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Lam, B.D.

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RAC1 LOCALIZATION, ACTIVATION AND SIGNALING IS REGULATED BY SEQUENCE AND POSITION OF THE HYPERVARIABLE C-TERMINUS

Younes Zoughlami¹, B. Daniel Lam¹, Trynette J. van Duijn, Eloise C. Anthony, Paula B. van Hennik and Peter L. Hordijk²

¹ These authors contributed equally
² to whom correspondence should be addressed

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The regulation of cell adhesion and motility requires precise, localized signaling to orchestrate coordinated cellular behavior. Among others, Rho-like GTPases are central regulators of cell adhesion and migration while at the same time these proteins are also activated upon engagement of adhesion receptors or cell stimulation with chemokines. RhoGTPases consist of a core G- (GTP binding) domain flanked at the C-terminus by a hypervariable region. The latter also harbors the cysteine to which a lipid membrane anchor is post-translationally attached. This C-terminus is generally considered to mediate specific targeting of Rho GTPases. Here we show that the hypervariable C-terminus of Rac1 is required, but not sufficient for proper targeting of activated Rac1 to Focal Adhesions (FAs). Selected, limited mutation of the hypervariable region did not prevent FA targeting or binding to the Rac1 effector IQGAP1, but did impair Rac1 induced cell spreading. In contrast, mis-positioning a normal, unmutated hypervariable region did prevent FA targeting of activated Rac1, and impaired the capacity of the protein to be activated by a co-expressed guanine nucleotide exchange factor. Finally, this construct also showed a reduction in Rac1-induced cell spreading.

In conclusion, these data underscore the relevance of the hypervariable region for Rac1 signaling and indicate that this region acts in conjunction with the switch I and switch II regions, exposed upon GTP-binding, in the subcellular targeting of Rac1.

SUMMARY

The regulation of cell adhesion and motility requires precise, localized signaling to orchestrate coordinated cellular behavior. Among others, Rho-like GTPases are central regulators of cell adhesion and migration while at the same time these proteins are also activated upon engagement of adhesion receptors or cell stimulation with chemokines. RhoGTPases consist of a core G- (GTP binding) domain flanked at the C-terminus by a hypervariable region. The latter also harbors the cysteine to which a lipid membrane anchor is post-translationally attached. This C-terminus is generally considered to mediate specific targeting of Rho GTPases. Here we show that the hypervariable C-terminus of Rac1 is required, but not sufficient for proper targeting of activated Rac1 to Focal Adhesions (FAs). Selected, limited mutation of the hypervariable region did not prevent FA targeting or binding to the Rac1 effector IQGAP1, but did impair Rac1 induced cell spreading. In contrast, mis-positioning a normal, unmutated hypervariable region did prevent FA targeting of activated Rac1, and impaired the capacity of the protein to be activated by a co-expressed guanine nucleotide exchange factor. Finally, this construct also showed a reduction in Rac1-induced cell spreading.

In conclusion, these data underscore the relevance of the hypervariable region for Rac1 signaling and indicate that this region acts in conjunction with the switch I and switch II regions, exposed upon GTP-binding, in the subcellular targeting of Rac1.
INTRODUCTION

The Rho family of small GTPases comprises 22 members, some of which show over 90% amino acid sequence identity (van Helden et al., 2012). RhoGTPases control the actin and microtubule cytoskeleton in addition to activating various protein kinases and regulating gene transcription (Tybulewicz and Henderson, 2009; Heasman and Ridley, 2008; Cernuda-Morollon and Ridley, 2006; Hall, 1998). It is imperative that such diversity in functions requires tight spatio-temporal regulation. Like many other small GTPases, RhoGTPases are activated upon binding to GTP, a step which is catalyzed by guanine nucleotide exchange factors (GEFs), a group of over 80 different members (Bos et al., 2007). GEF-mediated activation of RhoGTPases is accompanied by a conformational switch that allows interaction of the GTP-bound GTPase with a selection of effector proteins (Bishop and Hall, 2000). These effectors represent a large variety of cytoskeletal regulators (e.g. mDia), kinases (e.g. ROCK, PAK), enzymes such as the NADPH oxidase and adapter proteins, such as IQGAP. Binding to the activated GTPases promotes effector unfolding, translocation and/or activation, thus transmitting the signal down the pathway. Inactivation of Rho GTPases, in turn, requires GTP hydrolysis, an intrinsic activity that is promoted by so-called GTPase-activating proteins (GAPs) (Bos et al., 2007). Like the RhoGEFs, the RhoGAPs also represent a large (>80 members) and diverse group of regulators, of which only a few, such as bcr or NF1, have been characterized in more detail.

