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Tiam1 Is Recruited to $\beta 1$ -Integrin Complexes by 14-3-3 ζ Where It Mediates Integrin-Induced Rac1 Activation and Motility

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Abstract

14-3-3 is an adaptor protein that localizes to the leading edge of spreading cells, returning to the cytoplasm as spreading ceases. Previously, we showed that integrin-induced Rac1 activation and spreading were inhibited by sequestration of 14-3-3 ζ and restored by its overexpression. Here, we determined whether 14-3-3 ζ mediates integrin signaling by localizing a guanine nucleotide exchange factor (GEF) to Rac1-activating integrin complexes. We showed that GST-14-3-3 ζ recruited the Rac1-GEF, Tiam1, from cell lysates through Tiam1 residues 1–182 (N^{1–182} Tiam1). The physiological relevance of this interaction was examined in serum-starved Hela cells plated on fibronectin. Both Tiam1 and N^{1–182} Tiam1 were recruited to 14-3-3-containing $\beta 1$ -integrin complexes, as shown by co-localization and co-immunoprecipitation. Integrin-induced Rac1 activation was inhibited when Tiam1 was depleted with siRNA or by overexpression of catalytically inactive N^{1–182} Tiam1, which was incorporated into 14-3-3/ $\beta 1$ -integrin complexes and inhibited spreading in a manner that was overcome by constitutively active Rac1. Integrin-induced Rac1 activation, spreading, and migration were also inhibited by overexpression of 14-3-3 ζ S58D, which was unable to recruit Tiam1 from lysates, co-immunoprecipitate with Tiam1, or mediate its incorporation into $\beta 1$ -integrin complexes. Taken together, these findings suggest a previously unrecognized mechanism of integrin-induced Rac1 activation in which 14-3-3 dimers localize Tiam1 to integrin complexes, where it mediates integrin-dependent Rac1 activation, thus initiating motility-inducing pathways. Moreover, since Tiam1 is recruited to other sites of localized Rac1 activation through its PH-CC-EX domain, the present findings show that a mechanism involving its N-terminal 182 residues is utilized to recruit Tiam1 to motility-inducing integrin complexes.

Cell migration is critical for numerous physiological processes such as development, wound healing, hemostasis, lymphocyte trafficking, and angiogenesis, as well as pathological events such as atherosclerosis, tumor invasion, and metastasis. Migration occurs as membrane is extended, integrins anchor the front of the newly extended membrane to the matrix, and

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signals transmitted across ligated integrin result in signaling molecule assembly, generation of active Rac1, and the Rac1-induced polymerization of actin and assembly of focal complexes (Nobes and Hall, 1999; Gardiner et al., 2002; Itoh et al., 2002). The Rac1-activating integrin complexes have a short half-life, rapidly detaching from the matrix as actin polymerization propels the membrane forward and a new round of membrane extension and integrin attachment is initiated (Nobes and Hall, 1999; Geiger and Bershadsky, 2001; Zaidel-Bar et al., 2003).

One of the earliest events at sites of newly attached integrin along the leading edge is recruitment of a Rac1 guanine nucleotide exchange factor(s) (GEF), which activates membrane-associated Rac1 by facilitating its conversion from a GDP- to GTP-bound state (Schmidt and Hall, 2002). Once activated, Rac1 can interact with more than 20 downstream effectors, thereby initiating a variety of different signaling pathways and changes in cell behavior. The differential regulation of these pathways is accomplished by assembly of specific Rac1 effectors and downstream signaling molecules at the localized site of GEF recruitment. Many GEFs not only activate Rac1 but also participate in specifying which signaling pathways are activated by recruiting Rac1 effectors, making it important that GEFs are recruited in a site-specific manner (Schmidt and Hall, 2002).

Given the diversity of potential signals impinging on integrin adhesions at the leading edge, the composition of these complexes varies widely (Schwartz and Shattil, 2000; Geiger et al., 2001; Zamir and Geiger, 2001; Danen et al., 2002; Schwartz and Ginsberg, 2002; Zaidel-Bar et al., 2005, 2007). Thus, as with other signaling molecules, the GEF recruited to these complexes is determined by factors such as cell-specific GEF expression, identity of the ligated integrin, composition and rigidity of the matrix, and the complement of growth factors and other soluble stimuli available to covalently modify integrin cytoplasmic domains and potential focal complex-associated cytoskeletal, adaptor, and signaling molecules, including the GEFs and their docking proteins. Compared to other sites of stimulus-induced Rac1 activation, little is known about mechanisms that mediate adhesion-induced Rac1 activation in focal complexes. Thus, only a few of the more than 60 known GEFs have been implicated in integrin-mediated signaling (Gu et al., 2001; Marignani and Carpenter, 2001; Itoh et al., 2002; Gakidis et al., 2004; Sanchez-Martin et al., 2004; Katoh et al., 2006; Rosenberger and Kutsche, 2006; Chan et al., 2008; Gao and Blystone, 2009; Feng et al., 2010) and relatively little is known about regulatory mechanisms or factors determining the specificity of their recruitment to different sites of integrin-mediated adhesion.

Many GEFs are recruited to spatially restricted sites of assembled signaling molecules through the regulated recruitment of GEF-binding adaptor proteins to these sites (Schmidt and Hall, 2002). One adaptor protein that has been implicated in integrin-mediated Rac1 activation along the leading edge is 14-3-3. The 14-3-3 proteins comprise a family of at least seven mammalian isoforms that associate into a variety of hetero- and homodimers (Aitken et al., 2002; Gardino et al., 2006; Kjarland et al., 2006). Little is known about the functional significance of the different isoforms. While most are expressed in all tissues, their relative expression levels vary. While there appears to be little isoform specificity in interactions of binding partners that are recruited to the conserved ligand-binding pocket, not

all proteins bind at this site and examples of isoform specificity of proteins that bind at other sites have been described (Aitken et al., 2002; Kjarland et al., 2006; Jagemann et al., 2008).

Numerous studies have shown the adhesion-dependent recruitment of 14-3-3 isoforms from the cytoplasm to the leading edge and their return to the cytoplasm as membrane extension ceases and Rac1-activating focal complexes are replaced by anti-migratory Rho-activating focal adhesions (Garcia-Guzman et al., 1999; Han et al., 2001). A two-hybrid screen showed that 14-3-3 ζ interacts with the focal complex-associated adaptor protein, pCas¹³⁰, and the adhesion-induced co-localization and co-immunoprecipitation of 14-3-3 ζ and pCas¹³⁰ has been described (Garcia-Guzman et al., 1999). Two-hybrid screens, in vitro binding assays, and in vivo immunoprecipitation approaches have also demonstrated interactions of several 14-3-3 isoforms with β 1-, β 2-, β 3-, and β 4-integrins (Han et al., 2001; Fagerholm et al., 2002; Santoro et al., 2003; Nurmi et al., 2006). In the case of β 2- and β 4-integrins, phosphorylation of cytoplasmic domain serine/threonine residues has been identified as a potential regulatory mechanism and evidence that the interactions are required for integrin-induced cytoskeletal reorganizations and motility provided (Santoro et al., 2003; Fagerholm et al., 2005; Takala et al., 2008). In studies designed to identify mechanisms by which 14-3-3 influences integrin signaling, we demonstrated that sequestration of 14-3-3 ζ inhibited β 3 integrin-induced Rac1 activation and cell spreading while overexpression of 14-3-3 ζ restored these integrin-functions (Bialkowska et al., 2003).

14-3-3 dimers interact with numerous known proteins and influence intercellular signaling pathways by mechanisms that include localizing binding partners to sites at which they can participate in specific signaling pathways (Mackintosh, 2004; Aitken, 2006). Thus, as a first step in elucidating mechanisms by which 14-3-3 ζ participates in integrin-induced Rac1 activation, cytoskeletal reorganizations, and spreading, we examined the possibility that it facilitates the localized generation of active Rac1 at sites of newly attached integrin by recruiting a Rac1-activating GEF to these sites. Using in vitro pull-down and immunoprecipitation approaches we found that 14-3-3 ζ recruits the Rac1 GEF, Tiam1, to sites of newly attached β 1- or β 3-integrin in spreading cells and demonstrated that this is mediated by a novel membrane-interacting site at the N-terminus of Tiam1. Moreover, using siRNA, overexpression of a non-Tiam1-binding 14-3-3 ζ variant, and overexpression of a dominant-negative 14-3-3 ζ -interacting Tiam1 fragment, we obtained evidence that Tiam1 mediates β 1-integrin-induced Rac1 activation in serum-starved Hela cells spreading on fibronectin by a mechanism that requires its 14-3-3-mediated recruitment. Thus, we propose a model in which 14-3-3 induces β 1 integrin-mediated Rac1 activation by localizing Tiam1 to newly formed integrin adhesions at the leading edge, where Tiam1 generates active Rac1, thereby initiating downstream pathways leading to actin polymerization, membrane extension, and migration.

