Rac3 inhibits adhesion and differentiation of neuronal cells by modifying GIT1 downstream signaling

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Summary

Rac1 and Rac3 are highly homologous regulatory proteins that belong to the small GTPases of the Rho family. Previously, we showed that Rac3 induces cell rounding and prevents neuronal differentiation, in contrast to its close relative Rac1, which stimulates cell spreading and neuritogenesis. To explain these opposing effects, we investigated whether Rac1 and Rac3 interact with different proteins. Here, we show that both Rac1 and Rac3 interact with GIT1, a multifunctional Arf-GAP protein, which regulates cell-matrix adhesion, cell spreading and endocytosis. However, in contrast to Rac1, the Rac3-GIT1 interaction is not mediated by βPix. Interestingly, Rac3 expression severely attenuates the interaction between GIT1 and paxillin, accompanied by defective paxillin distribution, focal adhesion formation and disturbed cell spreading. Moreover, in Rac3-expressing cells, Arf6 activity is strongly reduced and the Arf6-GAP activity of GIT1 is required for Rac3 downstream signaling. Indeed, expression of wild-type Arf6 or the Arf6-GEF ARNO induced cell spreading in the otherwise rounded Rac3-expressing cells. Our data suggest that Rac3 and Rac1 oppose each other's function by differently modulating GIT1 signaling. Rac1 induces adhesion and differentiation by activating PAK1 and stimulating the GIT1-paxillin interaction, whereas Rac3 blocks this interaction and inactivates Arf6 by stimulating the GAP function of GIT1, thereby preventing cell spreading and differentiation.

Key words: Rac3, Rac1, GIT1, Paxillin, Arf6, Cell adhesion, Neurite outgrowth

Introduction

Rho GTPases are small regulatory proteins that act as molecular switches in signaling pathways upon receptor stimulation and adopt an active or inactive configuration by binding GTP or GDP, respectively. The activities of Rho GTPases are tightly regulated by guanine nucleotide exchange factors (GEFs; activators) and GTPase-activating proteins (GAPs; inactivators), which are complex multidomain proteins. GEFs stimulate the exchange of GDP for GTP, thereby activating Rho-like GTPases, whereas GTPase-activating proteins inactivate small GTPases by stimulating their intrinsic GTPase activity. In addition, there are also GDP dissociation inhibitors (GDIs) that prevent GDP dissociation.

The Rac proteins belong to the family of Rho GTPases, and in mammals they are represented by the ubiquitously expressed Rac1, the splice variant Rac1B, the hematopoietic-cell-specific Rac2 and the most recently described, Rac3, which is particularly strongly expressed in brain (Haataja et al., 1997). Of these, Rac1 is the best characterized Rac protein and has been implicated in the regulation of cytoskeletal rearrangements that lead to cell adhesion, cell spreading and migration, as well as cytoskeleton-independent processes. In neuronal cells, Rac1 regulates migration, neuritogenesis, growth cone stability and dendritic spine morphogenesis (Linseman and Loucks, 2008). Although highly homologous, with significant sequence difference only in the extreme C-terminus (Haataja et al., 1997), Rac1 and Rac3 are differentially expressed throughout tissues and during development, suggesting non-redundant functions. Rac3 is abundantly present in developing and adult brain, and mildly expressed in heart, placenta and pancreas (Haataja et al., 1997). Recent data obtained from *Rac3*$^{-/-}$ mice have shown that lack of Rac3 does not lead to any gross brain abnormalities. However, specific behavioural differences from the wild type, including superior motor coordination and learning, were observed (Corbetta et al., 2008; Corbetta et al., 2005). Rac3 gene expression is highest post natally in areas of the brain that contain projection neurons that are involved in long and complex neuronal networks, such as the hippocampus and cerebral cortex (Corbetta et al., 2005). In addition, a recently published study that identifies P-Rex1 (PREX1) as a GEF for Rac3 in neuronal cells (Waters et al., 2008), points out that it is likely that expression and activation of Rac3 takes place in a specific subset of neuronal cells and at a specific stage of neuronal development.

In spite of the high degree of homology, Rac1 and Rac3 induce strikingly opposing effects in neuronal cell morphology and differentiation (Hajdo-Milasinovic et al., 2007). Expression of Rac3 induces cell rounding, whereas Rac1 expression induces cell spreading and neuritogenesis. Similarly, depletion of Rac3 leads to cell spreading and unmitigated neuritogenesis, whereas depletion of Rac1 causes loss of cell-matrix adhesions and cell detachment. We identified the amino acid triplet just upstream of the CAAX box to be responsible for the striking functional difference between Rac1 and Rac3 (Hajdo-Milasinovic et al., 2007).

In neuronal cells, one of the most important proteins that mediate Rac1-induced cell adhesion, spreading and neuritogenesis, is G-protein-coupled receptor (GPCR) kinase interacting protein 1 (GIT1). GIT1 is a multifunctional scaffolding protein that is frequently found associated with p21-activated kinase 1 (PAK1)-interacting exchange factor (βPix; official symbol ARHG7). Together, these two proteins form multimers capable of binding and regulating various proteins (Hoefen and Berk, 2006). Rac1 participates in the GIT1-βPix-PAK1 complex, where it is presumably activated by βPix and subsequently stimulates PAK1 activation (Brown et al., 2002). PAK1 associates with the complex

via βPix, with the subsequent targeting of active PAK1 to cell-matrix adhesions where it exerts its role in formation of actin-based ruffles and lamellipodia (Zhao et al., 2000). The C-terminus of GIT1 harbors a paxillin-binding domain (PBD). Upon Rac1-PAK1 binding to GIT1-βPix, it is proposed that GIT1 adopts an open conformation that is required for GIT1-paxillin interaction (Totaro et al., 2007), which stimulates cell adhesion and spreading by recruitment of paxillin to novel, Rac1-induced focal complexes at the cell periphery (Di Cesare et al., 2000). GIT1 also harbors an Arf-GAP domain that exhibits GAP activity towards several Arf proteins, including Arf6 (Vitale et al., 2000). Arf6 has a major role in regulation of endosomal membrane trafficking and actin remodeling at the cell periphery (D'Souza-Schorey and Chavrier, 2006). GIT1 that lacks the Arf-GAP domain triggers formation of unusually enlarged recycling endosomes, implicating GIT1 as an important regulator of Arf6 activity (Di Cesare et al., 2000). Current understanding of the Arf6 cycle proposes that Arf6 is inactivated by its GAPs on the intracellular compartments, presumably on the tubulo-vesicular recycling endosome (D'Souza-Schorey and Chavrier, 2006). Since GIT1 is found predominantly in focal complexes, focal adhesions (FAs) and on membranous structures throughout the cell (Di Cesare et al., 2000; Paris et al., 2002; Zhao et al., 2000), it is likely that GIT1 exerts its Arf-GAP function on intracellular compartments. Thus, both the GIT1-paxillin interaction and the GAP function of GIT1 towards Arf6 might be of great importance for normal neuron morphology and development.

In the present study we have investigated the molecular mechanisms that mediate Rac3-induced cell rounding and inhibition of differentiation in neuronal cells. We show here that, in contrast to Rac1, Rac3 impinges on GIT1 and affects GIT1 signaling by inhibiting the GIT1-paxillin interaction and stimulating GIT1-GAP function towards Arf6, thereby preventing cell spreading and neuronal differentiation.

Results

Rac3 functionally interacts with GIT1 independently of βPix

When cultured in the presence of serum, N1E-115 neuroblastoma cells are rounded and adhere poorly. Withdrawal of serum initiates the process of differentiation, whereupon the adhesion to the surface increases, the cells spread and eventually produce neurite-like extensions. Expression of Rac1 in N1E-115 neuronal cells induces cell spreading and neurite outgrowth, whereas expression of Rac3 leads to rounded, poorly adherent cells. Concomitantly, downregulation of Rac1 initiates loss of cell-matrix adhesions and leads to cell detachment, whereas loss of Rac3 leads to outgrowth of neuronal-like protrusions (Fig. 1A) (Hajdo-Milasinovic et al., 2007). In the effort to understand these opposing functions of Rac1 and Rac3, we hypothesized that Rac1 and Rac3 could induce such different morphologies by binding to different pools of downstream effectors. We therefore performed pull-down experiments using glutathione S-transferase (GST)-tagged wild-type (wt) Rac1 or wt Rac3 and preloaded them with either GDP or GTP nucleotides to mimic the inactive and active conformation, respectively. By western blot analysis we searched for differences in binding to the proteins that have been previously implicated in regulation of cell adhesion. We analyzed the following proteins: kinases PAK1, PIP5, MAPK, DGK, PI3K (p85); Rho-GDI; adaptor proteins Crk and Nck; GEFs Tiam1, αPix and βPix; and GIT1. The majority of analyzed proteins bound equally well to either Rac1 or Rac3 (not shown), which is not unexpected considering the fact that these Rac proteins are highly homologous. However, Rac1 and Rac3 bound differently to scaffold protein GIT1 (Fig. 1B). Rac1 was found to interact with GIT1, as well as βPix, predominantly in its GDP-bound form. By contrast, Rac3 interacted with GIT1 only in the GTP-bound confirmation and independently of βPix (Fig. 1B, lanes 2, 5, 8). With respect to Rac1, our data correspond well to earlier studies that have shown that Rac1 associates with GIT1, and that this interaction depends on βPix-PAK1 participation in the complex (Daniels and Bokoch, 1999; Di Cesare et al., 2000).