Like many other GTP-binding proteins, RhoGTPases comprise a core G-domain and a hypervariable domain at their C-terminus (van Hennik et al., 2003). The remarkably high level of sequence homology between RhoGTPases occurs within the G-domain including the so-called effector domain in the N-terminus. This is the region that shows the largest conformational change upon activation and is generally accepted to mediate the interaction with effector proteins. As a consequence, some of the most highly related GTPases share effectors (Bishop and Hall, 2000). Yet, clear phenotypic differences are induced upon expression of activated GTPase mutants. A most striking example concerns Rac1 and Rac3 which show 100% sequence identity in the effector domain, but do induce opposite phenotypes upon expression in neuronal cells. These phenotypic differences could be directly linked to the GTPase-specific hypervariable C-terminus, as deduced from exchange experiments and the expression of chimeric proteins (Hajdo-Milasinovic et al., 2007).

The hypervariable C-terminus of RhoGTPases also encodes the CAAX box (C, Cysteine, A, aliphatic amino acid, X, any amino acid) at the very C-terminus of the protein. The CAAX box is post-translationally modified by the attachment of a lipid to the cysteine residue and removal of the last three amino acids, followed by methylation (Der and Cox, 1991; Cox and Der, 1992; Roberts et al., 2008). This lipid plays a key role in binding of most, but not all, inactive GTPases to the cytosolic chaperone RhoGDI, as well as in the binding of the activated RhoGTPases to vesicles or the plasma membrane (Boulter et al., 2010; Garcia-Mata et al., 2011; Roberts et al., 2008). Classically, subcellular localization of RhoGTPases has been claimed to be mediated by the lipid anchor in conjunction with a basic region that mediates charge-dependent association to negatively charged membranes or membrane domains. However, Tolias et al. were among the first to show that the hypervariable C-terminus of Rac1 can also mediate protein-protein interactions (Tolias et al., 1995; Tolias et al., 1998). Our group has performed a series of studies...
investigating this issue in more detail. The original hypothesis was that the hypervariable region would regulate specific binding of Rac1 to targeting proteins, certifying localized signaling. Whereas we could confirm this targeting function for the GEF β-PIX (Klooster et al., 2006), other proteins binding to the Rac1 hypervariable region, such as Caveolin1 and PACSIN2, play a role in Rac1 inactivation and ubiquitylation, rather than subcellular targeting (Nethe et al., 2010;de Kreuk et al., 2011).

This suggests that the role of the Rac1 hypervariable region is more complex than originally anticipated. We therefore analyzed the role of this domain in Rac1 localization, activation and signaling in more detail. Using mutations in the hypervariable region in the context of the full-length Rac1 protein, we show that mutating limited portions of this region did not impair the targeting of activated Rac1 mutants to Focal Adhesions (FAs) or the binding to the Rac1 effector, IQGAP1 (Fukata et al., 2002;Watanabe et al., 2004;Noritake et al., 2005). However, these mutations did affect a part of the downstream signaling of Rac1, as cells expressing these Rac1 proteins showed impaired cell spreading. We also generated a Rac1 version in which a normal hypervariable region was positioned away from the G-domain as a result of an insertion of GFP. This Rac1 variant did no longer localize to FAs and also showed impaired spreading. Together, these data suggest that the position and sequence of the hypervariable region in Rac1 plays an important role in the Rac1 targeting, the interaction with effector proteins, as well as the downstream signaling.