Materials and Methods

Reagents and antibodies

Polyclonal antibodies against 14-3-3 ζ (C-16), pan-14-3-3 (H-8), Tiam1 (C-16), and the C-terminal peptide used to raise the Tiam1 antibodies, were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against β 3-integrin were from Cell Signaling

(Beverly, MA) and normal rabbit IgG was from Sigma (St. Louis, MO). Chicken polyclonal antibodies against HA- and Flag-epitopes were from Bethyl Laboratories (Montgomery, TX). Monoclonal antibodies against Flag-epitope and its isotype control were from Stratagene (La Jolla, CA), against Rac1 and Vav1 from BD Transduction Laboratories (Lexington, KY), and against β 1-integrin from Santa Cruz Biotechnology. The pCMVTag2 vector and Quik-Change mutagenesis kit were from Stratagene and siRNAs from Dharmacon (Lafayette, CO). Lipofectamine reagents and horse serum were from Invitrogen (Grand Island, NY), Calcein AM, Alexa 663- and Alexa 594-phalloidin, and Alexa-coupled secondary antibodies from Molecular Probes (Eugene, OR), PAK-PBD-GST beads from Cytoskeleton (Denver, CO), protease inhibitor cocktail from Roche (Nutley, NJ), protein A-sepharose, glutathione-sepharose, full-range Rainbow markers, and enhanced chemiluminescence reagent from Amersham Biosciences (Piscataway, NJ), and Bradford reagent from Bio-Rad (Hercules, CA). Purified human fibronectin and fibrinogen, NP40, Triton X-100, deoxycholate, sodium orthovanadate, sodium fluoride, and sodium pyrophosphate were from Sigma and leupeptin, benzamidine, and okadaic acid from Calbiochem (San Diego, CA).

cDNA constructs

HA-tagged human 14-3-3 ζ was from Dr. Charles Abrams (University of Pennsylvania, Philadelphia), human Tiam1 cDNA from Dr. Gideon Bollag (Takeda, Inc., San Diego), and Q61LRac1 from Dr. Alan Hall (MRC Laboratory for Molecular Cell Biology, London). The S58D, S58A, S184D, and T232E 14-3-3 ζ variants were created with a Quik-Change mutagenesis kit. To generate GST-14-3-3 ζ fusion proteins, 14-3-3 ζ sequences were subcloned into BamHI and EcoRI sites of pGEX4T. Tiam1 fragments containing the PH-CC-EX region or residues 1-415 (N¹⁻⁴¹⁵ Tiam1) were generated by subcloning in-frame with a Flag-epitope in the pCMVTag2 vector. Those encoding residues 1-182 (N¹⁻¹⁸² Tiam1) and 182-415 (N¹⁸²⁻⁴¹⁵ Tiam1) were isolated from the N¹⁻⁴¹⁵ Tiam1 subclone by BamHI and EcoRI-SaII digestion, respectively, and ligated into pCMVTag2 vector. All constructs were fully sequenced prior to use.

Cells and transfections

Human platelets were isolated from freshly drawn blood by differential centrifugation (Fox and Phillips, 1982), suspended at $1-2 \times 10^9$ /ml at 37°C in 138 mM sodium chloride, 2.9 mM potassium chloride, 12 mM sodium bicarbonate, 0.36 mM sodium phosphate, 5.5 mM glucose, 0.25% BSA, 1.8 mM calcium chloride, 0.4 mM magnesium chloride, pH 7.4, and kept at 37°C for ~1 h prior to use. Hela and CHO cells were from American Type Culture Collection and human umbilical vein endothelial cells from Dr. Paul DiCorleto (Cleveland Clinic, Cleveland). Hela cells were maintained in MEM with 1% non-essential amino acids and 10% fetal calf serum and CHO cells in DMEM-F12 with 10% fetal calf serum, 1% glutamine, and 1% penicillin/streptomycin. Transient transfections were performed on 100 mm plates using Lipofectamine Plus. Lipofectamine 2000 and 30 nM siRNA were used for introduction of siRNAs. Cells were transfected in serum-free medium for 5 h then grown with 10% serum until they were 60-70% confluent. Unless otherwise stated, they were serum-starved for ~18 h to allow them to become quiescent before resuspension in serum-free medium for experiments.

GST-fusion protein pull-down assay

Adherent cells were rinsed, sedimented, and lysed with 25 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1% NP40, 0.25% deoxycholate, 1 mM EDTA and protease inhibitor cocktail at 4°C. Platelet suspensions were lysed by addition of a 2× solution of this buffer. Lysates were centrifuged at 15,000g for 10 min at 4°C. The protein concentrations of supernatants were determined (Bradford, 1976) and adjusted to that of the most dilute sample (typically 300–500 µg/ml). One ml aliquots were incubated for 1 h at 4°C with GST or GST-fusion proteins conjugated to glutathione-sepharose. Beads were isolated by centrifugation and washed three-times in lysis buffer. Bound material was solubilized in 50 µl of 2 Laemmli SDS buffer containing reducing agent and electrophoresed through 6% or 7.5% SDS-gels as described previously (Fox and Phillips, 1982). Proteins were transferred to nitrocellulose, blocked with 5% non-fat milk, and probed with antibodies of interest. Tiam1 was identified with commercially available Tiam1 (C-16) antibodies. As recommended by the suppliers, NIH3T3 extract was used to demonstrate reactivity of the antibodies with a single band of ~200 kDa. Specificity of the Tiam antibodies was demonstrated by the absence of the ~200 kDa band when C-16 immunizing peptide was included at a concentration 100-fold higher than that of the antibodies.

Immunoprecipitations

For immunoprecipitation from platelets, 1 ml aliquots ($1-2 \times 10^9$ platelets) were held in suspension or allowed to spread on 100 mm fibrinogen-coated dishes. At intervals, dishes were rinsed and platelets lysed by addition of 20 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1% Triton X-100, 1 mM calcium chloride, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 1 mM phenylmethylsulphonyl fluoride, 100 mg/ml leupeptin, 10 mM benzamidine, and protease inhibitor cocktail at 4°C. For platelet suspensions, 1 ml of 2× lysis buffer was added. To facilitate depolymerization of actin filaments, lysates were held at room temperature for 30 min (Fox, 1985). Remaining filaments were removed by centrifugation at 12,000g for 15 min at 4°C. Protein concentrations were determined (Bradford, 1976) and adjusted to that of the most dilute lysate (typically 300–500 µg/ml). Hela cells were exposed to 250 nM okadaic acid for 30 min, rinsed, and lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 10 mM EDTA, 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and protease inhibitor at 4°C. Lysates were centrifuged at 15,000g for 10 min at 4°C. Protein concentrations were determined (Bradford, 1976) and adjusted to that of the most dilute sample (100–300 µg/ml). Lysates were then pre-cleared with protein A-sepharose for 1 h at 4°C. For both Hela cells and platelets, lysates (1 ml) were incubated with antibody for 1 h at 4°C and then with protein A-sepharose for 1 h. Beads were washed in lysis buffer and bound material solubilized in 50 µl 2× Laemmli buffer. Samples were electrophoresed through SDS-gels as described previously (Fox and Phillips, 1982) and transferred to nitrocellulose. The nitrocellulose was cut into horizontal strips for individual analysis of proteins of interest.

