Rac1 meets ubiquitin: New insights in Rac1 signalling
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Chapter 3

Rac1 recruits Caveolin-1 to Focal Adhesions

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Summary
Directional cell migration is dependent on the spatiotemporal control of intracellular signaling events. These events regulate polarised actin dynamics, resulting in protrusion at the front of the cell and contraction at the rear. The actin cytoskeleton is regulated through signaling by Rho-like GTPases such as RhoA, which stimulates myosin-based contractility, and CDC42 and Rac1, which promote actin polymerization and protrusion. Here we show that Rac1 binds the adapter protein Caveolin-1 (Cav1). This interaction is mediated by the Rac1 hypervariable C-terminus and the scaffolding domain of Cav1. We show that in resting, polarised cells, Cav1 primarily localizes to the rear of the cell, with a small fraction trafficking from the leading edge towards the cell center. We find that Rac1 activation regulates this distribution and stimulates the accumulation of Cav1 in leading edge focal adhesions (FAs). This was observed following ectopic expression of an activated Rac1 protein, as well as following activation of endogenous Rac1 either with the cytotoxic necrotizing factor1 or after seeding of the cells on fibronectin. Finally, we show that the Rac1-Cav1 association is stimulated by cell adhesion. In conclusion, these data show that Rac1 activation recruits Cav1 to leading edge focal adhesions, suggesting that Cav1 localisation to FAs serves to promote cell spreading and migration.

Introduction
Directional cell motility is dependent on the spatiotemporal regulation of intracellular signaling events. These control cytoskeletal dynamics providing both driving force at the front of the cell and contraction at the rear. The actin cytoskeleton is central to these processes and is regulated through signaling by Rho-like small GTPases such as RhoA, which stimulates myosin-based contractility, and CDC42 and Rac1, which promote actin polymerization and cell protrusion (Ridley et al., 2003; Vicente-Manzanares et al., 2005).

Like most other small GTPases, Rho family members act as molecular switches, cycling between an active, GTP-bound state and an inactive, GDP-bound
state. These transitions are stimulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively (Bernards and Settleman, 2004; Bos et al., 2007; Rossman et al., 2005). Additionally, RhoGTPases cycle between the plasma membrane and the cytosol; in the cytosol they are bound to RhoGDI (guanine nucleotide dissociation inhibitor) (DerMardirossian and Bokoch, 2005; Dovas and Couchman, 2005). GEF-mediated GTPase activation and dissociation from RhoGDI takes place at cellular membranes, as most GEFs associate with membranes through PH-domain-mediated binding to phosphatidylinositol lipids (Bos et al., 2007; Rossman et al., 2005).

Recently, we and others have identified a series of regulatory proteins that associate with the hypervariable C-terminus of the Rac1 GTPase (Modha et al., 2008; ten Klooster et al., 2006; ten Klooster et al., 2007; van Hennik et al., 2003). With the exception of the effector kinase PRK (Protein kinase C-related kinase 1) (Modha et al., 2008), these proteins act as regulators, rather than as effectors, which usually associate to the Rac1 effector domain once Rac1 is activated. We have shown that the Rac1GEF β-PIX recruits Rac1 to focal adhesions (ten Klooster et al., 2006), probably prior to Rac1 activation at these sites. Another novel Rac1 interactor, the nuclear oncogene and PP2A inhibitor SET/I2PP2A, translocates from the nucleus to the cytosol upon Rac1 activation, cooperating with Rac1 in the control of cell motility (ten Klooster et al., 2007).