Rac3 interacts with GIT1 independently of βPix (Fig. 1B, lanes 3, 6, 9). Previous studies have shown that Rac1 interacts with βPix via the proline-rich stretch in the C-terminus of Rac1, which is not present in Rac3 (Ten Klooster et al., 2006), which could explain the lack of binding of βPix. Neither αPix nor shorter isoforms of βPix were found to mediate the Rac3-GIT1 interaction (not shown), suggesting that Rac3 participates in a GIT1 complex independently of Pix proteins.

To confirm that GIT1 preferentially binds GTP-bound Rac3 (see Fig. 1B), we performed immunoprecipitation experiments in HEK293 cells expressing N-terminally FLAG-tagged wt Rac3, FLAG-tagged Rac3N17 (constitutively inactive mutant) and FLAG-tagged Rac3V12 (constitutively active mutant), using monoclonal anti-FLAG antibody for immunoprecipitation. As shown in Fig. 1C, both wt Rac3 and Rac3V12 bound to GIT1 far more efficiently than Rac3N17, supporting our conclusion that Rac3 preferentially binds GIT1 in the GTP-bound confirmation and suggesting that GIT1 is a downstream effector of Rac3. Although it seemed that Rac3V12 and wt Rac3 showed similar affinity for GIT1, careful examination of various western blots revealed that, taking into consideration the differences in total levels of expressed proteins, there is a twofold increase in GIT1 binding to V12Rac3 versus wt Rac3 (Fig. 1C). Of note, in Rac activity assays we often observed that the wt Rac3 protein showed higher affinity for the CRIB-PAK domain when compared with Rac1. However, we were not able to determine whether endogenous Rac3 was more active in normal conditions, owing to the unknown affinities of the Rac1 and Rac3-specific antibody. Taken altogether, we conclude that Rac3 interacts with GIT1 and that this interaction is stimulated by GTP and is independent of βPix.

GIT1 functions in Rac3 downstream signaling

To investigate whether GIT1 has a role in Rac3 downstream signaling, we designed GIT1-specific shRNAs that were cloned into pSuper expression vector (Brummelkamp et al., 2002) and transiently expressed in parental or Rac3-expressing N1E-115 cells. Enhanced green fluorescent protein (eGFP) was coexpressed to identify the transfected cells. Most of the cells that contained high levels of shGIT1 (as estimated by high levels of eGFP expression) lost their adhesion to the matrix and detached, regardless of whether in parental or Rac3-expressing cells (data not shown). However, moderate downregulation of GIT1 expression, which did not affect parental N1E-115-ev cell morphology, led to cell spreading in otherwise rounded and poorly adherent N1E-115–Rac3 cells (Fig. 1D). The morphologies of fluorescent cells were scored as round or as spreading or neurite bearing, and the percentages of the latter were depicted in a bar diagram (Fig. 1D). Similar results were observed when we used DharmaFECT On-targetPLUS set of four duplex siRNA sequence against mGIT1 (data not shown). In addition, to verify the efficiency and specificity of the shGIT1 and siRNA pool, we treated Myc-GIT2-expressing N1E-115 cells with either shLuc,

