, were less stable in these cells than in RIAM-positive counterparts. These results are indicative of altered complex formation between these proteins upon RIAM depletion, suggesting that RIAM has adaptor roles during the assembly of adhesion complexes. Impaired talin-FAK association might arise from the deficient interaction between talin and the β1 integrin subunit because of RIAM knockdown (Herna´ndez-Varas et al., 2011), which might dictate subsequent incorrect interactions between the other FA proteins. In this scenario, Erk1/2 recruitment to adhesion complexes in RIAM-depleted cells might be deficient, which could result in the observed inhibition of FAK–paxillin association, leading to the assembly of FAs that are not competent to trigger integrin-mediated outside-in signalling. RIAM-dependent, RhoA-mediated activation of the MEK-Erk1/2 pathway is required for subsequent disassembly of FAs, which is crucial for cell migration. In addition, tight control of paxillin and FAK tyrosine phosphorylation by Src and PTP-PEST results in phosphorylation levels that are compatible with FA disassembly and cell migration. (B) In RIAM-knockdown cells, associations between FA proteins are defective, leading to anomalous FAs that cannot signal properly for activation of the MEK-Erk1/2 and RhoA pathways, causing deficient FA disassembly and impairment in cell migration. As a consequence of blockade of FA disassembly, paxillin becomes hyperphosphorylated by deregulated Src and PTP-PEST actions.

Fig. 8. Model for RIAM-regulated FA disassembly. (A) RIAM has an important adaptor role in the correct formation of adhesion complexes that are capable of triggering integrin-mediated outside-in signalling. RIAM-dependent, RhoA-mediated activation of the MEK-Erk1/2 pathway is required for subsequent disassembly of FAs, which is crucial for cell migration. In addition, tight control of paxillin and FAK tyrosine phosphorylation by Src and PTP-PEST results in phosphorylation levels that are compatible with FA disassembly and cell migration. (B) In RIAM-knockdown cells, associations between FA proteins are defective, leading to anomalous FAs that cannot signal properly for activation of the MEK-Erk1/2 and RhoA pathways, causing deficient FA disassembly and impairment in cell migration. As a consequence of blockade of FA disassembly, paxillin becomes hyperphosphorylated by deregulated Src and PTP-PEST actions.

RIAM controls focal adhesion turnover 5349
inhibition of FA turnover and increased paxillin tyrosine phosphorylation, indicate that paxillin hyperphosphorylation is not the cause, but rather a consequence of the blockade of FA disassembly.

Enhanced paxillin and FAK tyrosine phosphorylation in RIAM-depleted cells could be based on excessive kinase or deficient tyrosine phosphatase actions, or to both mechanisms. Our results suggest that both continuous Src kinase and deficient PTP-PEST actions cause paxillin and FAK hyperphosphorylation. Notably, we found that PTP-PEST overexpression in RIAM-knockdown cells led to recovery of Erk1/2 activation and formation of typical peripheral FAs, indicative of recovery of adhesion turnover, to rescue of paxillin and FAK normal tyrosine phosphorylation levels, and to restoration of cell migration and invasion. As mentioned above, MEK associates with paxillin, forming a platform for Erk1/2 recruitment to FAs, which then binds to paxillin and is later activated by MEK (Ishibe et al., 2004; Ishibe et al., 2003). Therefore, correction by PTP-PEST of paxillin tyrosine phosphorylation to levels that might be compatible with MEK-dependent paxillin-Erk1/2 association, could lead to the observed PTP-PEST-dependent rescue of Erk1/2 activation, which then could promote subsequent recovery of FA disassembly. Remarkably, PTP-PEST-null cells display increased paxillin and FAK tyrosine phosphorylation, accumulation of FAs on the central cell surface, as well as impaired cell migration (Angers-Loustau et al., 1999), closely resembling the RIAM-depleted melanoma cell phenotype. Thus, tight regulation of tyrosine phosphorylation of FA proteins is key for a FA turnover and, therefore, too much or too little phosphorylation might block adhesion disassembly, which is incompatible with cell migration.

Adhesions disassemble at the lamellipodium–lamellae interface and at the rear of migrating cells, a process that depends on actin dynamics (Parsons et al., 2010). RIAM binds VASP and profilin, two proteins that regulate actin polymerisation; thus, it was proposed that RIAM might indirectly control actin dynamics (Lafuente et al., 2004). In addition, regulated VASP interaction with RIAM controls RIAM–talin associations (Worth et al., 2010). We found that actin stress fibres in RIAM-depleted melanoma and breast carcinoma cells are thicker than in control counterparts, likely reflecting their increased FA size. Thus, RIAM interactions with talin and VASP might serve as functional bridges connecting two key events that are needed for FA disassembly: MEK-Erk1/2 activation following correct assembly of signalling-competent adhesion complexes, and VASP-regulated actin polymerisation.

In conclusion, our results define RIAM as a key adaptor molecule that supports the correct assembly of adhesion complexes needed for subsequent activation of MEK, a key pathway that is required for adhesion turnover and tumour cell invasion.