Localization of proteins by immunofluorescence microscopy

For visualization of proteins in spreading cells, serum-starved cells (1×10^5) were plated on fibronectin-coated coverslips in six-well dishes. For visualization of proteins in migrating cells, a scratch wound was created in serum-starved Hela cell monolayers and cells were allowed to migrate into the wound for 5 h. Cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, blocked in 4% horse serum, and stained with fluorescently labeled phalloidin for 1 h or antibodies against Tiam1, 14-3-3, or β 1-integrin for ~18 h. To ensure specificity, primary antibodies were omitted from some samples. For Tiam1 C-16 antibodies, specificity was also assessed by including samples in which the antibodies were incubated with 100 $\times$ excess immunizing peptide. Stained cells were visualized with a 63 $\times$ 1.4 oil objective using a Leica TCS-NT laser scanning confocal microscope. Laser intensities were adjusted to eliminate cross-talk between channels and images collected using Leica Confocal Software (version 2.5 Build 1227). To assess co-localization, images from confocal sections at the plane of attachment were acquired sequentially from each channel. For quantitative analysis, the ImagePro co-localization program (Media Cybernetics, Bethesda, MD) was used to assess the degree of overlap of Tiam/ β 1-integrin or 14-3-3/ β 1-integrin. Overlap was expressed by Pearson's coefficient; a value of zero indicates no overlap while a value of one indicates total overlap (Manders et al., 1992, 1993).

GTP-Rac1 pull-down assay

$1-2 \times 10^6$ cells were allowed to spread on 100 mm fibronectin-coated dishes. Incubations were terminated by briefly rinsing with ice-cold PBS and adding 1 ml ice-cold buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EDTA, and protease inhibitors to the adherent cells. Dishes were scraped and solubilized material centrifuged at 15,000g for 10 min at 4°C. The protein concentration of each supernatant was determined (Bradford, 1976) and adjusted to that of the most dilute sample (typically 100–300 μ g/ml). Active GTP-bound Rac1 was isolated from 1 ml of each supernatant by incubation with an immobilized GST fusion protein containing the PBD domain of the Rac effector, p21-activated serine/threonine kinase 1 (Pak1) for 45 min at 4°C (del Pozo et al., 2000). Beads were sedimented, washed three times with lysis buffer, and active Rac1 released by addition of 50 μ l of 2 $\times$ Laemmli buffer. Samples were electrophoresed as described (Fox and Phillips, 1982), transferred to nitrocellulose, and probed with Rac1 antibodies. Bands on autoradiographic films were quantified by densitometry, using the NIH Image or NIH ImageJ program. The GTP-Rac1 in each sample was normalized for the total Rac1 in the corresponding sample.

Cell spreading assay

Transiently transfected cells were serum-starved and $\sim 1 \times 10^5$ cells plated on fibronectin-coated coverslips in six-well dishes. At intervals, cells were fixed, permeabilized, and actin filaments stained with phalloidin and antibodies against epitopes of interest. Confocal sections were scanned at the plane of attachment and the surface-attached area of non-transfected cells or cells expressing proteins of interest quantified using the NIH ImageJ program.

Cell migration assay

Cells were transiently transfected and levels of expressed construct determined on Western blots. Serum-starved cells ($\sim 10^5$) expressing comparable amounts of constructs were allowed to migrate across fibronectin-coated Transwell inserts at 37°C. Cells that migrated to the lower surface were labeled with calcein AM, which was quantified in a plate reader and expressed as a percentage of the fluorescence of calcein-labeled inputs, as described previously (Buensuceso et al., 2005).

Statistical analysis

Statistical significance for between-group comparisons was determined by a two-tailed, two-sample, unequal-variance *t*-test. For all comparisons, the significance level was defined as *P* 0.05.

Results

Identification of Tiam1 as a GEF that is recruited by 14–3–3ζ

As a first step in determining whether 14–3–3ζ recruits a GEF, comparable amounts of GST-14–3–3ζ constructs coupled to glutathione–sepharose beads (Fig. 1A) were incubated with platelet lysates. Bound material was analyzed on Western blots using antibodies against the Rac1 GEFs, Vav1, and Tiam1. Vav1 was not detected in pull downs (Fig. 1B). In contrast, Tiam1 was recruited by GST-14–3–3ζ (Fig. 1B). Phosphorylations of 14–3–3ζ, at S58, S184, and T232, have been proposed as mechanisms for regulating the interaction of 14–3–3ζ with binding partners (Aitken et al., 2002; Aitken, 2006; Kjarland et al., 2006). Thus, for each of these residues, we generated a phosphomimic by replacing the serine/threonine with a negatively charged residue. While mutation of S184 (S184D) or T232 (T232E) had no effect on binding of Tiam1 to the GST-14–3–3ζ beads, Tiam1 was not recovered with beads in which S58 was mutated (S58D) (Fig. 1B). The same results were obtained when the GST-14–3–3ζ constructs were incubated with Hela cell (Fig. 1C) or human umbilical vein endothelial cell (data not shown) lysates. Substitution of S58 with a non-phosphorylatable alanine (S58A) did not decrease recovery of Tiam1 with the beads (Fig. 1C). The specificity of the recruitment of Tiam1 by GST-14–3–3ζ constructs was indicated by its absence in material recruited by GST-beads. The specificity of the Tiam1 antibodies was verified by Western blotting before and after absorption with immunizing peptide (Fig. 1D).

Mapping the region of Tiam1 required for recruitment by 14–3–3ζ

Like other Rac1 GEFs, Tiam1 contains a Dbp homology and adjacent PH domain (Dbp-PH in Fig. 2A), both of which are required for its GEF function. However, it contains a second PH domain in its N-terminal region, which, together with an adjacent coiled-coil (CC) and extended structure (EX), has been shown to contain a membrane-interacting region encompassing residues 431–670 (Mertens et al., 2003). Thus, we generated a fragment containing this region, expressed it in CHO cells as a Flag-tagged protein (Fig. 2B, upper blot), and determined whether it was recruited by GST-14–3–3ζ in a pull-down assay. As shown in Figure 2B (middle blot), this fragment did not interact with GST-14–3–3ζ beads. In

contrast, a fragment containing the N-terminal 1–415 residues (N^{1–415} Tiam1) was retained by these beads. As shown in Figure 2B, further fragmentation of N^{1–415} Tiam1 narrowed the 14–3–3 ζ -binding site to the N-terminal 182 residues. Thus a fragment containing residues 1–182 (N^{1–182} Tiam1), was retained by GST-14–3–3 ζ beads while one containing residues 182–415 (N^{182–415} Tiam1) was not, even when, as in Figure 2B, it was expressed at much higher levels than the fragments containing the N-terminal residues (Fig. 2B, middle blot). None of the Tiam1 fragments were recruited by GST-control beads (Fig. 2B, bottom blot).

Evidence that 14–3–3 ζ recruits Tiam1 to an integrin-containing complex in adherent cells

We next sought to determine whether Tiam1 is recruited by 14–3–3 in intact cells. Based on our studies implicating 14–3–3 ζ in integrin-mediated Rac1 activation (Bialkowska et al., 2003), we predicted that formation of such a complex would be restricted to periods of cell spreading and migration. Thus, we compared 14–3–3 ζ immunoprecipitates isolated from platelet suspensions with those from platelets that were spreading on fibrinogen, an adhesive ligand for the major platelet integrin, α IIb β 3 (Bennett, 2005) in the absence of soluble agonists. As shown in Figure 3A, Tiam1 was not detected in 14–3–3 ζ immunoprecipitates from platelets maintained in suspension (0 time). However, it co-immunoprecipitated with 14–3–3 ζ from spreading platelets. While small amounts of β 3-integrin were present in 14–3–3 ζ immunoprecipitates from suspended platelets, the amount was markedly higher in immunoprecipitates from spreading platelets (Fig. 3A). Thus, 14–3–3 ζ is incorporated into a complex that contains both β 3-integrin and Tiam1 in spreading cells.