MATERIALS AND METHODS

Antibodies and reagents
Antibodies against the following proteins were used: Anti-SET/I2PP2A (SC-25564, Santa Cruz Biotechnology), GFP (632381, Clontech), vinculin(V9131, Sigma), tubulin (T6199, Sigma), Rac1 (Transduction laboratories), IQGAP (Millipore), mouse anti-HA, rabbit anti-HA and rabbit anti-myc (all from Sigma-Aldrich), mouse anti-myc (Invitrogen), F-Actin was detected using Texas-Red- or Alexa-Fluor-633-labeled phalloidin (Invitrogen). Secondary Alexa-Fluor-labeled antibodies for immunofluorescence were from Invitrogen.

Cell culture and transfection
HeLa cells were maintained at 37°C and 5% CO2 in Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco) enriched with 10% heat inactivated Fetal Calf Serum (FCS, Life Technologies, Breda, The Netherlands) and 100 U/mL penicillin and streptomycin. Cells were passaged by trypsinization. HeLa cells were transfected using TransIT®-LT1 Transfection Reagent (Mirus Bio) according to the manufacturers’ recommendations (ratio TransIT:DNA = 3:1), and allowed to express protein for at least 24 hours.

Expression constructs
Constructs encoding GFP and GFP or mCherry fusions of Rac1 and Rac1 mutants have been described previously (de Kreuk et al., 2011). The Trio D1 construct used to induce Rac1 activation has been described previously (van Rijssel J. et al., 2012). For construction of myc-tagged Rac1Q61 GFP-C-term, eGFP was amplified by pcr from the clontech c1 backbone
using the following forward primer: 5’-GAGATCGGATCCGTGAGCAAGGGCGAGGAGC-3’ and the following reverse primer: 5’-CTACAAATGTGGTATGGC-3’. The N-terminal portion of myc- or HA-tagged Rac1 (myc-tagged wild type and HA-tagged Q61 and V12 versions, all in a pcDNA 3.1 vector) was amplified using the T7 forward primer and the following reverse primer: 5’- GAGATCGGATCCGACTGCTCGGATCGCTTCG-3’. The Rac1 C-terminus was amplified using the following forward primer: 5’-GAGATCCTCGAGCTCTCTGCCCGCCTCCCGTG-3’ and the following reverse primer: 5’- TAGAAGGCACAGTGCGAGG-3’. The C-terminal RKR mutation was introduced using the following reverse primer: 5’-GAGATCCTCGAGTTACAACAGGCATTTTGCCGCCGTCTTAC-3’. The PPP mutation was introduced using the following reverse primer 5’-GAGATCCTCGAGTTACAACAGCGATTTTCTCTTCCTCTCTCACGGCAGCCGCGA-3’. All fusion constructs were confirmed by sequencing.

**Confocal laser scanning microscopy**

Cells were seeded onto fibronectin-coated glass coverslips, transfected with the indicated plasmids and after 24 hours fixed with 3.7% formaldehyde (Merck) in PBS for 10 minutes and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. Coverslips were then incubated for 15 minutes with 2% BSA at 37°C. Immunostainings were performed at room temperature for 1 hour with the indicated antibodies. Fluorescent imaging was performed with a confocal laser-scanning microscope (LSM510/Meta; Carl Zeiss MicroImaging) using a 63× NA 1.40 or a 40× NA 1.30 oil lens (Carl Zeiss MicroImaging). Image acquisition was performed with Zen 2009 software (Carl Zeiss MicroImaging). For live-cell imaging, cells were seeded on fibronectin-coated glass coverslips, transfected with the indicated plasmids. After 24 hours, fluorescent imaging was performed. Figures were assembled using Adobe Photoshop CS5.