Here we show that the membrane-associated adapter protein caveolin-1 (Cav1) associates to Rac1, though the Rac1 hypervariable C-terminus. In Cav1-deficient fibroblasts, Cav1 has previously been implicated in Rac1 signaling as a regulator of internalization of membrane domains (i.e. rafts) (del Pozo et al., 2004). This has been linked to the control of integrin signalling and proposed to negatively regulate Rac1 (del Pozo et al., 2005). More recently, Cav1 was shown to negatively affect Rac1 and CDC42 while stimulating RhoA as part of a pathway regulating cell polarity (Grande-Garcia et al., 2007). In endothelial cells, Cav1 has also been suggested to negatively regulate Rac1 signaling (Gonzalez et al., 2004). Cav1 localizes to the rear of polarized cells and its phosphorylation on Tyr14 has been suggested to regulate focal adhesion dynamics (Goetz et al., 2008; Gonzalez et al.,

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The binding of Cav1 to Rac1 is independent of the phosphorylation of Cav1. Interestingly, Rac1 activation recruits endogenous Cav1 to peripheral focal adhesions, co-localizing with endogenous Rac1, paxillin and β1-integrins. Finally, the Rac1-Cav1 association is promoted by cell adhesion to fibronectin, suggestive for a regulatory role by integrins.

Results

The Caveolin-1 scaffolding domain associates to the C-terminus of Rac1

We recently identified Caveolin-1 (Cav1) as a Rac1-binding protein in the course of our studies on the ICAM-1-filamin-Cav1 complex (Kanters et al., 2008). This finding is in agreement with an earlier study (Zuluaga et al., 2007) that showed Rac1 binding to immunoprecipitated Cav1 in cardiomyocytes. Using biotinylated peptides, we found that endogenous Cav1, but not Cav2, binds to the Rac1 C-terminus, but does not associate to amino acids 17-32, a part of the Rac1 effector domain (Fig. 1A) (Vastrik et al., 1999). Peptides encoding the C-termini of RhoA and RhoC also bound to Cav1, complementing published data (Gingras et al., 1998; Lin et al., 2005), but the C-termini of CDC42, RhoG, Rac2 and RhoB did not (Fig. 1A). Bacterially-purified, full-length GST-Rac1, but not GST-Rac1ΔC, which lacks the Rac1 C-terminus, bound endogenous Cav1 (Fig. 1B), indicating that the Rac1 C-terminus is both necessary and sufficient for Cav1 binding.

Tyrosine phosphorylation of Cav1 on Tyr-14 (pYCav1) has been implicated in Cav1-mediated signalling as well as in its internalisation (del Pozo et al., 2005). Levels of tyrosine-phosphorylated Cav1 in cells are in the order of 1% only and we therefore induced higher levels of pYCav1 by treatment of cells with pervanadate (Grande-Garcia et al., 2007). This pYCav1, detected with a pY14Cav1-specific antibody, associated to the C-terminus of Rac1 (Fig. 1C), indicating that Cav1 phosphorylation does not interfere with its interaction with Rac1. Conversely, a mutant of Cav1, Y14FCav1 that cannot be phosphorylated on residue 14, also associated to the Rac1-C-terminus (Fig. 1D). Together, these data suggest that
tyrosine phosphorylation of Cav1 does not play a regulatory role in the interaction with Rac1.

The Rac1 C-terminal domain comprises two binding sites for protein-protein interactions (van Hennik et al., 2003). Use of mutant peptides showed that both the proline-stretch and the polybasic region are required for Rac1 binding to Cav1 (Fig. 1E,F). In this experiment, we used I1PP2A (inhibitor 1 of PP2A, (ten Klooster et al., 2007) as a control for binding to the Rac1 C-terminal peptide in which the prolines were mutated. The relevance of this sequence for Cav1 binding was further supported by the reduced binding of Cav1 to the C-terminus of Rac3, a closely related homologue of Rac1, of which the C-terminus also encodes three
consecutive proline residues but differs by two amino acids in the polybasic region (Fig. 1E,G). In agreement with the data that showed Rac1 binding to endogenous Cav1 (Fig. 1B), GST-Cav1, but not GST alone, bound to endogenous Rac1 (Fig. 1H). Many of the protein-protein interactions described for Cav1, which binds to a series of signalling proteins (Razani et al., 2002), are mediated by the ‘Caveolin Scaffolding Domain’ (CSD), encoded by amino acids 82-101. This sequence is divergent between Cav1 and Cav2 (Razani et al., 2002). Use of cavtratin, a cell-permeable peptide encoding the Cav1 CSD, showed that this region is sufficient to mediate the Cav1–Rac1 interaction (Fig. 1I). Conversely, the association of a myc-tagged version of Cav1 lacking the CSD (myc-Cav1 Δ80-100) showed reduced binding to the Rac1 C-terminal domain (Fig. 1J). The observed residual binding could be due to additional regions, outside the scaffolding domain of Cav1, contributing to the Cav1-Rac1 association. Together, these findings show that the Cav1 CSD is necessary and sufficient for optimal association to Rac1 and that additional regions in the Cav1 protein may further promote formation of the complex.