**Materials and Methods**

**Cells, antibodies and reagents**

B6, M3, and A375 human melanoma cell lines were cultured as described (Bartolomé et al., 2004). MDA-MB-468 breast carcinoma cells were grown in Leibovitz medium supplemented with fetal calf serum. Mock and stably RIAM-knockdown D11 and F8-Luc transfectants in BLM cells have been reported (Hernández-Varas et al., 2011). Antibodies for His-tag, β-actin, vinculin and talin were purchased from Sigma Aldrich (St. Louis, MO). Antibodies against Erk1/2 (Hernández-Varas et al., 2011). Antibodies for His-tag, knockdown D11 and F8-Luc transfectants in BLM cells have been reported. Leibovitz medium supplemented with fetal calf serum. Mock and stably RIAM-BLM, MV3 and A375 human melanoma cell lines were cultured as described.

**Vectors, RNA interference and transfection**

To overexpress RIAM, we used the pcDNA4MaxC-RIAM-His (RIAM-His) construct (a gift from Dr Vassiliki A. Boussiotis, Harvard Medical School, Boston, MA). The paxillin-DesRed vector was a gift from Dr Alan Rick Horwitz (University of Virginia School of Medicine, Charlottesville, VA). The MEK CA and MEK DN vectors have been previously described (Bao and Stromblad, 2004). The paxillin Y2E-YFP, Y2F-YFP and control YFP vectors were from Dr Benny Geiger (Weizmann Institute of Science, Rehovot, Israel). Vectors coding for GFP-fused forms of wild-type RhoA and activated V14-RhoA have been reported (Bartolomé et al., 2006), and a vector coding for wild-type PTP-PEST was a gift from Dr Rafael Pulido (Centro de Investigación Príncipe Felipe, Valencia, Spain). Phagosomes were transfected with the cells using Lipofectamine 2000 (Invitrogen, CA, France), following the manufacturer’s instructions. Control and RIAM siRNA sequences have been previously reported (Molina-Otiriz et al., 2009). Cells were transfected with siRNAs using X-tremeGENE (Roche Diagnostics, Mannheim, Germany) and transfectants tested in the different assays after 48 hours post-transfection.

**Confocal microscopy and FRAP analyses**

Cells on fibronectin (10 μg/ml)- or collagen (1 μg/ml)-coated slides were fixed and permeabilised with paraformaldehyde 1% Triton-X100/0.1% in PBS. After washing and blocking, cells were incubated with antibodies and samples imaged with a LEICA TCS-SP2-AOBS-UV or a Nikon A1R microscope with 63× oil immersion objective. Adhesion size, intensity and location values were extracted from confocal images using custom patch morphology analysis software (Digital Cell Imaging Laboratories, Belgium) developed for systems microscopy type of analyses (Lock and Strömblad, 2010). Adhesions were detected and segmented automatically using intensity-threshold and watershed-based algorithms. Identical confocal imaging conditions were used for image acquisition and identical algorithms were used for adhesion detection to ensure objective comparison of adhesion populations. For nucodazole studies, cells were incubated at 37°C with this compound (10 μM) (Sigma) and upon the wash-out period, cells were fixed and samples imaged as above. For FRAP analyses, cells were transfected with paxillin-DrRed2 and subsequently cultured on fibronectin-coated glass-bottom chambers (MatTek Corp., Ashland, MA). Samples were subjected to bleaching of the peripheral adhesions (561 nm at 100% laser intensity) for 2 seconds, and fluorescence recovery was monitored for the following 300 seconds using a LEICA TCS-SP5-AOBS-UV microscope coupled to a culture chamber with a 63× oil immersion objective.

**Immunoprecipitation, immunoblotting, GTPase and invasion assays**

For immunoprecipitation, cultured melanoma cells or cells attached on collagen (2 μg/ml) or fibronectin (10 μg/ml) were lysed in situ using a 1% digitonin-containing buffer as reported (Tecchioni et al., 2002), or alternatively in a buffer with 0.5% NP-40, 50 mM Tris-HCl pH 7.4, 150 mM NaCl and 1 mM EDTA. Cell extracts were incubated with antibodies followed by specific coupling to protein A- or G-Sepharose beads. After SDS-PAGE, proteins were subjected to immunoblotting with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies and detection with chemiluminescent substrate (Pierce, Rockford, IL). For Rho GTPase assays we essentially followed the described method (Bartolomé et al., 2004). Briefly, cells were attached onto FN in the presence of 1% serum, and upon cell lysis, aliquots from cell extracts were kept for total lyase controls and the remaining volume incubated with the GST-C21 fusion protein (Sandor et al., 1998), in the presence of glutathione agarose beads. After elution of bound proteins, they were subjected to immunoblotting with anti-RhoA antibodies. Densitometry of the resulting bands was performed using ImageJ software. Invasions across Matrigel (BD, Palo Alto, CA) were done as established (Bartolomé et al., 2004). The lower compartments of chambers were filled with invasion medium with or without CXCL12, and invasive cells were counted under a microscope.