**Rac1GTP pull-down assay**

HeLa cells transiently transfected with the indicated constructs (24 hours) were washed twice with ice-cold phosphate-buffered saline (PBS; Fresenius Kabi’s Hertogenbosch, The Netherlands) (supplemented with 1 mM CaCl2 and 0.5 mM MgCl2) and lysed in NP-40 lysis buffer (i.e. 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl2, 10% glycerol and 1% NP-40) supplemented with protease inhibitors (Complete mini EDTA, Roche, Mannheim, Germany), centrifuged at 20,000 g for 10 minutes at 4°C. To determine Rac1 activity, we used a CRIB-peptide based pull-down as described previously (Price et al., 2003). Shortly, the cell lysates were incubated with a biotinylated Pak1-CRIB peptide (20 µg) in the presence of streptavidin-coated beads at 4°C for 1 hour with rotation. Initial lysates and bound Rac1GTP were analyzed by SDS-PAGE and immunoblotting using specific monoclonal antibodies and HRP-coupled secondary antibodies.

**Immunoprecipitation**

HeLa cells were transiently transfected with the indicated myc or HA-tagged constructs and maintained for 24 hours. After lysing the cells as described under “Rac1GTP pull-down assay”, the lysates were then incubated with monoclonal anti-HA or anti-myc antibodies and protein G-agarose beads at 4°C for 2 hours. Samples were then washed 5 times with lysis buffer. Immunoprecipitated proteins were eluted by adding SDS sample buffer and heating at 95°C for 10 minutes. Protein association was assayed by SDS-PAGE and immunoblotting.
using mouse anti-IQGAP antibodies, rabbit anti-HA and rabbit anti-myc antibodies and HRP-coupled secondary antibodies.

**Electrical resistance measurements**

For ECIS (Electrical Cell-substrate Impedance Sensing)-based cell spreading experiments, gold ECIS electrodes (8W10E, Applied Biophysics) were coated with 10 µg/ml fibronectin in 0.9% NaCl for 1 hour at 37°C. Next, HeLa cells were seeded at a concentration of 100,000 cells per well in 400 µl IMDM with 10% FCS. Impedance was recorded continuously at 40 kHz using ECIS model Zθ (theta). The increase in cell spreading was measured as a function of impedance.

**RESULTS**

The hypervariable C-terminus of Rac1 is classically considered the key targeting domain controlling localized, Rac1-specific, signaling. We previously identified a series of Rac1 binding partners that associate to Rac1 through its C-terminus and showed differential requirements for the proline-rich portion versus the polybasic region. These studies were largely based on the use of small peptides, encoding this domain or mutated variants thereof. In this study, we analyzed the effects of mutating the Rac1 hypervariable domain in the context of full-length, constitutively activated Rac1. In addition, we generated a Rac1 mutant in which a normal, not mutated C-terminal domain was separated from the N-terminal part of the protein by insertion of a GFP-encoding sequence. The key Rac1 mutants used in this study are schematically depicted in Figure 1.

The first series of experiments were aimed at defining the localization of the different Rac1 mutants in HeLa cells. Previously, we and others showed that activated, but not wild-type (WT) Rac1 localized to FAs (Klooster et al., 2006; Chang et al., 2007; Chang et al., 2011; Nethe et al., 2010). We here compared the widely used Rac1Q61 mutant and the Rac1V12 mutant for their localization to FAs. Using vinculin as a bone fide FA marker, the results in Figure 2 show that both activated Rac1 localized to FAs. Since WT Rac1 does not localize to these structures, this suggests that activation of Rac1 is a central aspect of its subcellular targeting.