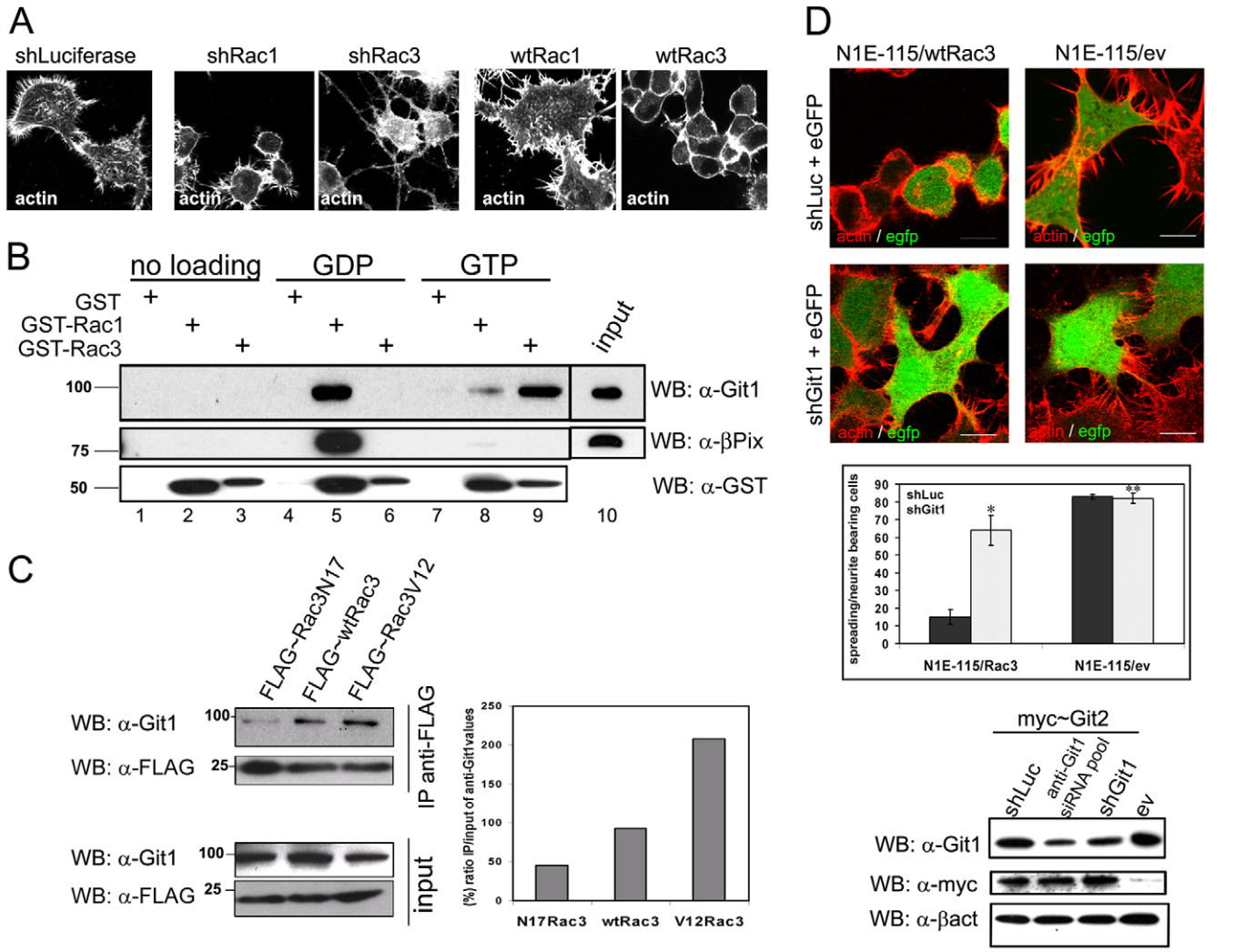

Fig. 1. Rac3 functionally interacts with GIT1 independently of βPix. (A) N1E-115 cells were transiently transfected with wt Rac1, wt Rac3, shRac1 or shRac3 (together with eGFP expression vector, ratio 10:1). 36 hours after transfection, cells were washed, fixed and stained with phalloidin (red) to visualize actin. Note the neurite-like protrusions in Rac3-depleted cells, and cell rounding in Rac3-expressing cells. (B) Pull-down assay performed with GDP, GTP or non-loaded GST-wt Rac1, GST-wt Rac3 or GST only. The experiment is representative of three independent experiments, using either newborn mice brain lysate or N1E-115 lysate (N1E-115 lysate-based experiment shown here). Rac3 interacts with GIT1 (lane 9) and βPix does not participate in this complex, as it does in the case of Rac1 (lane 5). (C) Immunoprecipitation assay in N1E-115 cells expressing wt Rac3, N17Rac3 or V12Rac3. GIT1 co-precipitates efficiently with wt Rac3 and Rac3V12, but weakly with Rac3N17. Relative amounts of precipitated GIT1, compared with total protein, and normalized for amount of protein in input blot, were scored and depicted in the bar diagram. (D) N1E-115–ev and N1E-115–Rac3 cells were transiently transfected with shLuciferase (shLuc) or shGIT1 together with eGFP expression vector (ratio eGFP:shRNA construct was 1:10). Note the spreading of normally rounded Rac3- expressing cells when GIT1 is depleted. Scale bars: 25 μm. Bar diagram represents the percentage of spreading or neurite bearing cells (50-100 cells counted in at least two independent experiments). **P*=0.001 for shGIT1 versus shLuc in N1E-115/Rac3; ***P*=0.448, shGIT1 versus shLuc in N1E-115–ev. Bottom, the cells transiently transfected with shLuc, shGIT1 or empty vector (efficiency about 60%), or treated with siRNA pool following the protocol provided by the manufacturer, were harvested, lysed and submitted to western blot analysis. Upper blot indicates the level of depletion of endogenous GIT1 by siRNA pool (lane 2) or shGIT1 (lane 3). Middle blot represents the levels of (exogenous) myc~GIT2, that is not affected by these depletion agents. Note that downregulation of GIT1 is moderate because of the 60% transfection efficiency.

siRNA pool against GIT1 or shGIT1. Cells were harvested, lysates prepared and investigated by western blot analysis. As shown in the lower part of Fig. 1D, the upper panel reveals the level of GIT1 downregulation, whereas the middle panel shows that GIT2 was unaffected by either shGIT1 or siRNA pool against GIT1. Note that the western blot (Fig. 1D) shows a moderate level of downregulation of GIT1 because of a large variation in expression of the interference constructs and that not all cells expressed these constructs. Based on these results, we conclude that GIT1 is required for Rac3-mediated cell rounding and prevention of differentiation.

Rac3 severely attenuates GIT1-paxillin interaction

To further characterize the nature and consequences of the Rac3-GIT1 interaction, we investigated whether the presence of Rac3 influences the composition of the GIT1 complexes. Since Rac3 does not interact with βPix (see Fig. 1B), we assessed whether Rac3 binding to GIT1 alters the GIT1-βPix interaction. To this end, we expressed N-terminally XPRESS-tagged full-length GIT1 in the presence or absence of Rac3 in HEK293 cells and analyzed the amounts of endogenous βPix bound to GIT1 after immunoprecipitation with anti-XPRESS antibody. We found that the amount of βPix that interacted with GIT1 was not significantly

affected by coexpression of Rac3 (Fig. 2A), suggesting that Rac3 binding to GIT1 does not influence its capacity to interact with βPix. Similar results were observed in N1E-115 cells (not shown). Based on these data, it is tempting to speculate that two separate GIT1 complexes exist in cells: an abundant one that contains βPix, Rac1 and PAK, and another that harbors Rac3 instead of Rac1, where βPix is possibly substituted by a GEF that is specific for Rac3.

Rounded Rac3-expressing N1E-115 cells show defects in FA formation and diffuse distribution of paxillin (Fig. 2C, middle panel) (Hajdo-Milasinovic et al., 2007). As GIT1 contains a functional PBD in its C-terminus (Turner et al., 1999), and has been proposed to regulate distribution of paxillin to the cell periphery and FAs (Di Cesare et al., 2000; Zhao et al., 2000), we investigated whether Rac3 influences the interaction between GIT1 and paxillin. To this end, we expressed FLAG-tagged Rac3, EGFP-tagged paxillin and XPRESS-tagged GIT1 in HEK293 cells, and performed an immunoprecipitation assay by using anti-XPRESS antibody. Strikingly, Rac3 coexpression severely attenuated the GIT1-paxillin interaction (Fig. 2B, lanes 3 and 4). As GIT1 interaction with paxillin is necessary for localization of GIT1-βPix and paxillin to FAs (Brown et al., 2002; Matafora et al., 2001), these results suggest that Rac3 blocks the GIT1-paxillin interaction, which might influence the proper localization of GIT1-βPix and, most importantly, paxillin, in FAs. A consequence of Rac3-induced inhibition of the GIT1-paxillin interaction could thus be that the FA formation is disturbed, which could contribute to the rounding off of Rac3-expressing N1E-115 cells.