In order to be able characterize the 14–3–3 ζ -mediated recruitment of Tiam1 and investigate its function through expression of recombinant proteins, we used HeLa cells, a human cancer cell line in which a role for Tiam1 in motility and invasiveness has been well documented (Minard et al., 2004). These cells were transiently co-transfected with HA-tagged 14–3–3 ζ and Flag-tagged N^{1–415} Tiam1. Cells were plated on fibronectin, a ligand for HeLa cell α 5 β 1 (Albiges-Rizo et al., 1995; Takino et al., 2006), lysed, and N^{1–415} Tiam1 immunoprecipitated with antibodies against the Flag-epitope. Examination of the N^{1–415} Tiam1 immunoprecipitates on Western blots revealed the presence of both 14–3–3 and β 1-integrin (Fig. 3B). The co-immunoprecipitation of these proteins with the N-terminal Tiam1 fragment was specific, as shown by their absence in immunoprecipitates obtained with isotype-specific control antibodies. To gain insight into the possibility that 14–3–3 ζ is responsible for recruiting Tiam1 to integrin complexes, we immunoprecipitated Flag-tagged N^{1–415} Tiam1 from cells expressing comparable amounts of wild-type HA-14–3–3 ζ and the 14–3–3 ζ S58D phosphomimic that failed to recruit Tiam1 in pull-down assays (Fig. 1B,C). Comparison of N^{1–415} Tiam1 immunoprecipitates from lysates expressing the two forms of 14–3–3 ζ showed that the amount of 14–3–3 that co-immunoprecipitated with N^{1–415} Tiam1 was markedly reduced in cells expressing 14–3–3 ζ S58D. Moreover, β 1-integrin was not detected in the immunoprecipitates from 14–3–3 ζ S58D expressing cells (Fig. 3B). Taken together, these findings provide evidence that a complex between 14–3–3 ζ , Tiam1, and integrin forms in intact cells, show that Tiam is recruited through its N-terminal region in adherent cells, and provide evidence that recruitment of Tiam1 to integrin complexes is dependent on its interaction with 14–3–3.

Evidence for the transient localization of 14–3–3 and Tiam1 to the leading edge

We next determined whether the complexes detected by co-immunoprecipitation reflected localization of 14–3–3 and Tiam1 to the transient integrin complexes that initiate Rac1-induced actin polymerization and membrane extension. To eliminate the possibility that translocation to the membrane resulted from signals induced by soluble agonists, cells were serum-starved and plated in the absence of serum. Under these conditions, cells spread rapidly, with the most active period occurring during the first ~10–20 min. Isolation of GTP-bound Rac1 with immobilized PBD domain of Pak1 (del Pozo et al., 2000) showed that levels of active Rac1 were elevated during this spreading phase (Fig. 4A). Moreover, levels of active Rac1 did not increase when cells were plated on the non-integrin ligand, poly-L-lysine (Fig. 4A), confirming that the adhesion-induced increase in cells plated on fibronectin resulted entirely from integrin-mediated attachment. To determine whether Tiam1 and 14–3–3 localized to the membrane under these conditions, cells were fixed and examined by immunofluorescence microscopy. Regions of extending membrane were identified by the presence of submembranous actin filaments (Fig. 4B, top part). Staining for endogenous Tiam1 revealed its presence at the membrane, where it was specifically localized to regions at which submembranous actin filaments had formed (Fig. 4B, top part). As reported previously (Han et al., 2001), endogenous 14–3–3 also localized to sites of membrane extension (Fig. 4C). Comparison of 14–3–3 and Tiam1 staining revealed that these proteins localized to the same regions of membrane (Fig. 4C).

After about ~1 h, some cells continued to show regions of membrane extension although others had ceased spreading and become firmly attached, as demonstrated by the presence of stress fibers. 14–3–3 was no longer submembranous in these cells (Fig. 4D, upper row) and was clearly excluded from the β 1 integrin-containing focal adhesions that had formed around the periphery (upper row, right-hand part). As with 14–3–3, Tiam1 failed to localize to these sites of integrin-mediated adhesion in spread cells (Fig. 4D, lower row).

Evidence that 14–3–3 and Tiam1 are recruited to focal complexes in migrating cells

Since integrin-mediated Rac1 activation is critical for directed migration, we also examined the distribution of endogenous 14–3–3 and Tiam1 in migrating Hela cells, using a model in which migration was initiated by wounding serum-starved monolayers (Fig. 5). This method allowed the simultaneous visualization of regions at which densely packed β 1-integrin focal complexes had induced submembranous actin filaments and membrane extension (examples outlined by squares) and regions that remained attached through stress fiber-associated focal adhesions (examples outlined by rectangles). Staining for 14–3–3 (Fig. 5A) or Tiam1 (Fig. 5B) showed that both proteins localized to restricted regions of the membrane. These regions were the same as those at which β 1-integrin and submembranous actin filaments were concentrated, indicating that they were sites of integrin-induced Rac1 activation. Determination of Pearson's correlation coefficient for 14–3–3 and β 1 (Fig. 5A, bar graph) showed a high degree of overlap (0.50 ± 0.07). The specificity of 14–3–3 recruitment to Rac1-activating β 1-integrin was demonstrated by the low level of overlap with β 1-integrin at other membrane regions (Pearson's coefficient of 0.04 ± 0.02). Determination of Pearson's coefficient for Tiam1 (Fig. 5B, bar graph) revealed that values were similar to those for 14–

3-3, with the degree of overlap with $\beta 1$ -integrin being high in the leading edge (0.52 ± 0.02) and low at other regions of the membrane (0.12 ± 0.03).

Evidence that Tiam1 mediates $\beta 1$ -integrin-induced Rac1 activation

Having identified Tiam 1 as a GEF recruited from cell lysates by 14–3–3 ζ and provided both immunofluorescence and biochemical evidence that both Tiam1 and 14–3–3 are components of focal complexes, we next examined the functional consequence of recruitment of Tiam1 to these sites of integrin-mediated Rac1 activation. In initial experiments, we used RNA interference technology to determine whether Tiam1 was required for the integrin-induced generation of active Rac1. Consistent with the rapid turnover of Tiam1 (Habets et al., 1994) and known efficiency of siRNAs in HeLa cells (Elbashir et al., 2001; Yano et al., 2004; Bohil et al., 2006), Tiam1 was typically reduced below detection levels by Tiam1-specific siRNA (Fig. 6A, top part). In contrast, levels were not affected by a non-targeting siRNA (Fig. 6A, top part). To determine whether depletion of Tiam1 affected the ability of $\beta 1$ -integrin to generate active Rac1, HeLa cells were plated on fibronectin in the absence of serum and levels of GTP-Rac1 determined. As shown in Figure 6A, depletion of Tiam1 was accompanied by a marked reduction in the amount of active Rac1 generated by the adherent cells, as compared to non-transfected cells or cells transfected with non-specific siRNA. Quantification of data from three separate experiments showed that levels of active Rac1 in cells in which Tiam1 had been depleted were $58 \pm 12\%$ (mean $\pm$ SD; $P < 0.05$) lower than those in non-transfected cells. We confirmed the specificity of the effect by showing that levels in cells transfected with non-targeted siRNA were comparable to those in non-transfected cells (Fig. 6B).

Inhibition of integrin-signaling by overexpression of non-Tiam1-recruiting 14–3–3 ζ S58D

Having shown that Tiam1 mediated integrin-induced Rac activation, we next sought to determine whether this functional activity of Tiam1 was dependent on its recruitment to 14–3–3. To do this, we overexpressed the 14–3–3 ζ S58D variant that we had shown to lack the ability to recruit Tiam1 in pull-down experiments (Fig. 1), to co-immunoprecipitate with Tiam1 (Fig. 3B), or to recruit Tiam1 into integrin complexes (Fig. 3B). We compared the effects of this non-Tiam1-binding variant with those of either WT 14–3–3 ζ or 14–3–3 ζ in which S58 was mutated to a non-phosphorylatable residue (14–3–3 ζ S58A), both of which recruited Tiam1 in pull-down experiments (Fig. 1).