**Figure 1. Schematic overview of the various Rac1 mutants used in this study.** Mutations are all made in the context of the full length Rac1 WT of Rac1 Q61 proteins. Mutants include Rac1 proteins in which: the three consecutive prolines in the hypervariable region are changed into alanines; three basic residues (RKR) from the polybasic region are changed into alanines; a GFP-encoding sequence is inserted in between the core G-domain and the unmutated hypervariable region.
In a previous study, we showed that mutating the C-terminal cysteine residue blocks membrane localization of Rac1 and unmasks a nuclear localization signal present in the polybasic region in the C-terminus (Lanning et al., 2004). We next tested whether mutating the two regions in the Rac1 C-terminus upstream of the CAAX box, i.e. the polybasic region and the proline-rich region (van Hennik et al., 2003; Saci et al., 2011), would affect Rac1 targeting to FAs. These mutations were analyzed in the context of full-length, active Rac1Q61 tagged at the N-terminus by mCherry. Unexpectedly, mutating part of the polybasic region to alanines (Rac1Q61 RKR, Figure 1, Figure 3A) did not impair targeting of activated Rac1 to FAs. Similarly, mutation of the three consecutive proline residues to alanines (Rac1Q61 PPP; Figure 3B) also did not impair targeting of activated Rac1 to FAs. This data is surprising, since all protein-protein interactions, we and others previously identified, that involve the Rac1 C-terminus, require the RKR sequence (Nethe et al., 2010; de Kreuk et al., 2011; de Kreuk et al., 2011; van Hennik et al., 2003; van Duijn et al., 2010; Saci et al., 2011; Modha et al., 2008), whereas some also require the proline-rich region. The latter involve interactions through SH3 domains, such as for β-PIX, PACSIN2 and CD2AP (Klooster et al., 2006; de Kreuk et al., 2011; van Duijn et al., 2010).

Figure 2. Localization of activated Rac1 mutants. HeLa cells were transfected with constructs encoding mCherryRac1Q61 (panel A) or HA-tagged Rac1V12 (panel B) proteins (in red). After 24 hrs, cells were fixed and immunostained for the Rac1 mutants and for vinculin (green), as a marker for Focal Adhesions (FA) and Focal Contacts (FC). Both Rac1 activated mutants localized to peripheral FC. Rac1 activity in the transfected cells prevented formation of large, intracellular FA.
Figure 3. Localization of activated Rac1 C-terminal mutants. HeLa cells were transfected with constructs encoding activated mCherry Rac1Q61 proteins that carried mutations in the hypervariable region. (A) Two examples of the Rac1Q61 RKR mutant protein (red) are shown. This mutant of Rac1 does not induce a typical Rac1 phenotype characterized by extensive cells spreading (e.g. compare to Figure 2), but does localize to peripheral focal contacts and more centrally localized focal adhesions, identified by vinculin staining (green). (B) The Rac1Q61 PPP mutant protein induces a moderate spreading phenotype and, like the RKR mutant in (A), localizes to peripheral FC and more centrally localized FA.
The Rac1 hypervariable C-terminus has originally not been considered an integral part of the overall 3D structure of Rac1, which is largely governed by the folding of the so-called G-domain (Schweins and Wittinghofer, 1994; Wittinghofer and Vetter, 2011). NMR-based analysis, however, has suggested that some of the residues in the Rac1 C-terminus, in particular the RKR sequence, can interact with Pro73 and Gln74 in the Rac1 Switch 2 region (Modha et al., 2008). In addition, these authors showed that the Rac1 C-terminus can interact with the Rac1 effector Protein kinase C-Related kinase 1 (PRK1). This suggests that the hypervariable domain does form an integral part of the overall Rac1 structure.

To test this in more detail, we constructed an activated Rac1Q61 mutant that has the sequence encoding GFP inserted just before the hypervariable C-terminus (Rac1Q61 GFP-C-term), separating the hypervariable region from the core G-domain. No other mutations were introduced in this construct. In Figure 4A/B, we compared the FA targeting of the Rac1Q61 protein with the localization of the Rac1Q61 GFP-C-term protein. Most notably, the Rac1Q61 GFP-C-term protein was not observed in FAs. The protein localized clearly to the nucleus, but this was also observed for the other Rac1Q61 variants. In addition, part of the protein was localized in the cytosol. The Rac1Q61 GFP-C-term protein showed a tendency to associate to actin cables, in particular at the cell cortex, which is unusual for activated Rac1, which is normally found in FAs. Together, these findings suggest that the positioning of the Rac1 C-terminus relative to the core G-domain is relevant for proper intracellular targeting to FAs.