**Rac1 activation recruits endogenous Caveolin-1 to peripheral focal adhesions**

Detection of endogenous Rac1 has previously been hampered by the quality and the specificity of available antibodies. We recently tested a novel anti-Rac1 antibody for its specificity on Western blots and immunostainings. The antibody recognizes a single band of approximately 22 kDa on Western blots, which is strongly diminished when lysates are used from cells transfected with a Rac1 siRNA (Fig. 2A).
Figure 2. Detection and localisation of endogenous Rac1. (A) Western blot of cell lysates from control and Rac1 siRNA transfected HeLa cells was stained for Rac1, to demonstrate the specificity of the antibody. (B) Immunostainings for Rac1 of HeLa cells transfected with control siRNA or Rac1 siRNA (bar, 40 μm) show lack of detectable staining in the siRNA-treated cells. In cells in stationary culture, endogenous Rac1 localizes to membrane ruffles, the nucleus and to FAs, whereas endogenous Cav1 primarily localizes to the contractile rear of polarized cells and to a perinuclear area, partly colocalizing with a fraction of Rac1. (C) Basal and apical confocal sections of semi-confluent HeLa cells, stained with monoclonal anti-Rac1 and polyclonal anti-Cav1 antibodies to define the (co)localisation of the endogenous Rac1 and Cav1 proteins. Bar, 10 μm. (D) Analysis of GFP-Cav1-positive vesicle trafficking from the leading edge of the cell as depicted by still images (t=sec) of a zoom of a time-lapse analysis of GFP-tagged Cav1 in a polarised HeLa cell. Bar, 10 μm.

Subsequent analysis by immuno-staining in combination with confocal microscopy showed that cells, transfected with a Rac1 siRNA were negative in immunostaining, further confirming the specificity of the Rac1 detection (Fig. 2B). Endogenous Rac1 could be detected in membrane ruffles, focal adhesions (FAs) and a perinuclear compartment (Fig. 2B,C). Colocalisation of Rac1 with endogenous Cav1, which concentrates at the contractile rear end of polarised cells with little Cav1 in membrane ruffles (Fig. 2C), was limited. However, live-cell imaging of polarised cells revealed that a portion of the pool of GFP-Cav1, which, like endogenous Cav1 accumulates in the rear of the cells, translocates from the front of the cell, back towards the centre, likely on vesicles (Fig. 2D).

Interestingly, expression of an activated mutant of Rac1, Rac1Q61L, in HeLa cells induced a marked re-localisation of endogenous Cav1 to Rac1Q61L-positive, peripheral sites of adhesion (Fig. 3A). Similarly, activating endogenous
Rac1 with CNF-1 (Flatau et al., 1997; Pop et al., 2004), induced the accumulation of endogenous Cav1 at peripheral adhesions that also stained positive for the FA marker paxillin (Fig 3B,C). Immunostainings of CNF-1-treated human umbilical vein endothelial cells (HUVEC) confirmed that also in this cell type, activation of endogenous Rac1 promotes Cav1 accumulation at paxillin-positive FAs (Fig. 3D). In these adhesion structures, Cav1 colocalizes with endogenous Rac1, as well as with β1-integrin (Fig. 3B,E). Unlike Cav1, the localization of clathrin did not change upon CNF-1-mediated Rac1 activation (not shown).