Immunofluorescence microscopy showed that like endogenous 14–3–3 (Fig. 4), overexpressed HA-wild-type, S58A, and S58D forms of 14–3–3 ζ were each recruited to the membrane when cells were plated on fibronectin (data not shown). Within 2 h, endogenous (Fig. 4D), HA-wild-type 14–3–3 ζ (Fig. 7A, top part), and 14–3–3 ζ S58A (data not shown) had redistributed to the cytoplasm and cells had ceased to spread. In contrast, cells expressing the non-Tiam1-binding S58D variant remained relatively unspread and the expressed protein continued to localize at the membrane (Fig. 7A, bottom part). Quantification of cell areas demonstrated that the mean ($\pm$ SEM) surface-attached area of cells expressing HA-14–3–3 ζ S58D was significantly reduced compared to that of non-expressing cells ($27,014 \pm 4,104$ pixels for expressing cells compared to $66,347 \pm 4,148$ for non-expressing cells) (Fig. 7B) while that of cells expressing the Tiam1-binding HA-WT or

HA-S58A control constructs ($71,713 \pm 11,456$ and $76,484 \pm 11,036$, respectively) was not significantly different to that of non-expressing cells (Fig. 7B).

As another readout of the consequence of expression of non-Tiam1-binding 14-3-3 ζ on Rac1-dependent integrin functions, we quantified its effect on migration. As shown in Figure 7C, migration to the lower surface of fibronectin-coated Transwell inserts was significantly reduced in cells expressing the non-Tiam1-binding 14-3-3 ζ S58D variant ($9.9 \pm 2.0\%$) compared to cells expressing comparable amounts of either wild-type ($19.6 \pm 1.2\%$) or S58A ($23.0 \pm 5.9\%$) Tiam1-recruiting 14-3-3 ζ controls.

The inhibitory effects of 14-3-3 ζ S58D were exerted upstream of Rac1 activation, as shown by a marked decrease in the adhesion-induced generation of active Rac1 in cells expressing 14-3-3 ζ S58D compared to the 14-3-3 ζ S58A control (Fig. 7D, left-hand part).

Quantification of data from three separate experiments showed that levels of active Rac1 in cells transfected with the non-Tiam1-recruiting S58D phosphomimic were only $39.6 \pm 10.1\%$ of those in cells transfected with 14-3-3 in which introduction of S58A ensured that this residue remained in a non-phosphorylated state (Fig. 7D, right-hand part).

Inhibition of integrin-signaling by overexpression of 14-3-3 interacting dominant-negative Tiam1 fragments

As an independent approach to determining whether recruitment of Tiam1 to 14-3-3 is required for it to mediate integrin-induced Rac1 activation, we examined the functional consequence of overexpression of the N-terminal Tiam1 fragments that we had shown to contain the region that interacts with 14-3-3 ζ in pull-down assays (Fig. 2B) and co-immunoprecipitates with integrin-associated 14-3-3 ζ from adherent cells (Fig. 3B). Since these fragments lack the db1 domain through which Tiam1 activates Rac1 (Fig. 2A), we reasoned that they would act as dominant-negative inhibitors of GEF-dependent functions of Tiam1 at sites of recruitment to 14-3-3.

Examination of cells transiently transfected with these Tiam1 fragments showed that at times at which most non-expressing cells were well-spread, those expressing N¹⁻¹⁸² Tiam1 (Fig. 8A, upper part) or N¹⁻⁴¹⁵ Tiam1 (data not shown) remained unspread, with both Tiam1 fragments and endogenous 14-3-3 continuing to localize at the periphery of the cell (Fig. 8A, lower part). Quantification showed that even 2 h after plating, the surface-bound area of cells expressing N¹⁻¹⁸² Tiam1 was only 38.6% of that of non-expressing cells ($443.88 \pm 27.5 \mu\text{m}^2$, N=77 compared to $1150.23 \pm 39.99 \mu\text{m}^2$, N=89; mean $\pm$ SEM, * $P < 0.001$) (Fig. 8B, right-hand graph). In contrast, the mean area of cells expressing the non-14-3-3-binding N¹⁸²⁻⁴¹⁵ Tiam1 fragment was not significantly different to that of non-expressing cells (Fig. 8B, right-hand graph).

The inhibitory effect of the N¹⁻¹⁸² Tiam1 fragment on spreading was rescued by co-transfection with Q61LRac1, a constitutively active form of Rac1 (Fig. 8C), indicating that the Tiam1 fragment inhibited cell spreading by inhibiting Rac1 activation. Direct evidence that 14-3-3 ζ -binding Tiam1 fragments acted in this way was provided by measuring active Rac1 when cells expressing 14-3-3 ζ -interacting and non-interacting Tiam1 fragments were plated on fibronectin. As shown in the representative Western blots in Figure 8D, levels of

active Rac1 in spreading Hela cells were markedly decreased in cells expressing the 14–3–3 ζ -binding N^{1–415} or N^{1–182} Tiam1 fragments while the non-binding N^{182–415} fragment had little effect. Quantification of data from four experiments showed that GTP-Rac1 levels in cells expressing N^{1–415} Tiam1 were only 51±25% (mean±SD) of those in vector-transfected cells (Fig. 8D, right-hand part). Levels of N^{1–182} Tiam1 expression in Hela cells were consistently lower than those of longer or more C-terminal fragments (e.g., Flag-epitope blot in Fig. 8D). Despite its lower expression levels, cells transfected with this fragment showed adhesion-induced GTP-Rac1 levels that were only 44±22% of those in cells transfected with vector alone while cells expressing the non-14–3–3 ζ -binding N^{182–415} Tiam1 fragment had GTP-Rac1 levels that were comparable to those in the vector transfectants (Fig. 8D, right-hand part).

Discussion

Several studies have implicated a role for 14–3–3 proteins in motility, showing that they localize to the leading edge, where transient integrin complexes generate activate Rac1, but not to the stable anti-migratory focal adhesions that mediate firm adhesion of stationary cells (Garcia-Guzman et al., 1999; Han et al., 2001). Previously, we showed that sequestration of 14–3–3 ζ prevented integrin-induced Rac1-activation and spreading and that these integrin functions were restored by overexpression of 14–3–3 ζ (Bialkowska et al., 2003). Here, we provide a mechanistic explanation for these findings by showing that 14–3–3 ζ recruits the Rac1-GEF, Tiam1, to sites of transient integrin attachment at the leading edge and that this allows Tiam1 to mediate integrin-induced Rac1 activation, thereby regulating lamellipodia extension, spreading, and migration.

Several lines of in vitro and in vivo evidence supported this model. Thus, evidence that 14–3–3 ζ recruits Tiam1 came from pull-down studies in which GST-14–3–3 ζ beads recruited endogenous Tiam1 from platelet, Hela, and HUVEC cell lysates and recombinant Tiam1 from CHO cell lysates. Second, evidence that 14–3–3 ζ and Tiam1 translocate to integrin complexes was provided by the adhesion-induced co-immunoprecipitation of endogenous 14–3–3 ζ , Tiam1, and β 3-integrin from platelets and co-immunoprecipitation of 14–3–3 ζ , β 1-integrin, and an overexpressed 14–3–3 ζ -binding Tiam1 fragment from Hela cells. The adhesion-induced complex presumably formed at the leading edge, as shown by the specific localization of 14–3–3 and Tiam1 to regions of densely packed integrin complexes at sites of membrane extension in cells spreading on fibronectin or migrating into a wound. Third, evidence that recruitment to integrin complexes was mediated by 14–3–3 came from identification of a 14–3–3 ζ phosphomimic, S58D, that failed to recruit Tiam1 from cell lysates or to co-immunoprecipitate with Tiam1 from spreading cells. Unlike wild-type 14–3–3 ζ , when overexpressed, this non-Tiam1-binding 14–3–3 ζ variant failed to incorporate β 1-integrin into Tiam1 immunoprecipitates. Finally, evidence that the 14–3–3 ζ -induced localization of Tiam1 allowed this GEF to activate Rac1 at sites of integrin-mediated adhesion, came from the demonstration that adhesion-induced activation of Rac1, membrane extension, and spreading were significantly reduced by expression of the non-Tiam1-binding 14–3–3 ζ variant that lacked the ability to incorporate Tiam1 into β 1-integrin immunoprecipitates or by expression of a catalytically inactive, 14–3–3 ζ -binding Tiam1 fragment.