RhoGTPase activation requires GEFs that are usually equipped with membrane-anchoring domains, such as PH domains (Bos et al., 2007). Specific RhoGTPase signaling may well be governed by local activation by one or more GTPase-specific GEFs. To test if the hypervariable C-terminus plays a role in the activation of Rac1, we expressed a series of C-terminal mutants in the context of fluorescent protein (FP)-tagged, Rac1WT and examined whether these could be efficiently activated by a co-transfected GEF. For this we used a fragment of the large RhoGEF Trio, encoding the first GEF domain of Trio, i.e. TrioD1 (van Rijssel J. et al., 2012). We then detected Rac1GTP levels using a peptide encoding the PAK CRIB domain (Price et al., 2003). These experiments showed that TrioD1 efficiently activated FP-tagged Rac1 WT, inducing an up to 10-fold increase in GTP-Rac1 (Figure 5A). Mutating the Rac1 C-terminus either at the proline-rich region or the polybasic region had little effect on the efficiency of Trio D1-mediated activation. However, the construct in which the C-terminus was separated from the G-domain by the GFP insertion, was much less efficiently activated. There were some differences in expression level of the various mutants, but these did not explain differences in activation. In addition, co-expression with Trio D1 increased expression of the Rac1WT PPP and Rac1WT RKR mutants, but this increase was corrected for in the quantization of these experiments (quantification in Figure 5B).

To test the extent to which binding to an established Rac1 effector, IQGAPI, was dependent on an intact C-terminus, we performed a series of immunoprecipitation experiments using various Rac1 mutants and detected their association to endogenous IQGAPI. Figure 6A shows that activated Rac1V12, but not Rac1WT, associated efficiently with IQGAPI. Interestingly, a Rac1V12 PBQR mutant, in which all 6 basic residues in the Rac1 C-terminus are replaced by Gln residues, did not associate to IQGAPI. We analyzed this association in more detail by expressing the Rac1V12 PPP and Rac1V12 RKR mutants. These experiments showed that these mutations
Figure 4. Localization of activated Rac1 requires proper positioning of the C-terminus. HeLa cells were transfected with constructs encoding activated mCherry Rac1Q61 protein (A) and the Rac1Q61 GFP-C-term mutant (B), in which a GFP moiety is inserted in between the G-domain and the hypervariable C-terminal region. This Rac1 version, does no longer localize to FA, as the Rac1 Q61 version does (A). Moreover, the Rac1Q61 GFP-C-term mutant shows association to actin cables, in particular at the periphery of the cell (zoomed images in panel B).
Finally, we used a series of Rac1Q61 mutants in which each individual amino acid of the hypervariable domain was mutated separately to alanine (Figure 6C). In line with the data in Figure 6B, these mutants all associated efficiently to endogenous IQGAP1. As a control, we show that the inactive Rac1N17 mutant does not interact with IQGAP1. Of note, separate experiments using a biotinylated peptide encoding the Rac1 hypervariable domain showed that this peptide does not bind endogenous IQGAP1 (data not shown). Together, these data suggest that the entire Rac1 C-terminal domain is required, in conjunction with the conformation of the activated Rac1 G-domain, for binding to the effector IQGAP1.