Figure 3. Active Rac1 recruits Cav1 to peripheral FAs. (A) HeLa cells were transfected with myc-tagged Rac1Q61L (arrows) and stained for (co)localisation of Rac1Q61L and endogenous Cav1 (bar, 20μm). Untransfected cells are indicated with asterisks and zoomed images are included to underscore the Rac1-Cav1 colocalization at the periphery of the cells. (B) Rac1 activation in control or CNF-1-treated HeLa cells was analysed by a Pak-Crib pull-down assay (upper panel), parallel to imaging the (co)localisation of endogenous Rac1 and Cav1 in CNF-1-treated HeLa cells. Bar, 10μm. (C) (Co)localisation of endogenous Cav1 with paxillin was analysed by immunostainings in control and CNF-1-treated HeLa cells (bar, 10μm). Bar diagram shows percentage of cells in which Cav1 co-localises with paxillin-marked sites of adhesion upon stimulation with CNF-1, as analysed by Zen co-localisation software (Zeiss) (50 cells/condition; n=3). (D) Immunostainings of CNF-1 treated HUVEC cells for F-actin, endogenous Cav1 and paxillin (bar, 10μm) or (E) endogenous β1-integrin in Hela cells. Bar, 20 μm. Rac1 activation by CNF1 also recruits Cav1 to peripheral adhesions in endothelial cells, underscoring that this effect is not cell-type specific. Co-staining with β1-integrin underscores the notion that Cav1 localizes to FAs following Rac1 activation.
We did not pursue analysis of pY14Cav1 localization by immunofluorescence, as previous claims of its localization to FAs have been questioned as a result of cross-reactivity of the phospho-Cav1 antibody with phospho-paxillin (Hill et al., 2007). Alternatively, we used live-cell imaging analysis of HeLa cells cotransfected with mCherry-tagged Rac1Q61L and either GFPCav1 or GFPCavY14F. These data showed that GFPCav1Y14F is unable to colocalize with active Rac1 at sites of adhesion, suggesting that phosphorylation of Cav1 on Tyr-14 is required for Cav1 localization at FAs (data not shown). As phosphorylation on Tyr-14 of Cav1 does not appear to be required for Rac1 binding, this finding further suggests that in addition to Rac1, other proteins further promote the localization of Cav1 at FAs.

**Cav1 recruitment by Rac1 is independent of microtubules and RhoA**

Since Cav1 traffic has been suggested to be microtubule (MT) dependent (Conrad et al., 1995), cells were pre-treated with the MT-disrupting agent nocodazole prior to treatment with CNF1 to activate endogenous Rac1. In the absence of CNF1 treatment, nocodazole did not induce Cav1 re-localization to peripheral adhesions (not shown). CNF1 treatment of nocodazole-treated cells induced endogenous Cav1 to relocalize to the cellular periphery, as in untreated cells, which shows that this effect is independent of microtubules (Fig. 4).

CNF1 also mediates activation of RhoA (Schmidt et al., 1997), which, together with its downstream effector Rho-associated, coiled-coil-containing protein
kinase (ROCK), is involved in the formation of FAs (Bershadsky et al., 2006). To test whether RhoA-ROCK signaling is required for the targeting of Cav1 to peripheral FAs, we treated cells with CNF1 in the presence of the ROCK inhibitor Y27632. Under these conditions, CNF1 induced the formation of a rim of paxillin-positive, small adhesions at the periphery of the cells, with a marked loss of central FAs (Fig. 5A).

Figure 5. RhoA or CDC42 do not regulate Cav1 targeting. (A) Immunostainings for endogenous Cav1 and paxillin in HeLa cells treated with CNF-1 in the presence of the ROCK inhibitor Y27632, show that Cav1 targeting to the periphery is not inhibited by blocking ROCK. Bar, 20 μm. (B) HeLa cells were transfected with myc-tagged active G14VRhoA and stained for (co)localisation of G14VRhoA and endogenous Cav1 (bar, 20 μm). Overexpression of active RhoA correlated with an increased formation of F-actin stress fibers. Cav1 could be seen along these fibers; however, no accumulation of Cav1 at peripheral adhesions was induced. (C) HeLa cells were transfected with myc-tagged active Q61L CDC42 and stained for (co)localisation of CDC42Q61L and endogenous Cav1 (bar, 20 μm). In contrast to active Rac1, activated CDC42 does not recruit Cav1 towards the cell periphery.