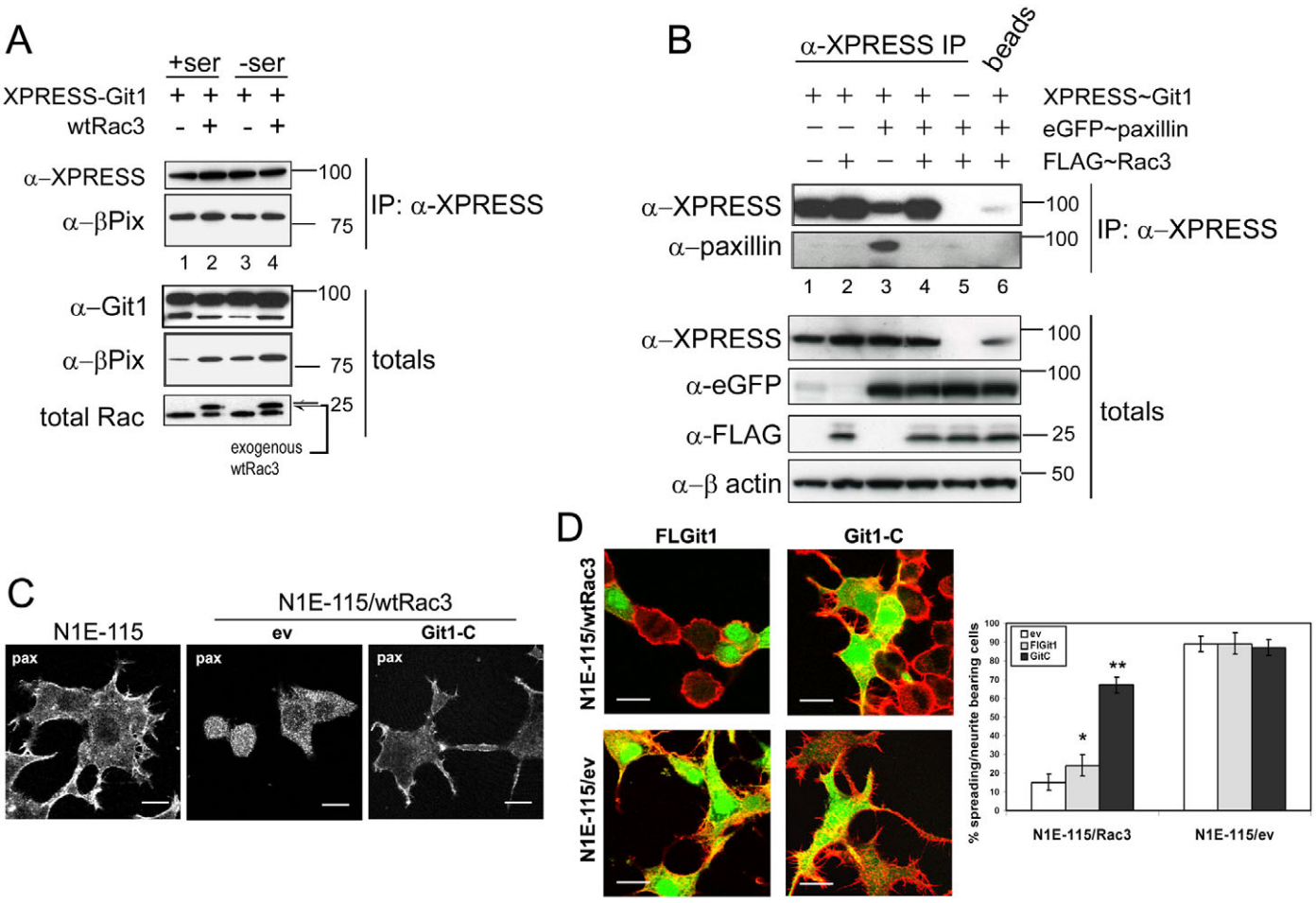

Fig. 2. Rac3 severely attenuates the interaction between GIT1 and paxillin. (A) HEK293 cells were transfected with XPRESS-tagged full-length GIT1, with or without FLAG-tagged Rac3. Immunoprecipitation was performed with anti-XPRESS antibody, and the amount of complexed endogenous βPix was visualized with anti-βPix antibody. Rac3 does not affect GIT1 and βPix interaction. (B) HEK293 cells expressing XPRESS-tagged GIT1 and EGFP-tagged paxillin, together or not with FLAG-tagged Rac3 were lysed and immunoprecipitation assay was performed using monoclonal anti-XPRESS antibody. Comparison of lanes 3 and 4 on anti-paxillin stained blot (upper panels) reveals that the presence of Rac3 (lane 4) strongly attenuates paxillin-GIT1 binding. (C) N1E-115–ev (left) and N1E-115–Rac3 (right) cells were transfected with empty vector or a construct expressing GIT1-C mutant, which contains freely accessible paxillin-binding domain (PBD), together with 1/10 eGFP-expressing vector. 36 hours after transfection, cells were sorted by FACS and replated to uncoated coverslips. After 8 hours, cells were washed, fixed and stained with monoclonal anti-paxillin antibody. Note the enrichment in peripheral paxillin staining in N1E-115–Rac3 cells transfected with GIT1-C mutant, in contrast to diffuse paxillin staining in ev-transfected N1E-115–Rac3 cells. Scale bars: 25 μm. (D) N1E-115 (right) and N1E-115–Rac3 (middle) cells were transiently transfected with a construct expressing GIT-C mutant together with 1/5 eGFP. As a control, N1E-115–Rac3 cells were transfected with a construct expressing full-length GIT1 (FLGit1), combined with 1/5 eGFP (left panels). 36 hours after transfection, cells were washed, fixed and actin was visualized by phalloidin staining. Scale bars: 25 μm. Note that GIT1-C expression reverts otherwise rounded Rac3-induced morphology into adherent and stretching morphology that resembles the one of parental cells. Full-length GIT1 does not have a significant effect on the morphology of either cell line. Bar diagram represents the percentage of spreading or neurite-bearing cells (50 transfected cells counted in two independent experiments). **P*=0.246 for full-length GIT1 versus ev in N1E-115–Rac3; ***P*=0.005, GIT1-C versus ev in N1E-115–Rac3.

Restoration of GIT1-paxillin binding induces adhesion in Rac3-expressing cells

It has been proposed that GIT1 is regulated by an intramolecular mechanism, whereby binding of βPix and PAK1 stimulate an open conformation, which allows paxillin to access the C-terminus of GIT1 (Brown et al., 2002; Di Cesare et al., 2000; Matafora et al., 2001; Totaro et al., 2007). As we hypothesized that Rac3-induced cell rounding was at least partially caused by an impaired GIT1-paxillin interaction, we made use of a GIT1 mutant with a deletion in the N-terminus, so that the PBD is fully accessible for paxillin binding (GIT1-C) (Brown et al., 2002; Di Cesare et al., 2000; Matafora et al., 2001; Totaro et al., 2007). Expression of full-length GIT1 did not lead to any morphological changes in N1E-115 cells of parental or Rac3 background (Fig. 2D, right panels). However, expression of GIT1-C in N1E-115–Rac3 cells was sufficient to induce an adhesive and stretching morphology in otherwise poorly adherent, rounded Rac3-expressing cells (Fig. 2C,D), suggesting that restoration of GIT1-paxillin binding at least partially rescued the cell adhesion capacity in Rac3-expressing cells. By analyzing paxillin distribution under these conditions we found that in adherent N1E-115 cells, paxillin was partially localized to the plasma membrane and often enriched in spiky protrusions (Fig. 2C, left panel). However, in Rac3-expressing N1E-115 cells, paxillin was distributed throughout the cell, but not at the plasma membrane (middle panel). GIT1-C mutant expression in Rac3 background rescued the localization of paxillin to the cell periphery, resembling the paxillin distribution of parental cells. How Rac3 prevents GIT1-paxillin binding remains to be established, but it is tempting to speculate that Rac3 binding prevents the established conformational change of the GIT1 protein and therefore the accessibility of its C-terminus to paxillin.

Rac3-induced cell rounding depends on a functional GAP domain of GIT1

Next to its role in FA turnover, which is dependent on the Rac1-βPix-PAK1-paxillin complex, GIT1 exerts yet another important role in neuronal cells through its N-terminally localized GAP domain (see Fig. 3A). It has been shown that GIT1 has a role in Arf6-mediated endosomal membrane recycling and actin remodeling (de Curtis, 2001; Hoefen and Berk, 2006) and functional Arf6 is necessary for cell spreading (Song et al., 1998) and neurite outgrowth (Albertinazzi et al., 2003; Hernandez-Deviez et al., 2004). We therefore investigated whether the GAP function of GIT1 was necessary for or affected by Rac3 signaling. To this end, we expressed either full-length GIT1 or a GIT1 mutant lacking the GAP domain (GIT1ΔGAP) (Fig. 3A) in both parental N1E-115–ev and N1E-115–Rac3 cells. As shown in Fig. 3B and Fig. 2D, full-length GIT1 does not affect the morphology of either parental or Rac3-expressing cells. However, deletion of the GAP domain of GIT1 strongly overrode Rac3-induced cell rounding in N1E-115–Rac3 cells and induced a similar morphology and a similar degree of spreading to that seen in parental N1E-115 cells. Since the GIT1ΔGAP mutant is missing a substantial portion of the N-terminus, it is possible that it not only lacks GAP activity but possibly also potently binds paxillin, similarly to the GIT1-C mutant. To determine whether the rescue is genuinely due to the GIT1 GAP activity and is not paxillin binding related, we used a GIT1 mutant with a point mutation that abolishes Arf-GAP activity but leaves paxillin-binding properties untouched (GIT1R39K) (Fig. 3A). As the bottom panels of Fig. 3B reveal, GIT1R39K was also capable of rescuing Rac3-specific morphology and induced cell spreading, to a similar extent as the