Finally, we tested the effects of the C-terminal mutations on Rac1-induced cell spreading. This was analyzed in a quantitative fashion using ECIS (Nethe et al., 2010; de Kreuk et al., 2011). The data in Figure 7A show that, as expected, expression of GFP-tagged Rac1Q61 promoted cell spreading as compared to the GFP-transfected controls. Expression of the various Rac1 C-terminal mutants (Figure 7B) did, to different extents, impair cell spreading. The PPP and RKR mutations in the context of the Rac1Q61 protein significantly impaired cell spreading and also the Rac1Q61-GFP-C-term protein was not as efficient in inducing cell spreading as compared to the Rac1Q61 protein. These data show that the induction of cell spreading requires an intact Rac1 hypervariable region, which likely needs to be properly positioned relative to the core G-domain.
The ruffling behavior of GFP-C-term cells was analysed, using the method described in Chapter 5. There is no significant difference in number (#) and frequency of the ruffles, but the speed is slightly higher and length shorter in the GFP-C-term mutant. This indicated that ruffles start at the same moment, but they do not persist, which is consistent with the finding that Rac1 activity is highest in this phase of the ruffle (Machacek et al., 2009).

DISCUSSION

The present analysis shows that the hypervariable C-terminus of Rac1 plays a complicated role in Rac1 function. Both the sequence as well as the position of this hypervariable region controls, in various ways and to different extent, the localization, activation and output of Rac1.
Rho GTPases harbor a post-translationally added C-terminal lipid anchor, which is, in inactive GTPases, important for the binding to the cytosolic chaperone RhoGDI (Hall, 1992; Olofsson, 1999). For active GTPases, the same lipid anchor mediates membrane association, which is essential for proper downstream signaling and likely also for efficient GEF-dependent activation. These lipid anchors come in different forms (e.g. geranyl-geranyl; palmitoyl, etc) that do confer some specificity to GTPase signaling as these lipids direct the association to some, but not all membranes or membrane domains (Hancock, 2003; Hancock and Parton, 2005). The same is true for constitutively membrane-associated GTPases such as those of the Ras family, that have two cysteine residues that are lipid modified. Most recently, also for Rac1 a second cysteine at position 178 was shown to be targeted for lipidation, in this case by palmitate (Navarro-Lerida et al., 2012). This modification did not affect GDI binding, but was found to be required for efficient Rac1-GTP loading and for consequent cell spreading and migration. In addition, a Rac1-mutant that was palmitoylation-deficient showed a reduced portioning into so-called liquid-ordered, cholesterol-rich membrane domains. These are the domains to which activated Rac1 translocates to following stimulation of cells with growth factors or adhesion to matrix proteins (del Pozo et al., 2004; del Pozo et al., 2000; del Pozo et al., 2002).

Although lipid anchor-controlled GTPase association to membrane subsets may well drive signaling specificity, the fact that the C-terminus of different, highly homologous RhoGTPases, shows such high sequence variability has generally been assumed to be a key aspect that is instrumental in driving protein association-mediated specific downstream signaling. Work from our lab and others has identified an extensive range of proteins that associate to the Rac1 hypervariable domain. The original hypothesis was that such proteins would be instrumental for the subcellular targeting of Rac1. One of the most discrete localizations of activated Rac1 is