However, the CNF1-induced concentration of Cav1 at these peripheral adhesions was not impaired. These data suggest that ROCK does not mediate the CNF1-induced accumulation of Cav1. Since activation of RhoA by nocodazole-induced MT depolymerization (Enomoto, 1996) also did not induce re-localization of Cav1 (not shown), the Rho-ROCK pathway appears not to be required for the targeting of Cav1 to peripheral adhesions. This is further supported by the expression of an activated mutant of RhoA(G14VRhoA) which did not induce recruitment of Cav1 to

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FAs, despite induction of abundant actin stress fibers (Fig. 5B). Similarly, expression of activated mutant of another CNF-1 target CDC42 (Q61LCDC42) also did not induce recruitment of Cav1 (Fig. 5C). These data are in good agreement with the notion that CNF-1 treatment of HeLa cells induces a Rac1, rather than a CDC42 or RhoA phenotype.

**Cell adhesion promotes Cav1 targeting to focal adhesions as well as Rac1-Cav1 association.**

Finally, we tested the effects of Rac1 activation by an endogenous stimulus. For this purpose we chose to use integrin-mediated adhesion as a Rac1-activating event, which is induced by seeding cells on fibronectin (Price et al., 1998). Also this means of activating endogenous Rac1 induced the accumulation of Cav1 at paxillin-positive peripheral FAs (Fig. 6A). Moreover, we found cell adhesion to fibronectin to promote the Rac1 C-terminal interaction with Cav1, whereas the interaction was largely absent in detached cells, kept in suspension (Fig. 6B). Thus, activation of endogenous Rac1 following cell adhesion promotes both the Rac1-Cav1 interaction and the accumulation of Cav1 at peripheral adhesions.

**Discussion**

Members of RhoGTPase family are characterized by their high sequence homology, in particular in between isoforms, such as Rac1,2 and -3. Yet, these isoforms can induce specific phenotypes in cells, indicative for differential activation of signalling
pathways (Hajdo-Milasinovic et al., 2007). Effector interactions are mediated by the so-called effector domain in the N-terminus, which surprisingly is identical in the three Rac isoforms. The most divergent region between the Rac1 proteins is the very C-terminal region just proximal to the C-terminal cysteine residue and this domain has been linked to the control of signalling specificity. The Rac1 C-terminus has been proposed to mediate Rac1 subcellular targeting, in part by its polybasic nature and by the C-terminal lipid moiety, covalently linked to the terminal cysteine (Joseph et al., 1994; ten Klooster and Hordijk, 2007). More recently, several studies from our group and others have established that the Rac1 C-terminal domain not only interacts with charged lipids in the membrane, but also selectively associates to a number of proteins involved in Rac1 signaling (Modha et al., 2008; ten Klooster et al., 2006; ten Klooster et al., 2007; van Hennik et al., 2003). We previously showed that the Rac1/CDC42 GEF β-PIX binds the Rac1 C-terminus and recruits Rac1 to leading edge FAs, one of the cellular locations of activated Rac1 (ten Klooster et al., 2006). In the current study, we show that activated Rac1, in turn, recruits endogenous Cav1 to such peripheral FAs. This was observed following ectopic expression of Rac1Q61L, as well as following activation of endogenous Rac1 in epithelial and endothelial cells, either using the CNF-1 toxin or following the induction of cell spreading.

CNF1 treatment of the cells induced a phenotype resembling that induced by activated Rac1. Yet, since CNF1 also activates RhoA, we analyzed the contribution of RhoA signalling to Cav1 accumulation at FAs. Neither the activation of endogenous RhoA by MT depolymerization nor the transfection of an activated mutant of RhoA mimicked the CNF1 or activated Rac1-induced accumulation of Cav1 at FAs. Similarly, inhibition of ROCK, an effector of activated RhoA, did not prevent Cav1 to accumulate at peripheral adhesions, despite a significant change in the distribution of these structures. Thus, RhoA-ROCK signalling does not contribute to the CNF1- or Rac1-induced translocation of Cav1. Cav1 recruitment to FAs is also independent of MTs, which suggests that the Cav1 is recruited from other regions within the plasma membrane or from the Golgi (Conrad et al., 1995). Cav1 has been suggested previously to associate to integrins (Wary et al., 1998),
which is in line with our data showing that integrin-mediated adhesion, like 
activation of Rac1 by CNF1, targets Cav1 to FAs. Moreover, cell adhesion 
stimulated the interaction between Rac1 and Cav1, suggesting that Rac1 and 
integrins co-operate in the targeting of Cav1 to FAs, where Cav1 functions in 
integrin- and Rac1-mediated signalling by regulating the internalization of specific 
membrane domains (del Pozo et al., 2004; del Pozo et al., 2005).