14-3-3 proteins are dimers that interact with numerous binding partners and regulate cellular functions through mechanisms that include localizing a signaling molecule to a site at which it can participate in a specific signaling pathway (Mackintosh, 2004; Aitken, 2006; Messaritou et al., 2010). Thus, we propose a model in which one subunit of a 14-3-3 dimer interacts with a binding partner at sites of newly attached integrin and the other recruits Tiam1, thus bringing Tiam1 into proximity with localized Rac1 effectors and signaling molecules specific for Rac1-induced actin polymerization and lamellipodia extension at the leading edge. Interactions between 14-3-3 and Rho family GEFs have been described previously. However, these prior reports suggest that binding to 14-3-3 provides a mechanism for negatively regulating the functional activity of each of the 14-3-3-binding GEFs identified to date (Zhai et al., 2001; Diviani et al., 2004; Zenke et al., 2004; Angrand et al., 2006; Chahdi and Sorokin, 2008). Thus, the present study identifies a novel function of 14-3-3 as an adaptor protein that can participate in the differential regulation of Rac1-induced changes in cell behavior by localizing a GEF to a specialized site of Rac1-induced signaling.

Once activated, Rac1 can interact with numerous downstream effectors, with the resulting modulation of a variety of different signaling pathways and cellular functions. The differential regulation of these pathways is determined by the nature of the Rac1 effectors and downstream signaling molecules localized at the site of GEF recruitment (Schmidt and Hall, 2002). In addition to generating active Rac1, many GEFs interact with downstream effectors or scaffold protein complexes on which path-specific signaling molecules are assembled (Schmidt and Hall, 2002). Thus, the specificity of GEF translocation can be critical in determining that appropriate downstream pathways are activated at a specialized site of Rac1 activation. Evidence that Tiam1 influences signaling in this way comes from the demonstration that its interaction with the scaffold proteins IB2/JIP2 results in Rac1-induced activation of scaffold-associated components of the p38 mitogen-activated protein kinase cascade (Buchsbau et al., 2002) while its interaction with spinophilin specifies that spinophilin-associated p70 S6 kinase is activated downstream of Rac1 (Buchsbau et al., 2003). In addition, Tiam1 interacts directly with the Arp2/3 complex (ten Klooster et al., 2006). Since this is a key component of pathways by which Pak1 induces filament networks (Pollard and Borisy, 2003), recruitment of Arp2/3 may be another way in which Tiam1 influences the differential activation of Rac1-induced pathways in integrin complexes. Thus, by recruiting Tiam1 to newly attached integrin at the leading edge, 14-3-3 may function not only to induce localized generation of active Rac1 but also to participate in the selection of Rac1-induced pathways at these specialized sites of actin polymerization-driven membrane extension.

In our previous studies, we found that sequestration of 14-3-3 ζ reversibly inhibited the integrin-induced activation of both Rac1 and cdc42 (Bialkowska et al., 2003). Since Tiam1 is a Rac1-specific GEF, mechanisms other than the absence of Tiam1 must have been responsible for the inability of integrin adhesion complexes to activate cdc42 upon 14-3-3 ζ sequestration. While the cdc42/Rac-GEF, β -Pix, is recruited by 14-3-3 β (Angrand et al., 2006; Chahdi and Sorokin, 2008), the protein kinase A-induced binding of this GEF decreases rather than increases its Rac1-activating function and does not appear to participate in regulation of its cdc42-activating function (Chahdi and Sorokin, 2008). Thus,

mechanisms by which 14-3-3 influences integrin-mediated cdc42 activation remain unknown. We also do not know how 14-3-3 interacts with integrin adhesion complexes. Based on reports of interactions of several 14-3-3 isoforms with $\beta 1$ -, $\beta 2$ -, $\beta 3$ -, and $\beta 4$ -integrins (Han et al., 2001; Fagerholm et al., 2002; Santoro et al., 2003; Nurmi et al., 2006) and regulation of some interactions by phosphorylation of cytoplasmic domain serine/threonine residues (Santoro et al., 2003; Fagerholm et al., 2005; Nurmi et al., 2007; Takala et al., 2008), one possibility is that 14-3-3 ζ interacts directly with ligated integrin. However, it appears equally feasible that it is recruited to ligated integrin through an adaptor protein, such as pCas¹³⁰, which has been identified as a 14-3-3 ζ -interacting protein in $\beta 1$ integrin-containing focal complexes (Garcia-Guzman et al., 1999). Another unknown is the mechanism by which Tiam1 is recruited to 14-3-3. We do not yet know whether this interaction is direct or whether it is mediated by an intermediate protein(s). Moreover, further studies will be needed to determine whether 14-3-3 isoforms other than the zeta isoform used in the present study recruit this GEF to activate Rac at sites of integrin-mediated membrane attachment.

Yet another question that remains to be answered is whether regulation of Tiam recruitment occurs at the level of modulation of 14-3-3. Thus, several studies have shown that phosphorylation of 14-3-3 ζ at S58, S184, or T232 differentially regulates recruitment of specific binding partners, at least in vitro (Aitken et al., 2002; Tzivion and Avruch, 2002; Aitken, 2006). We did not detect an effect of mutating S184 or T232. However, while 14-3-3 ζ in which S58 was replaced with a non-phosphorylatable alanine retained its ability to recruit Tiam1 from cell lysates, 14-3-3 ζ in which a phosphomimic was created at this residue (14-3-3 ζ S58D) was unable to recruit Tiam1 from cell lysates, to co-immunoprecipitate with Tiam1, or to recruit Tiam1 to integrin complexes. Evidence has been provided that S58 can be phosphorylated by PKCd, Akt, and mitogen-activated protein kinase-activated protein kinase 2 and that this results in dissociation of 14-3-3 ζ into monomers that are no longer able to recruit certain binding partners (Tzivion and Avruch, 2002; Woodcock et al., 2003; Aitken, 2006). Thus, it will be of interest to determine whether phosphorylation of S58 provides a physiologically relevant mechanism for modulating the ability of 14-3-3 ζ to recruit Tiam1 to integrin complexes.

The present findings also extend our understanding of functions of Tiam1. Previously this GEF has been shown to activate Rac1 at a variety of different subcellular locations such as cell-cell contacts where it mediates the cadherin-induced activation of Rac1 leading to tight-junction formation; the cytoplasmic domain of activated EphB2 receptor, where it mediates the Rac1-induced effects on dendritic spine development (Tolias et al., 2007), and the periphery of cells stimulated by growth factors and lipid products such as lysophosphatidic acid, where active Rac1 induces membrane ruffles (Mertens et al., 2003). While one study reported that $\alpha 3\beta 1$ -mediated keratinocyte matrix deposition was dependent on Tiam1-induced Rac1 activation (Hamelers et al., 2005), molecular mechanisms were not elucidated. Thus, while the role of Tiam1 in motility and invasiveness of tumor cells is well known (Minard et al., 2004) and its role in growth factor-induced lamellipodia formation has been studied in detail (Mertens et al., 2003), little is known about the involvement of Tiam1 in the critical migration-inducing activation of Rac1 in integrin complexes. In the present study, we provide direct evidence that Tiam1 mediates integrin-induced signaling by showing that $\beta 1$ -

integrin-induced Rac1 activation was inhibited when Tiam1 function was blocked, either by expression of a 14–3–3 ζ -interacting dominant-negative N^{1–182} Tiam1 fragment, which co-immunoprecipitated with 14–3–3 ζ and β 1-integrin, or by depletion of Tiam1 with siRNA. Together with the inhibitory effects of the 14–3–3 ζ phosphomimic that was unable to incorporate Tiam1 into integrin complexes, these data provide compelling evidence that the adhesion-induced activation of Rac1 at transient sites of β 1-integrin-mediated attachment of newly extended membrane was accomplished by the 14–3–3-mediated recruitment of Tiam1 to these sites.