**Statistical analyses**

Unless specified, all experiments were performed at least three times. Data were analysed by one-way ANOVA followed by Tukey–Kramer multiple comparisons. In all analyses, the minimum P-value level considered statistically discernable was P≤0.05.
RIAM controls focal adhesion turnover


Fig. S1. RIAM-silencing leads to altered distribution of focal adhesions. (A) Fibronectin-attached mock or D11 cells were analyzed by confocal microscopy using anti-paxillin antibodies (green). Nuclei were visualized by DAPI staining (blue). (B) Cells transfected with paxillin-DsRed2 vector were seeded on fibronectin-coated glass-bottom plates. After allowing the cells to attach, adhesion dynamics were analyzed by time-lapse microscopy using a Live Cell Imaging microscope (Leica AF6000 LX type DMI6000B). Shown are still images taken at different times. (C) Cells were stained with anti-paxillin antibodies and with Alexa568 phalloidin to visualize F-actin. (D-F) MV3 melanoma and MDA-MB-468 breast carcinoma cells were transfected with control or RIAM siRNA and transfectants subjected either to immunoblotting with antibodies to RIAM, or to talin or vinculin to control for protein loading, or analyzed by confocal microscopy with anti-paxillin or anti-vinculin antibodies, or with Alexa568 phalloidin to visualize F-actin. Bars, 25 μm.
**Fig. S2.** Microtubule re-growth following nocodazole wash-out in mock and RIAM knockdown melanoma cells. Cells plated for 24 h on glass coverslips coated with fibronectin were serum-starved and subsequently treated with nocodazole (10 μM, 4 h). The drug was then washed-out for the indicated times and following fixation, cells were immunostained with anti-tubulin antibodies (red) and analyzed by confocal microscopy. Nuclei were stained with DAPI (blue). Representative cell fields are illustrated. Bars, 25 μm.
Fig. S3. RIAM depletion leads to altered paxillin and FAK tyrosine phosphorylation. (A) Mock and D11 cells were analyzed by confocal microscopy using anti-phospho-tyrosine antibodies (pTyr) (red). (B) BLM melanoma cells were transfected with control or RIAM siRNA, and transfectants subjected either to immunoblotting with anti-paxillin or with anti-phospho-paxillin Tyr118 antibodies (left), or analyzed by confocal microscopy (right). (C, D) Cells were tested by immunoblotting (C) or by confocal microscopy (D) with antibodies to phospho-FAK Tyr397, phospho-FAK Tyr925 or total FAK (Santa Cruz Biotech. (Santa Cruz, CA). (E) Mock and D11 cells were transfected with control or RIAM-His vectors and transfectants subjected to western blotting with antibodies to phospho-FAK Tyr576 or total FAK (left), or analyzed by confocal microscopy for phospho-FAK Tyr576 expression (middle). (Right) Quantitative analyses of percentage of cells with altered FA phenotype (**p<0.001). (F, G) MV3 melanoma and MDA-MB-468 breast carcinoma cells were analyzed by immunoblotting or confocal microscopy with antibodies to the indicated proteins. Bars, 25 μm.
**Fig. S4. Control transfection of YFP-containing vectors.** Mock and D11 cells were transfected with YFP (empty vector) and transfectants analyzed by confocal microscopy for YFP and paxillin expression.
Fig. S5. RIAM silencing in B-Raf V660E-carrying A375 cells leads to defective Erk1/2 activation and altered FA distribution. A375 cells were transiently transfected with two different RIAM siRNA (Mission siRNA, Sigma), and subjected to immunoblotting with antibodies to the indicated proteins (left), or analyzed by confocal microscopy using anti-paxillin antibodies (right). Bar, 25 μm.

Fig. S6. Inhibition of MEK1 causes altered distribution of FAs in mock cells. Mock and D11 cells were incubated with or without UO126, and analyzed by immunoblotting using anti-Erk1/2 antibodies (left), or by confocal microscopy with anti-paxillin antibodies (right).
Fig. S7. Constitutively active MEK rescues normal FA phenotype in RIAM-depleted cells. Quantitative determination of the cell area covered by focal adhesions from confocal images of mock and D11 cells transfected with control, MEK CA or MEK DN vectors, that had been either left untreated, incubated with nocodazole, or exposed to this inhibitor following by its removal (wash-out).
Fig. S8. PTP-PEST actions on paxillin tyrosine phosphorylation in melanoma cells. Mock and D11 cells were transfected with PTP-PEST vector and analyzed by confocal microscopy for phospho-paxillin Tyr118 expression. Insets display localization of PTP-PEST-transfectants (red staining).

Fig. S9. Overexpression of PTP-PEST in RIAM-silenced cells recovers cell migration. Control or PTP-PEST transfectants were subjected to cell migration in wound healing assays. For this, wounds were done across confluent cell monolayers, and cultures incubated in medium with serum. Right panel shows quantification of transfectant migratory speed. Migration speed was determined from the distance covered by cells in 24 h. (**p<0.001). Bars, 100 μm.
Movie 1 and 2. Mock (movie 1) and D11 (movie 2) cells transfected with paxillin-DsRed2 vector were seeded on fibronectin-coated glass-bottom plates. After allowing the cells to attach, they were subjected to FRAP analyses as indicated in the Methods, and fluorescence recovery was monitored for the following 300 sec.