Figure 7. Mutations in the Rac1 hypervariable C-terminus impair Rac1-induced spreading. HeLa cells were transfected with GFP or the indicated Rac1 mutants and were seeded, 24 hrs after transfection, on gold ECIS electrodes for quantitative analysis of cell spreading. (A) Cells expressing empty GFP (black), GFP-Rac1Q61 (red) or Rac1Q61 GFP-C-term (blue) show that whereas activated Rac1Q61 promotes cell spreading, the mutant carrying the GFP insertion show reduced cell spreading within the first 0.5 - 4.5 hrs after seeding. (B) Cells expressing GFP-Rac1Q61 (black), GFP-Rac1Q61 PPP (red), GFP-Rac1Q61 RKR (green) or Rac1Q61 GFP-C-term (blue) show that all mutants of the Rac1 C-terminus impair cell spreading.
at FAs (Nethe et al., 2010), where integrins mediate the adhesion of the cell to the underlying extracellular matrix. Integrin activation induces Rac1 activation, so the targeting of Rac1 appears functionally relevant (del Pozo et al., 2000; del Pozo et al., 2002). Moreover, we found the FA-associated Rac1-GEF β-PIX to mediate targeting of Rac1, at least towards the plasma membrane (Klooster et al., 2006). However, our current analysis shows that relatively large mutations within the Rac1 C-terminus (3 out of 10 amino acids), in the context of full-length activated Rac1, have little effect on the targeting of the protein to FAs. This includes mutations in the proline-rich region, which is required for binding of Rac1 to β-PIX. It is important to underscore in this context that activated GTPases are not supposed to bind GEFs, suggesting that the targeting of Rac1 by β-PIX is particularly relevant prior to Rac1 activation (Etienne-Manneville and Hall, 2002; Bos et al., 2007). These data further suggests that the Rac1 C-terminus only plays a limited role in subcellular targeting to these sites. Since wild-type, not activated Rac1, does not associate to FAs, it appears the activity as such and/or the location of the GEFs is a more critical determinant for the localization of activated Rac1.

Also the activated Rac1 mutant with a RKR-AAA mutation in the hypervariable region did not show impaired localization of Rac1 to FAs. This is surprising, since this sequence is, based on the peptide-binding assays that we have performed, required for the association of Rac1 to most, if not all, C-terminus-interacting proteins. This would suggest that these proteins do not serve as Rac1 targeting proteins. Similarly, this would also indicate that the hypervariable C-terminus does not serve a major role in Rac1 targeting. To address this issue, we mispositioned the normal, unmutated Rac1 C-terminus, by insertion of a GFP-encoding sequence in the background of both wild-type and activated Rac1 mutants. This configuration resembles that of the so-called Raichu probes that have been widely used for the analysis of Rac1 activation in live cells (Itoh et al., 2002; Nakamura et al., 2006). Surprisingly, this Rac1Q61-GFP-C-term
protein did not localize to FAs, indicating that activity of Rac1 per se is not sufficient for Rac1 localization, but that the relative position of the hypervariable domain is important. In addition, a wild-type version of Rac1 was found to be less efficiently activated by the GEF Trio. The PPP and RKR mutants of wild type Rac1 were activated normally. This data further underscores the relevance of the positioning of the C-terminus for GEF-mediated activation and also shows that a normal Rac1 hypervariable region is, unlike the corresponding region of Ras, insufficient for proper intracellular targeting.

The various activated Rac1 mutants that we used in this study all showed reduced induction of cell spreading. This was also found for the PPP and RKR mutants that showed normal FA targeting. These results also separate localization, e.g. at FAs, from the full induction of a phenotype, in this case cell spreading. This may be, to a limited extent, due to effector binding. We addressed this, analyzing the binding activity of the mutants to endogenous IQGAP1 as a proof of principle. IQGAP1 binds very efficiently and selectively to activated Rac1. The PPP and RKR mutants of activated Rac1 bound to IQGAP1 efficiently, but when a PBRQ mutant was used, in which all basic residues in the C-terminus are mutated to Gln residues, IQGAP1 binding was lost. The Rac1 C-terminal peptide does not bind to IQGAP1, which shows that the hypervariable C-terminus is required, but not sufficient, for IQGAP1 binding.

Together, these data suggest that the hypervariable C-terminus of Rac1 is required for efficient effector binding and signaling. The position of the C-terminus relative to the core G-domain is important for proper localization of Rac1, even in a constitutively activated Rac1 protein. This is in agreement with the findings data of Modha et al., (Modha et al., 2008), who previously proposed that the hypervariable C-terminus of Rac1 can contact residues in the switch II region, which shows conformational change upon GTP loading of Rac1. Therefore, this study uncovers an unsuspected role of the Rac1 hypervariable region, acting in concert with the G-domain to mediate the activation, the FA-targeting and the downstream signaling of Rac1.
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