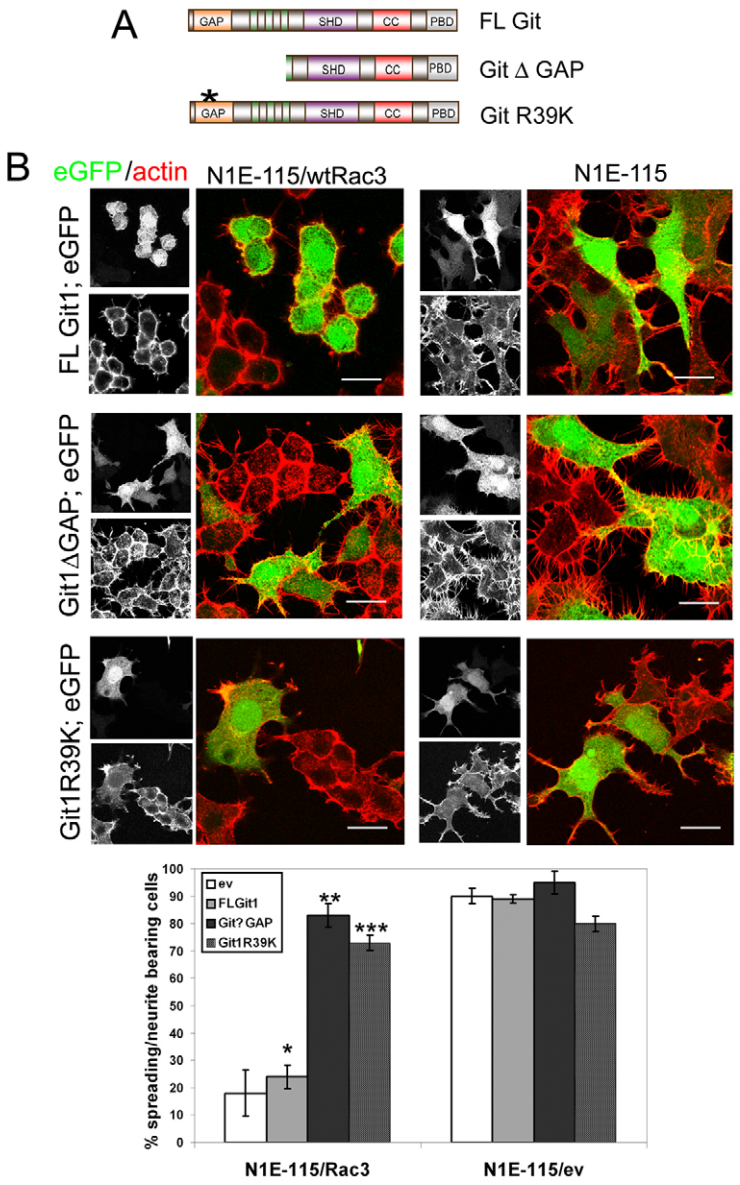

Fig. 3. Rac3-induced signaling is dependent on GAP function of GIT1. (A) Schematic representation of full-length GIT1 and mutants GIT1ΔGAP and GIT1R39K. (B) N1E-115 and N1E-115–Rac3 cells were transiently transfected with vector expressing either full-length GIT1, GIT1ΔGAP or GIT1R39K, together with 1/10 eGFP. 36 hours after transfection, cells were washed, fixed and stained with phalloidin (red). Note that both GIT1ΔGAP and GIT1R39K induce parental morphology in normally poorly adherent and rounded N1E-115–Rac3 cells, in contrast to full-length GIT1, which does not affect the morphology of either parental or Rac3-expressing N1E-115 cells. Scale bars: 25 μm. The morphology of the cells was scored as either rounded, or spreading or neurite bearing, and the percentages of the latter morphology of three independent experiments (50-100 cells counted per experiment) are depicted in the bar diagram. **P*=0.107 for full-length GIT1 versus empty vector (ev) in N1E-115–Rac3; ***P*=0.003, GIT1ΔGAP versus ev in N1E-115–Rac3; ****P*=0.046, GITR39K versus ev in N1E-115–Rac3.

GAP deletion mutant. Quantification is shown in the bar diagram. From these data, we conclude that Rac3-induced cell rounding in N1E-115 cells is functionally dependent on the Arf-GAP activity of GIT1.

Rac3 expression severely attenuates Arf6 activity

Previous studies have established that GIT1 participates in the regulation of Arf6 activity (Albertinazzi et al., 2003; de Curtis and Paris, 2005; Meyer et al., 2006). As the GAP domain of GIT1 is essential for Rac3 downstream signaling, which leads to cell rounding, we speculated that Rac3-induced morphology might also be influenced by disturbed GIT1-mediated Arf6 regulation. To test this hypothesis, we analyzed Rac3-dependent Arf6 activity (Schweitzer and D'Souza-Schorey, 2002), using GST-coupled MT2 protein as bait. In both HEK293 cells (Fig. 4A) and N1E-115 cells (Fig. 4B) we observed a striking loss of Arf6 activity in cells expressing Rac3 (Fig. 4A, lanes 1 and 2; Fig. 4B, lanes 2 and 4). The capacity of constitutively active Arf6 (Arf6L61) (Fig. 4A) to bind GST-MT2 was not affected by Rac3, indicating that Rac3 genuinely affected nucleotide loading of wt Arf6 protein. In addition, expression of Rac1 did not have any effect on Arf6 activity (Fig. 4B, lanes 2 and 3). These results show that Rac3, but not Rac1, leads to inhibition of Arf6 activity. Since we have already shown that Rac3 binds to GIT1 and that GIT1 participates in Rac3 downstream signaling, these data suggest that the interaction between Rac3 and GIT1 stimulates Arf-GAP activity of GIT1 towards Arf6, which eventually results in the reduced activity of Arf6.

We also investigated the localization of Arf6 in N1E-115–ev and N1E-115–Rac3 cells. Active Arf6 localizes predominantly to the plasma membrane and by interacting with specific GAPs it becomes inactivated on internal membranes, presumably on the tubulovesicular recycling endosome (D'Souza-Schorey and Chavrier, 2006). As shown in Fig. 4C, 4 hours of serum starvation in N1E-115–ev cells induced mild cell spreading, accompanied by plasma membrane localization of endogenous Arf6 (panels left). However, serum-starved N1E-115–Rac3 cells remained rounded and Arf6 did not localize to the plasma membrane (Fig. 4C, panels right), even after prolonged serum starvation (our unpublished data). Most active Arf6 localizes at the plasma membrane, although GDP-bound Arf6 can also be found at the plasma membrane (Macia et al., 2004). However, based on the Arf6 activity assays and the localization of Arf6 in parental and N1E-115–Rac3 cells, we conclude that endogenous Arf6 is predominantly inactive in N1E-115–Rac3 cells. Rac3 thus attenuates Arf6 activation and thereby presumably prevents its localization to the plasma membrane.

We also investigated whether Rac3-induced attenuation of Arf6 activity is dependent on the Arf-GAP function of GIT1. To this end, we performed an Arf6 activity assay using N1E-115 cells expressing either mutant GIT1ΔGAP or GIT1R39K in parental and Rac3-expressing cells. We used both GAP-deficient mutants to discriminate whether the possible effects were due to the lack of GAP activity only (point mutant) or to lack of GAP activity together with the increased paxillin binding (GAP deletion mutant). As shown in Fig. 4D, both mutants were capable of increasing Arf6 activity several fold above the normal level in parental cells. More importantly, they both also rescue Arf6 activity in Rac3-expressing cells (Fig. 4D, lanes 1, 2, 3 and 4, upper panel). From these data, we conclude that Rac3 inhibits Arf6 activity, and that the Arf-GAP activity of GIT1 is required for this inhibition.

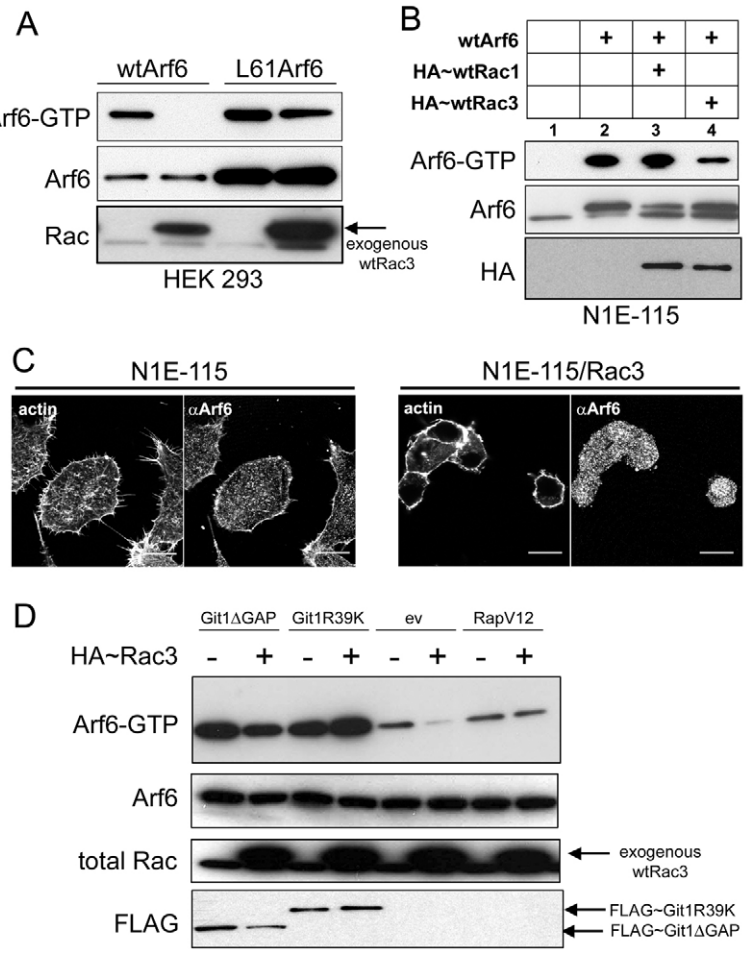

Fig. 4. Rac3 expression severely attenuates Arf6 activity. (A) HEK293 cells expressing wt Arf6 or Arf6L61 mutant, coexpressed or not with Rac3, were used for Arf6 activity assay, where GST-MT2 protein was used as bait. There is a strong reduction in Arf6 activity when Rac3 is coexpressed (lanes 1 and 2, upper blot). (B) Arf6 activity assay was also conducted in N1E-115 cells, transiently expressing either wt Rac1 or wt Rac3 together with wt Arf6. Also in these cells presence of wt Rac3 strongly inhibits Arf6 activity, whereas wt Rac1 does not have any effect (lanes 2, 3 and 4). (C) N1E-115 cells (upper panels) and N1E-115–Rac3 cells (lower panels) were seeded on glass coverslips, allowed to adhere during 12 hours and subsequently serum starved for 4 hours. Cells were washed, fixed and stained with phalloidin (left) and polyclonal anti-Arf6 antibody to visualize localization of endogenous Arf6 protein (right). Note the absence of Arf6 from the plasma membrane in Rac3-expressing cells. Scale bars: 25 μm. (D) Similarly to A and B, N1E-115 cells were transfected with indicated constructs, allowed to express the proteins for 36 hours. Subsequently, cells were lysed and processed for Arf6 activity analysis. Upper blot shows that both GIT1ΔGAP mutant and GIT1R39K mutant are capable of increasing Arf6 activity, in both parental and Rac3-expressing cells. Expression of RapV12 increases the Arf6 activity in Rac3-expressing cells to the levels of the parental cells (last two lanes).