Another novel finding was that Tiam1 localized to integrin complexes through a region in its N-terminal 182 residues. The membrane-localizing region of Tiam1 was originally mapped in cells in which translocation of overexpressed Tiam1 to the membrane was induced by signals from growth factors and lipid products present in serum-containing culture media (Michiels et al., 1997; Stam et al., 1997). These studies identified the PH-CC-EX domain as the critical membrane-localizing region. Subsequently PH-CC-EX has been shown to interact with receptors such as CD44 (Bourguignon et al., 2000b) and the EphB2 receptor (Tolias et al., 2007), with membrane-associated proteins such as α -actinin (Bourguignon et al., 2000a) and the Arp2/3 complex (ten Klooster et al., 2006), and with the scaffolding proteins, IB2/JIP2 (Buchsbaum et al., 2002) and spinophilin (Buchsbaum et al., 2003). These PH-CC-EX-mediated interactions mediate the functional activities of Tiam1, as shown by the inhibitory effects of a fragment containing this region on serum-induced Rac1 activation, membrane ruffling, C-Jun NH2-terminal kinase activation, and tumor cell invasion and migration (Michiels et al., 1997; Stam et al., 1997; Bourguignon et al., 2000a) and on Rac1 activation in response to CD44 or EphB receptor-induced signals (Bourguignon et al., 2000b; Tolias et al., 2007). Thus, a model has emerged in which the PH-CC-EX-mediated recruitment of Tiam1 provides a common mechanism for localizing this GEF to the different subcellular locations at which it initiates Rac1-mediated signaling.

Based on this model of membrane-localization, we expected that the PH-CC-EX domain would also mediate the interaction of Tiam1 with 14–3–3. Surprisingly, however, 14–3–3 ζ failed to interact with this region in pull-down assays. Rather, the interaction was mapped to a region within the N-terminal 182 residues. This N-terminal region was responsible for the functional interaction of Tiam1 with integrin complexes, as shown by recruitment of fragments containing residues 1–182 to the membrane, where they were incorporated into 14–3–3-containing β 1-integrin complexes, as shown by co-immunoprecipitation, and inhibited adhesion-induced Rac1 activation and spreading in a manner that could be overcome by constitutively active Rac1. The N-termini of many GEFs, including Tiam1, impose conformational constraints that must be released for activation of their GEF function (Schmidt and Hall, 2002). These constraints can be released by phosphorylation, which has been implicated in PH-CC-EX-mediated localization and activation of Tiam1 in response to a number of stimuli (Fleming et al., 1997, 1999; Buchanan et al., 2000; Servitja et al., 2003; Tolias et al., 2007). However, interaction of the N-terminus with other proteins can also release N-terminal auto-inhibitory constraints (Schmidt and Hall, 2002). Thus, it will be of interest to determine whether the N-terminus-mediated recruitment of Tiam1 to its integrin-associated docking site serves not only to localize Tiam1 adjacent to Rac and site-specific

Rac1 effectors but also to provide an integrin-specific mechanism for activating its GEF function.

It is of interest that an interaction with the N-terminal residues of Tiam1 has been implicated as a mechanism by which the metastasis suppressor, nm23H1 (Leone et al., 1991; Kantor et al., 1993), exerts its inhibitory effects on motility and tumor invasiveness (Otsuki et al., 2001). When overexpressed, nm23H1 co-immunoprecipitated with Tiam1, decreased the amount of Tiam1 that translocated to the membrane as cells were plated on fibronectin, and inhibited Tiam1-induced Rac1 activation and motility. In contrast, it failed to co-immunoprecipitate with C1199, a truncated form of Tiam1 that lacks its N-terminal 392 residues and has been widely used to investigate regulation of Tiam1 localization and function (Michiels et al., 1997; Stam et al., 1997; van Leeuwen et al., 1997; Sander et al., 1998; Bourguignon et al., 2000a,b; Fleming et al., 2004, 2000; ten Klooster et al., 2006). The inability of nm23H1 to associate with C1199 was accompanied by lack of an effect on its membrane localization or functional activities (Otsuki et al., 2001). While these findings were interpreted as evidence that the inhibitory effects of nm23H1 on motility are dependent on its ability to interact with the N-terminal 392 residues of Tiam1 (Otsuki et al., 2001), they have been difficult to understand within the context of a paradigm of membrane-localization being mediated by PH-CC-EX. However, based on the present findings, a model can now be envisioned in which nm23H1 competes with the Tiam1-recruiting protein in integrin complexes for interaction with the N-terminal region of Tiam1. Thus, taken together, the current evidence suggests a critical role for the 14–3–3 mediated recruitment of Tiam1 to integrin complexes in the invasiveness and metastasis of tumor cells and points to the potential benefits of a therapeutic approach in which the 14–3–3 mediated recruitment of Tiam1 to integrin complexes is prevented.

To date, only a few of the more than 60 known GEFs have been implicated in integrin-mediated Rac1 activation and little is known about mechanisms regulating the differential recruitment of these GEFs to the diverse array of potential integrin adhesions (Gu et al., 2001; Marignani and Carpenter, 2001; Itoh et al., 2002; Gakidis et al., 2004; Sanchez-Martin et al., 2004; Katoh et al., 2006; Rosenberger and Kutsche, 2006; Chan et al., 2008; Gao and Blystone, 2009; Feng et al., 2010). The present study adds to currently known possibilities by identifying a mechanism in which 14–3–3 translocates Tiam1 to integrin complexes. While factors regulating utilization of this mechanism remain to be investigated, it is of interest that there are many reports in which adhesion-induced localization of 14–3–3 (Garcia-Guzman et al., 1999; Han et al., 2001; Santoro et al., 2003) or Tiam1 to the leading edge has been noted (Clark et al., 1998; Otsuki et al., 2001, 2003; Arthur et al., 2004; ten Klooster et al., 2006; Desai et al., 2008). The present study shows that this mechanism mediates β 1 integrin-induced Rac1 activation in Hela cells spreading on fibronectin in the absence of serum. The inhibitory effects of nm23H1 in the study of Otsuki et al. (2001) suggest that it can also mediate Rac1 activation by β 1-integrin in Rat1 fibroblasts. Our previous finding that 14–3–3 ζ was required for α v β 3-induced Rac1 activation in CHO cells spreading on von Willebrand factor (Bialkowska et al., 2003), together with the present demonstration of the adhesion-induced association of 14–3–3, Tiam1, and β 3-integrin in platelets spreading on fibrinogen, suggest that the 14–3–3/Tiam1 mechanism may also participate in Rac1 activation by ligated β 3-integrin.

To summarize, the present study describes a previously unrecognized 14–3-3/Tiam1-dependent mechanism for generating active Rac1 at sites of integrin-mediated attachment. In contrast to its PH-CC-EX-mediated localization to other specialized sites of Rac1 activation, Tiam1 was recruited to 14–3-3 ζ -containing integrin complexes through a region within its N-terminal 182 residues. Integrin-induced Rac1 activation and motile behaviors were inhibited by decreasing Tiam1 expression with siRNA, blocking its function with dominant-negative N-terminal Tiam1 fragments, or blocking its recruitment to integrin complex by overexpression of a non-Tiam1-binding 14–3-3 variant. Activation of Rac1 in the transient integrin complexes that anchor extending membrane to the matrix initiates signaling required for migratory responses of cells. Thus, the present study suggests that blocking the N-terminal interaction of Tiam1 with 14–3-3 in Rac1-activating integrin-complexes may be an effective therapeutic approach for pathological conditions such as tumor invasiveness and metastasis.

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Fig. 1.

Identification of Tiam1 as a GEF recruited by GST-14-3-3 ζ . GST-14-3-3 ζ variants were quantified by Coomassie-blue staining of SDS gels (A) and incubated with platelet (B) or HeLa cell (C) lysates. Proteins pulled down by the GST-14-3-3 ζ beads or GST-beads alone were analyzed by Western blotting using Vav1 or Tiam1 (C-16) antibodies. D: The specificity of the Tiam1 antibodies was verified on Western blots of NIH3T3 and HeLa cell lysates by probing Western blots with antibodies in the absence (left-hand part) or presence (right-hand part) of a 100-fold excess of C-16 immunizing peptide.

Fig. 2.

Tiam1 interacts with 14–3–3 ζ through its N-terminal region. A: Schematic representation of Tiam1 and fragments utilized in pull-down assays. PH denotes Pleckstrin homology domain; CC, coiled-coil region; EX, extended structure; PDZ, PSD-95/DlgA/ZO-1 domain; and Dbl, Dbl homology domain. B: CHO cells were transiently transfected with the indicated Flag-tagged Tiam1 fragments. The top blot shows expression levels of Flag-tagged fragments in comparable amounts of CHO cell lysates; the middle blot shows Flag-tagged fragments recruited by GST-14–3–3 ζ beads; the bottom blot shows the absence of Flag-tagged fragments in material recruited by control GST-beads.

Fig. 3.