Several previous studies have suggested that Cav1 tyrosine phosphorylation 
localizes Cav1 to FAs. However, these findings were questioned when it was shown 
that the commonly used mouse antibody to tyrosine-phosphorylated Cav1 cross-
reacted with tyrosine-phosphorylated paxillin, which also resides in FAs (Hill et al., 
2007). For this reason, we did not pursue immunostainings for pYCav1, but rather 
stained for endogenous, total Cav1. We did not detect significant levels of tyrosine-
phosphorylated Cav1 on western blots of cell lysates, unless the cells were pre-
treated with pervanadate. This pYCav1 also associated to the Rac1 C-terminal 
domain, indicating that tyrosine phosphorylation did not interfere with Rac1 
binding. We also did not find detectable levels of pYCav1 in Rac1 C-terminal pull-
down assays (data not shown), which indicates that there is no preferential binding 
of phosphorylated Cav1 to Rac1. Similarly, a phospho-deficient mutant of Cav1 did 
bind to Rac1, but was inefficiently targeted to FAs, even in the presence of an 
activated mutant of Rac1, suggesting that Rac1 activity cooperates with a 
phosphorylation-dependent mechanism to accumulate Cav1 at FAs. Previously, 
tyrosine-phosphorylated Cav1 has been implicated in FAK stabilization, RhoA 
activation and FA turnover (Goetz et al., 2008; Joshi et al., 2008) Moreover, Cav1 
tyrosine phosphorylation has been suggested to promote membrane order in FAs 
(Gaus et al., 2006) Thus, although phosphorylation of Cav1 is not critical to Rac1 
association, it appears important for Cav1 targeting to FAs and FA stability, and as 
such does play an important role in cell spreading and cell motility.

In conclusion, our data show that Rac1 activation induces the recruitment 
of a fraction of endogenous Cav1 to leading edge FAs. Since this recruitment as well 
as the association of Cav1 to Rac1 is promoted by cell adhesion, these findings
further support the relevance of Cav1 in regulating integrin function as well as Rac1 signaling.

Materials and Methods

**Antibodies and reagents** The following antibodies were used: anti-Caveolin-1 (610493), anti-Caveolin2 (610684), anti-p(Y14)Caveolin-1 (610059), anti-Paxillin (610619) (all from BD Bioscience) anti-Rac1 (clone 23A8) and anti-beta-pix (A B3829) (both from Milli pore), anti-RhoA (sc-418) and I1PP2A (Santa Cruz), anti-p(Y14)Paxillin (44-722G) (Invitrogen), anti-GFP (JL-8; Clon tech), anti-c-myc (Zymed). F-actin was stained with rhodamine-labeled phalloidin (Invitrogen). The Rac1 inhibitors EHT1864 and NSC23766 were obtained from Sigma and Calbiochem, respectively. Confocal imaging of immunostained samples as well as live cell imaging was performed with a ZEISS LSM510 Meta system in combination with ZEISS Zen software for analysis and processing. All data are representative for at 3 or more experiments, unless otherwise indicated. Movies are representative for at least 3-10 cells, analysed individually.

**Cell culture** HeLa cells were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM; BioWhittaker) containing 10% (v/v) heat-inactivated fetal calf serum (FCS) (Bodinco), 2 mM L-glutamine and penicillin/streptomycin (all purchased from PAA Cell Culture Company). Cells were passed by trypsinization. HUVEC were obtained from Lonza and cultured in EGM2 medium (Lonza) prepared according to the manufacturer’s instructions. All cells were grown at 37°C in a humidified atmosphere containing 5% CO2.