Restoration of Arf6 activity rescues the Rac3 phenotype

As our data suggest that inhibition of Arf6 activity is causal in the Rac3-induced cell rounding, we analyzed whether the restoration of Arf6 activity could induce cell spreading in N1E-115–Rac3 cells. As shown in Fig. 5A,C, transient expression of wt Arf6 did not notably change parental N1E-115 cell morphology. However, exogenous wt Arf6 fully abolished the characteristic rounded cell morphology induced by Rac3 in N1E-115 cells (Fig. 5A, left upper panel). This change in morphology was also accompanied by plasma membrane localization of wt Arf6 (Fig. 5C). In addition, restoration of Arf6 activity by expressing an activator of Arf6, Arf-GEF ARNO also induced cell spreading in the otherwise rounded Rac3-expressing cells (Fig. 5A, lower panels). Thus, either lack of the GAP activity of GIT1 or the restoration of Arf6 activity directly is sufficient to abolish the rounded morphology of N1E-115–Rac3 cells. We conclude therefore that Rac3-induced inactivation of Arf6, as a result of increased Rac3-induced GAP activity of GIT1, is a key element of the Rac3-induced cell rounding.

Furthermore, we investigated whether restoration of Arf6 activity influenced Rac3-induced inhibition of the GIT1-paxillin interaction and defective paxillin distribution. For this, we immunoprecipitated GIT1 and analyzed paxillin binding to GIT1, in the presence or absence of Rac3 and wt Arf6 (Fig. 5D). Although expression of wt Arf6 was capable of rescuing cell rounding induced by Rac3 (Fig. 5A), it was not capable of restoring the GIT1-paxillin interaction in Rac3-expressing cells (Fig. 5D, paxillin blot, lanes 1-4). However, restoration of Arf6 activity was sufficient to ensure peripheral distribution of paxillin in Rac3-expressing cells, similarly to its distribution in parental cells (Fig. 5B), suggesting that the distribution of paxillin is not only dependent on its interaction with GIT1.

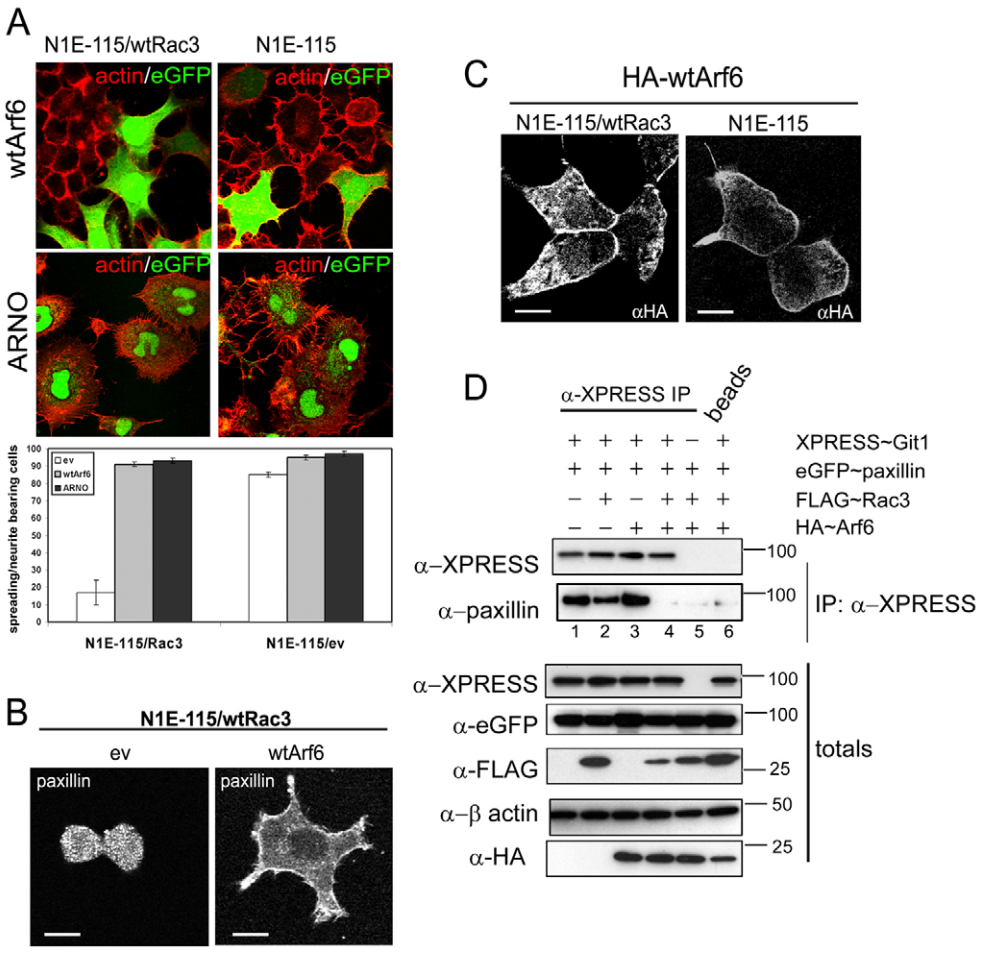

Fig. 5. Restoration of Arf6 activity rescues Rac3 phenotype. (A) N1E-115–Rac3 and N1E-115 cells were transiently transfected with vector expressing wt Arf6 (upper panels) or full-length ARNO (lower panels) cDNA, together with eGFP expression vector (ratio eGFP:construct was 1:10). After 36 hours, cells were washed, fixed and stained with phalloidin to visualize actin (red). Note the spreading of otherwise rounded N1E-115–Rac3 cells when Arf6 activity is restored (left panels). The percentage of spreading or neurite-bearing cells is depicted in the bar diagram below (two independent experiments, 50-100 cells counted in each experiment). (B) N1E-115–Rac3 cells were transiently transfected with wt Arf6 (right panel) or with ev (left), and 36 hours after transfection, cells were washed, fixed and stained with anti-paxillin antibody (Invitrogen). Note that wt Arf6 expression relocates paxillin back to the cell periphery in N1E-115–Rac3 cells. (C) N1E-115 and N1E-115–Rac3 cells were transiently transfected with vector expressing HA-wt Arf6 (together with eGFP, 1/10). 36 hours after transfection, cells were washed, fixed and stained with anti-HA antibody to visualize exogenously expressed Arf6. Note the plasma membrane localization of HA-tagged wt Arf6 in both parental and Rac3 backgrounds, as well as the flat morphology of the cells. Scale bars: 25 μm. (D) HEK293 cells expressing XPRESS-tagged GIT1 and EGFP-tagged paxillin, with or without FLAG-tagged Rac3 and wt Arf6, were lysed and immunoprecipitation assays were performed by using monoclonal anti-XPRESS antibody. Comparison of lanes 1, 2, 3 and 4 on anti-paxillin stained blot (upper panels) reveals that the presence of Arf6 (lanes 3 and 4) does not rescue the strongly attenuated paxillin-GIT1 binding induced by Rac3.

Discussion

In this study we investigated the mechanisms behind the strikingly opposing effects of Rac1 and Rac3 in neuronal spreading and differentiation. Rac3 induces cell rounding, weak cell-matrix adhesions and blocks neurite outgrowth and cell differentiation of neuronal cells. By contrast, Rac1 promotes cell spreading, lamellipodia formation and neurite outgrowth. Our experiments here show that Rac3, in contrast to Rac1, binds to GIT1 in a βPix-independent manner. Upon this binding, Rac3 modulates downstream signaling of GIT1 by both inhibiting the GIT1-paxillin interaction as well as inhibiting Arf6 activity, probably by utilizing Arf-GAP activity of GIT1.