Adhesion-induced co-immunoprecipitation of Tiam1, 14-3-3, and integrin. **A:** Platelets were lysed in suspension or allowed to spread on fibrinogen for the indicated times prior to lysis. Lysates were immunoprecipitated with 14-3-3 ζ antibodies or control IgG and immunoprecipitates analyzed on Western blots probed with antibodies against 14-3-3 ζ , Tiam1, and β 3-integrin. **B:** HeLa cells were cotransfected with Flag-tagged N¹⁻⁴¹⁵ Tiam1 fragment and either HA-tagged 14-3-3 ζ or non-Tiam1-recruiting 14-3-3 ζ S58D. Lysates were immunoprecipitated with antibodies against Flagepitope or isotype-specific control IgG. Western blots of immunoprecipitates were probed with antibodies against Flag-epitope, pan-14-3-3, and β 1-integrin.

Fig. 4.

14-3-3 and Tiam1 localize to regions of extending membrane in spreading HeLa cells. A: HeLa cells were serum-starved and plated on fibronectin (Fn) or poly-L-lysine (PLL). At intervals, cells were lysed and levels of active Rac1 determined. The fibronectin and poly-L-lysine samples were run on the same gel but irrelevant intermediate lanes were deleted. B: Cells that were spreading on fibronectin were fixed and stained for actin filaments and Tiam1. Tiam1 localized to regions of extending membrane, which were identified by the presence of submembranous actin filaments. C: Tiam1 co-localized with 14-3-3 at the

leading edge of spreading cells. In (B,C), examples of extending membrane are indicated with arrows. D: Cells were fixed 2 h after they were plated on fibronectin. At this time, spreading had ceased and both 14-3-3 and Tiam1 were absent from the membrane. Examples of focal adhesions are indicated with arrow heads; neither protein localized to these sites of integrin-mediated adhesion. Bars, 10µm.

Fig. 5.

14-3-3 and Tiam1 translocate to the leading edge in migrating HeLa cells. Confluent monolayers of HeLa cells were wounded, allowed to migrate into the wound for 5 h, fixed, and stained for actin filaments, $\beta 1$ -integrin, and either 14-3-3 ζ (A) or Tiam1 (B). Examples of leading edge regions are shown with squares and regions at which stress fibers terminate at focal adhesions with rectangles. Co-localization of 14-3-3 ζ (A) or Tiam1 (B) with $\beta 1$ integrin was assessed by quantification of Pearson's coefficient (Manders et al., 1993). Values are shown in bar graphs as mean $\pm$ SEM. An asterisk indicates a significant difference between Pearson's coefficient at the leading edge and that at other membrane regions, $P < 0.0001$. Bars, 10 μ m.

Fig. 6.

Tiam1 generates active Rac1 during β 1-integrin-mediated spreading. A: Western blot of a representative experiment in which non-transfected Hela cells (C) or cells transfected with Tiam1-specific (si) or non-specific siRNA (NS) were plated on fibronectin in the absence of serum for 15min. The top blot shows Tiam1 levels. The lower blots show levels of active and total Rac1 in lysates of the corresponding cells. B: Levels of active Rac1 were normalized for total Rac1 and those in transfected cells expressed as a percentage of those in non-transfected cells. Data from three separate experiments were pooled; values shown are mean $\pm$ SEM. The asterisk indicates a significant difference between levels in transfected compared to non-transfected cells, $P < 0.05$.

Fig. 7.

Inhibition of focal complex functions by a non-Tiam1-binding 14-3-3 ζ variant. A: HeLa cells were transiently transfected with HA-tagged wild-type (WT) 14-3-3 ζ , Tiam1-binding S58A 14-3-3 ζ , or non-Tiam1-binding S58D14-3-3 ζ . Cells were serum-starved, plated on fibronectin for 2 h, fixed, and stained with HA antibodies and phalloidin. Arrows indicate cells expressing HA-14-3-3 ζ proteins. Bar, 10 μ m. B: The mean area of non-expressing cells and cells expressing HA-14-3-3 ζ proteins was quantified 60 min after plating on fibronectin; mean $\pm$ SEM values for the indicated number of cells are shown. An asterisk

indicates a significant difference between non-expressing cells (Con) and those expressing an HA-14-3-3 ζ construct, $P < 0.0003$. Similar results were obtained in four different experiments for HA-WT and HA-S58D 14-3-3 ζ and two different experiments for HA-S58A 14-3-3 ζ . C: Cells expressing comparable amounts of HA-wild-type, S58A, or S58D 14-3-3 ζ were allowed to migrate across fibronectin-coated Transwell inserts for 1 and 4 h. Cells that migrated to the lower surface were quantified. For each bar, values shown represent the mean $\pm$ SEM of six samples pooled from two different experiments. An asterisk indicates a significant difference between cells expressing wild-type 14-3-3 ζ and a 14-3-3 ζ variant, $P < 0.02$. D: Cells expressing comparable amounts of HA-S58A or HA-S58D 14-3-3 ζ were plated on fibronectin for 10 min, lysed, and levels of total and active Rac1 determined. The left-hand part shows Western blots from a representative experiment. The top blot shows expression levels of Tiam1-binding 14-3-3 ζ S58A and non-Tiam1-binding 14-3-3 ζ S58D. The lower blots show levels of active and total Rac1 in the corresponding lysates. In the right-hand part, active Rac1 was normalized for total Rac1 and the level in 14-3-3 ζ S58D-transfected cells expressed as a percentage of that in cells transfected with the Tiam-binding 14-3-3 ζ S58A control. Mean $\pm$ SEM values from three different experiments are shown. An asterisk indicates a significant difference between cells expressing non-Tiam1-binding 14-3-3 ζ S58D and Tiam1-binding S58A control, $P < 0.004$.

Fig. 8.

Inhibition of focal complex functions by a 14-3-3-binding fragment of Tiam1. **A:** HeLa cells that had been transiently transfected with Flag-tagged N¹⁻¹⁸²Tiam1 fragment were plated on fibronectin. At intervals, cells were fixed and actin filaments stained with phalloidin, Tiam1 fragment with Flag antibodies, and endogenous 14-3-3 with pan 14-3-3 antibodies. The upper part shows cells fixed 1 h after plating. In contrast to the N¹⁻¹⁸² Tiam1-expressing cell indicated with an arrow, non-transfected cells were well spread. In the lower part, a representative transfected cell, fixed at 2 h, is shown at higher magnification. The N-terminal

Tiam1 fragment and endogenous 14-3-3 continued to localize with β 1-integrin at the periphery of the cell, which remained relatively unspread even 2 h after plating. Bars, 10 μ m. B: Cells were transiently transfected and plated on fibronectin for 1 h. In the left-hand graph, the mean area of 89 non-expressing and 77 Flag-tagged N¹⁻¹⁸² Tiam1-expressing cells was quantified. In the right-hand graph, the mean area of 80 non-expressing and 20 Flag-tagged N¹⁸²⁻⁴¹⁵ Tiam1-expressing cells was quantified. Bar graphs show mean $\pm$ SEM. An asterisk indicates a significant difference between expressing and non-expressing cells, $P < 0.001$. C: Cells that had been co-transfected with Flag-tagged N¹⁻¹⁸² Tiam1 and HA-Q61LRac1 were serum-starved, plated on fibronectin for 1 h, fixed, and stained for actin filaments, Flag-tagged N¹⁻¹⁸² Tiam1 (green), and HA-Q61LRac1 (cyan). The inhibitory effect of N¹⁻¹⁸² Tiam1 on spreading was rescued by co-expression of Q61LRac1. Bar, 10 μ m. D: Hela cells that had been transiently transfected with vector or Flag-tagged Tiam1 fragments were serum-starved, plated on fibronectin for 10 min, lysed, and levels of total and active Rac1 determined. In the left hand part, Western blots from a representative experiment show expression of Tiam1 fragments, levels of active Rac1, and levels of total Rac1 in the corresponding samples. In the right-hand part, levels of active Rac1 were normalized and expressed as a percentage of that in cells transfected with vector alone. Values shown in the bar graph are the mean $\pm$ SEM from four different experiments. An asterisk indicates a significant difference between cells transfected with a Tiam1 fragment and those transfected with vector, $P < 0.05$.