**Cell transfection and DNA constructs** HeLa cells were transiently transfected with FuGene (Roche) as described by the manufacturer. Briefly; 1 μg DNA was mixed with 6 μl of Fugene in 100 μl of Opti-mem® I (Invitrogen) and incubated for 30 min at RT, after which the transfection mix was applied to 50% confluent cells. The following constructs were used: GFP-Cav1 (kind gift from M.A. Schwartz, University of Virginia, Charlottesville VA, USA), GFP-Cav1Y14F was constructed by site-directed mutagenesis (Stratagene). GST-Rac, GST-RacΔC (a kind gift from R. Ahmadian, European Molecular and Cell Biology Laboratory, Heidelberg, Germany; Haeusler et al., 2003); GST-Cav1 was kindly provided by Dr. Elsa Regan-Klapisz, Utrecht University, Utrecht, The Netherlands). Myc-Cav1 and myc-Cav1A80-100 constructs were kindly provided by D. Anderson (Machleidt et al., 2000). CNF1 was isolated as previously described (Schmidt et al., 1997). Cells were incubated with 500 ng/ml purified GST-CNFI toxin, as indicated.

**Design and transfection of siRNA oligos** HeLa cells (4.10e5) were passaged by trypsinisation in a 10 cm dish, neutralised with complete IMDM, and cultured o/n in Opti-mem® I medium (Invitrogen). After 18 hrs, siRNA transfection mix containing siRNA primers (87 μl of 20 μM) and Oligofectamine (87ul) was prepared in Opti-mem® I medium according to the manufacturer’s protocol (Invitrogen) and incubated for 30' at RT. During this incubation the Opti-mem® I medium on the cells was replaced for IMDM complete medium. Transfection medium was removed 24 hrs after transfection by replacing the culture medium with complete IMDM. The following chemically synthesized, double-stranded siRNAs, with 19-nt duplex RNA and 2nt 3’dTdT , purchased from Eurogentec in non-modified and desalted form, were used: Rac1 siRNA primers GAGGAAGAGAAAA UGCCU (5’-3’) and CAGGCAUUUUCUCUUCCUC (5’-3’) (Noritake et al., 2004). As a nonspecific control in this study, we used siRNA against a luciferase reporter gene: CGUACGCGGAAUCUUCGA (5’-3’) and GCAUGCGCCUUAUGAACGU (5’-3’).

**Pull-down assays** Peptide pull-down assays were performed as described previously (ten Klooster et al., 2006). In short, each assay was performed with 5 μg of indicated biotin-labelled peptide, 25 μl streptavidin-coated beads (Sigma-Aldrich) in NP-40 lysisbuffer (50mM Tris-HCl, 150mM NaCl, 10mM MgCl₂, 10% glycerol, 1% NP-40). All peptides were fused to a protein transduction domain sequence: YARAAARQARA , that was also used as a control in the pull-down experiments (van Hennik et al., 2003). GST-fusion proteins were purified from BL21 bacteria as described (ten Klooster et al., 2006) after which 100 μg of indicated GST-fusion constructs were used in each pull-down. Rac1 activation was assayed by a CRIB-peptide pull-down approach as previously described (Price et al., 2003). Mass spectrometry analysis for the identification of Cav1 was done as described (Kanet al., 2008).

**Peptide synthesis** Peptides were synthesized on a peptide synthesizer (Syro II) by Fmoc solid phase chemistry. Peptides encoded a biotinylated protein transduction domain (Biotin-YARAARQARAG) (Ho et al., 2001) followed by the 10 amino acids proceeding the CAAX domain for all used RhoGTPase peptides. The sequences of the Rac1 (P-A) and the Rac1 (RKR-AAA) mutants are: CAAAVKKRKRK
and CPPPVKAAAK, respectively. The biotinylated protein transduction domain for the Cavitratin peptide (Gratton et al., 2003) was followed by 20 amino acids corresponding to the scaffolding domain (amino acids 82-101) of Cav1: DG1WKSFTFTTVKTYFWYR.

References


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