Upon dual expression of Rac1 and Rac3, the Rac3-induced rounded morphology prevails. Although exogenous Rac1 yields somewhat lower levels of expression when compared with Rac3 (we suspect degradation), in general we found that Rac3 overrules the Rac1 phenotype, suggesting that competition for the same binding partner but with a different outcome might be the root of the opposing morphologies we observed. We performed elaborate analyses of binding partners of Rac1 and Rac3 and these experiments revealed that both the preference and intensity of protein interactions are predominantly similar for both Rac proteins. Rac1 and Rac3 share a high degree of homology, most importantly the effector binding region is 100% homologous (Haataja et al., 1997); therefore, it is not surprising that they mostly behave similarly with respect to the nature of their interactions. However, Rac1 and Rac3 differ at the C-terminus, and we have shown before that a triplet of amino acids just before the CAAX box is responsible for intracellular localization of Rac1 and Rac3, and the distinct effects they have on (neuronal) cell morphology (Hajdo-Milasinovic et al., 2007). Here, we have established that Rac3, similarly to Rac1, is able to bind the multifunctional scaffolding Arf-GAP protein GIT1. However, the Rac1 interaction with GIT1 is mediated by βPix (Brown et al., 2002), whereas our results show that the Rac3 interaction with GIT1 does not involve βPix. In the past, others have also shown that the Rac1-βPix interaction is determined and stabilized by the Rac1 C-terminus (Ten Klooster et al., 2006), through a stretch of amino acids not shared between Rac1 and Rac3. Our data support this observation, and show that this lack of a Rac3-βPix interaction does not prevent Rac3 participation in the GIT1 complex. Because of the high degree of homology between Rac1 and Rac3, it is likely that Rac3 participates in a GIT1 complex via a GEF different from the Pix family, possibly a GEF specific to Rac3.

Participation of active Rac1 in βPix-GIT1-PAK1 complex induces an open conformation of GIT1, which stimulates binding of paxillin to the C-terminus of GIT1. This complex subsequently translocates to FAs, where it stimulates FA formation and turnover (Brown et al., 2002; Di Cesare et al., 2000; Matafora et al., 2001; Totaro et al., 2007). Our data indicate that Rac3 has a different role in GIT1 signaling and that Rac3 strongly attenuates the GIT1-paxillin interaction. We have investigated Rac1 and Rac3 binding to different PAK proteins and found that both are equally capable of binding and activating these proteins (data not shown). However, since Rac3 takes part in the GIT1 complex without intermingling of βPix, it is tempting to speculate that Rac3 participation in the GIT1 complex somehow changes the complex in such a way that the conformational change of GIT1 is prevented. The consequence is a 'covered' GIT1 C-terminus, which is not accessible for interaction with paxillin. The loss of GIT1-paxillin interaction in Rac3-expressing cells probably contributes to loss of proper FA formation and/or turnover. Support for this hypothesis is provided by the experiments presented in Fig. 2C,D, where the expression of a paxillin-binding mutant of GIT1 with a constitutively open conformation induces spreading and stretching in otherwise rounded and poorly adherent N1E-115–Rac3 cells.

Our data indicate that another feature of GIT1 signaling, its GAP activity towards Arf6, is also affected by Rac3 binding. We found that the presence of Rac3 strongly inhibits Arf6 activity and, simultaneously, affects its subcellular localization (Fig. 4). The expression of either a deletion mutant of GIT1 that lacks the GAP

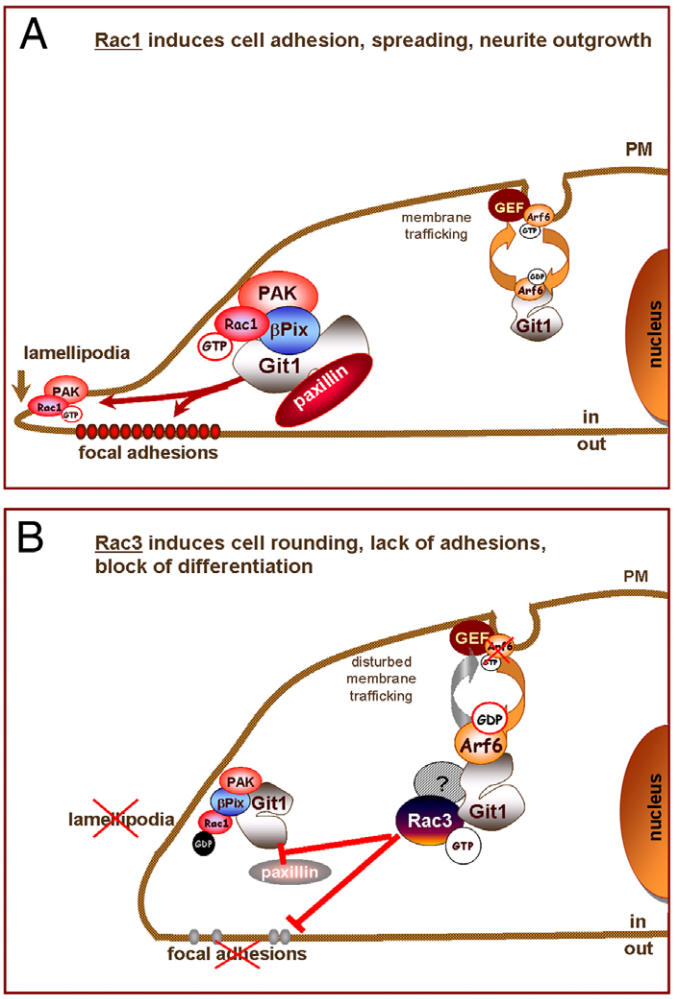

Fig. 6. Proposed model of Rac3 inhibition of adhesion and differentiation of neuron-like cells by its interaction with Arf-GAP-GIT1, and modification of downstream signaling. (A) As a result of cell-matrix adhesions, βPix binds Rac1 and activates this GTPase. PAK1 interacts with the Rac1-βPix-GIT1 complex and stimulates an open conformation of GIT1. This leads to binding of paxillin to GIT1 and targeting of the PAK1-βPix-GIT1-paxillin complex to FAs or focal complexes at the cell periphery, with peripheral actin rearrangements and cell spreading as a consequence. (B) Rac3 interacts directly or indirectly with GIT1, and thereupon the accessibility of GIT1 to paxillin is severely reduced; subsequently, the complex is not redistributed to the FAs, leading to poor cell-matrix adhesion. In addition, Rac3 binding might stimulate the GIT1-GAP activity towards Arf6, resulting in severe reduction of Arf6 activity in N1E-115–Rac3 cells, which leads to defective adhesions and a lack of cell spreading. Both the impaired GIT1-paxillin interaction and the reduction of Arf6 activity seem to contribute to the Rac3-mediated defective adhesion, because restoration of one of these pathways rescues cell spreading in N1E-115–Rac3 cells.

domain (GIT1ΔGAP), or a mutant with a point mutation that abolishes GAP activity but does not affect other properties of the molecule (GIT1R39K), or the expression of exogenous wt Arf6 or an activator of Arf6 show increased adhesion, spreading and induced a full rescue of the Rac3-induced rounded cell morphology. These data strongly suggest that the Rac3-GIT1-mediated effect on Arf6 activity causes Rac3-induced cell rounding, because three different means of re-establishing Arf6 activity are fully capable of rescuing the Rac3-induced rounded morphology.

What about the Rac3-induced inhibition of GIT1-paxillin interaction? As shown in Fig. 5D, Arf6 activity induced cell spreading and proper paxillin distribution in a Rac3 background, but does not have an effect on the Rac3-induced inhibition of GIT1-paxillin interaction. This indicates that paxillin distribution is not dependent only on its interaction with GIT1. It is unlikely that the lack of a GIT1-paxillin interaction is characteristic of rounded N1E-115 cell morphology per se. N1E-115 cells cultured in full serum show poor adhesion and lack of spreading, similarly to Rac3-expressing cells. However, when investigating the GIT1-paxillin interaction in N1E-115 cells grown in full serum, we have never observed effects on this interaction as seen in Rac3-expressing cells. This suggests that it is not cell rounding per se, but the presence of Rac3 that truly attenuates the GIT1-paxillin interaction. Our data suggest that Rac3 attenuation of GIT1-paxillin interaction is an early event in the Rac3-GIT1 signaling pathway. It is probably Rac3 participation in the GIT1 complex (direct or indirect) that causes differential folding of GIT1 and thereby prevents the GIT1-paxillin interaction. The cell spreading induced by restoring Arf6 activity could be due to the independent effects of Arf6 on cell adhesion and FA formation, or could be due to signaling that impinges downstream of GIT1-paxillin interaction, but upstream of FA formation.

Based on literature and the presented data, we propose a model as depicted in Fig. 5C. Upon Rac1 binding to the βPix-GIT1 complex and its subsequent activation by βPix, PAK1 interacts with this complex and stimulates an open conformation of GIT1. This allows the binding of paxillin to GIT1 and subsequent targeting of the PAK1-Rac1-βPix-GIT1-paxillin complex to the FAs or focal complexes at the cell periphery. As a consequence, this stimulates cell-matrix adhesions, peripheral actin rearrangements and cell spreading (Fig. 6A) (de Curtis, 2001; Frank and Hansen, 2008; Hoefen and Berk, 2006). However, when Rac3 interacts with GIT1, the accessibility of GIT1 to paxillin is severely reduced, and the GIT1-paxillin complex is not redistributed to the FAs (Fig. 6B). As a matter of fact, the formation of FAs per se is probably prohibited, leading to poor cell-matrix adhesion (Hajdo-Milasinovic et al., 2007). At the same time, Rac3 binding might stimulate the GIT1 Arf-GAP activity, resulting in reduced Arf6 activity (Fig. 4A,B), as well as lack of plasma membrane localization of endogenous Arf6 in Rac3-expressing cells (Fig. 4C). Owing to this dual effect of Rac3 on GIT1 signaling, the expression of Rac3 results in cell rounding and impaired differentiation of neuronal N115 cells.

Taken together, our data imply that Rac3 and Rac1 oppose each other by differently modulating GIT1 function. We propose that Rac1 and Rac3 each stimulate a different branch of GIT1 signaling in neuronal cells, leading to strikingly different morphological outcomes. Rac1 binds to βPix and GIT1 and stimulates GIT1-paxillin interaction, thereupon triggering FA formation, cell spreading and differentiation. By contrast, Rac3 interacts with GIT1 independently of βPix and prevents GIT1-paxillin interaction and prevents Arf6 activation, probably by stimulating GIT1-GAP function. Both GIT1-mediated pathways lead to lack of cell spreading and differentiation in neuronal N1E-115 cells.

Materials and Methods

Cell culture and transfection

N1E-115 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (both from Invitrogen) and antibiotics. The N1E-115–Rac3 cell line, stably expressing Rac3, has been described previously (Hajdo-Milasinovic et al., 2007). To induce differentiation, cells were washed with PBS and cultured for 16 hours in serum-free DMEM supplemented with antibiotics. When indicated, to induce spreading or neurite outgrowth, the cells were treated with 50 ng/ml nerve growth factor (mNGF, Alomone labs) for 2 hours or 8 hours, respectively. The protocol for transient transfection assays was performed as described previously (Hajdo-Milasinovic et al., 2007).

Plasmids

The pcDNA3-FLAG constructs of human Rac3, the constitutive active Rac3 (G12V) mutant and dominant negative Rac3 (N17) mutant were generated by PCR and cloned via *Bam*HI and *Eco*RI restriction sites (N-terminal FLAG tag). Human cDNAs of wt Rac1 and wt Rac3, cloned in vector pcDNA3.1 (Invitrogen) and N-terminally tagged with HA sequence were obtained from UMR cDNA Resource Center, University of Missouri, Rolla, MO. To obtain GST-coupled proteins, wt Rac1 and wt Rac3 were cloned into pGEX-6P-2 vector (GE Healthcare), by using *Sma*I-*Xho*I and *Pme*I-*Xho*I digestion sites for vector and inserts, respectively. For pSuper/shGIT1 construct, we used the targeting sequence against rat GIT1, essentially described by Zhang and colleagues (Zhang et al., 2005), which was adjusted to target a mouse GIT1 sequence. The oligonucleotides were annealed and cloned into the *Bgl*II-*Eco*RI site of pSuper vector (Brummelkamp et al., 2002) (15 /id). Sequences of the primers were: shGIT1 forward:, 5′-CGGGATCCCCGCGCTCAGCAACCGGCTTTTTCAAGAGAAA-AGCCGGTTGCTGAGGGCTTTTTGGAAAAGCTTGGG-3′; shGIT1 reverse, 5′-CCCAAGCTTTTCCAAAAAGCGCTCAGCAACCGGCTTTTCTCTTGAAAAA-GCCGGTTGCTGAGGGCGGGGATCCCG-3′.

Alternatively, GIT1 expression was suppressed by using DharmaFECT On-targetPLUS set of four duplex siRNA sequence against mGIT1 (LQ-065632-00-0002; Dharmacon RNA technologies), used as a pool of four target sequences, and following the protocol provided by the supplier.

Other constructs used were pFLAG-GIT1-C, pFLAG-GIT1-C2 and pFLAG-GIT1R39K (GIT1-C, GIT1ΔGAP and GIT1R39K, respectively), kindly provided by Ivan de Curtis (HSR, Milan, Italy); pFLAG-FLGIT1, kindly provided by Alan Rick Horwitz (UVSM, Charlottesville, VA); pFLAG-ARNO, kindly provided by Julie Donaldson (NIH, Bethesda, MD); pXS-Arf6, pXS-Arf6Q67L and pXS-Arf6T27N, kindly provided by Victor Hsu (BWH, Boston, MA); pEGFP-paxillin, kindly provided by Kenneth Yamada (NIH, Bethesda, MD); pCDNA3-XPRESS-GIT1, a kind gift from Bradford C. Berk (URSMD, Rochester, NY).

Immunofluorescence and confocal microscopy

Immunofluorescence staining was carried out essentially using a protocol reported previously (Hajdo-Milasinovic et al., 2007). F-actin was visualized by incubating the cells with 0.2 μM Alexa Fluor 568-labeled phalloidin (Invitrogen) for 45 minutes. The following primary monoclonal antibodies were used: anti-paxillin (BD Transduction Laboratories); anti-paxillin mAb (Invitrogen); anti-Arf6 (Santa Cruz Biotechnology); anti-HA (clone 3F10, Sigma); anti-Rab11 (Upstate); anti-EEA1 (BD Transduction Laboratories). Images were obtained by confocal microscopy (model TCS NT; Leica).

Production of GST-fusion proteins and in vitro binding studies

The production of GST fusion proteins was essentially performed as described previously (Sander et al., 1998). GST-protein-binding experiments were preformed as described (Villalonga et al., 2001). Briefly, GST, GST-Rac1 and GST-Rac3 proteins were incubated in exchange buffer, supplied with GDP, GTP or no nucleotide, and exchange reaction was performed for 30 minutes at 30°C. Loading was terminated by addition of $MgCl_2$ to 15 mM final concentration. Subsequently, beads were washed and directly used in pull-down experiments. We used either N1E-115 cells or freshly prepared newborn mice whole brain, snap-frozen in liquid nitrogen and pulverized using a homogenizer. Cell material was lysed (lysis buffer: 0.5% NP-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$ and protease inhibitor cocktail), centrifuged and the supernatant was incubated with previously prepared, nucleotide loaded GST-fusion proteins. After incubation for 2 hours at 4°C, the beads were precipitated by centrifugation, washed and dried. The precipitated proteins were resolved in sample buffer, boiled for 5 minutes and analyzed by western blotting.

Immunoprecipitation and western blotting

Immunoprecipitation was performed using either mouse monoclonal anti-FLAG antibody (M2, Sigma) or mouse monoclonal anti-XPRESS antibody (Invitrogen). For starting material, HEK293 or N1E-115 cells were seeded in 10 cm dishes, allowed to adhere for 12 hours, transfected with indicated constructs, and 36 hours after transfection, the cells were briefly cooled, washed and lysed in ice-cold RIPA buffer

(150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH 7.4). For further procedures, the protocols provided by the antibody suppliers were followed.

For western blotting, the following primary monoclonal (mAb) and polyclonal (pAb) antibodies were used: mouse hybridoma 12CA5 for detection of HA-tag; anti-FLAG mAb (M2, Sigma); anti-XPRESS mAb (Invitrogen); anti-GFP mAb (Roche Applied Science); anti-βactin mAb (Sigma-Aldrich); anti-Arf6 mAb (Santa Cruz Biotechnology); anti-GIT1 mAb (BD Transduction Laboratories); anti-paxillin mAb (BD Transduction Laboratories); anti-βPix pAb (Chemicon); for detection of both Rac1 and Rac3, anti-Rac1 mAb (Upstate) was used. Specific binding was detected using a secondary peroxidase-conjugated antibody (GE Healthcare) followed by chemiluminescence.

Arf6 activity assay

HEK293 or N1E-115 cells were seeded in 10 cm dishes, transfected with indicated constructs and 36 hours after transfection the cells were lysed and processed. Arf6 assay was performed as described previously using GST-MT2 (Schweitzer and D'Souza-Schorey, 2002). The GST-MT2 construct was kindly provided by Crislyn D'Souza-Schorey (UND, Notre Dame, IN).

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