Rac1 and Rac3 in cell morphology and adhesion: brothers and foes
Hajdo-Milašinovi, A.

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Chapter 5

Summary and discussion
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Guanosine triphosphatases (GTPases) of Ras superfamily represent a large family of small regulatory enzymes. By binding either GDP or GTP nucleotide, they adopt an inactive or active conformation, respectively, and act therefore as convenient molecular switches that are often employed in the regulation of dynamic cellular processes. Upon activation, small Ras-like GTPases can interact with a large number of downstream effectors and by doing so affect and regulate a wide variety of processes in the cell, including growth, proliferation, cellular morphology and differentiation, cell movement and vesicle transport. Although the small GTPases share a great degree of structural homology, especially within the distinct families/subfamilies, they exert their function in a very specific and precise manner (for detailed overview, see Chapter 1). Considering their importance in so many different aspects of cell biology, it is not surprising that understanding the biochemical context of the Ras GTPases action has became a hot topic, with implications for health and disease.

This thesis focuses on the specific novel roles of small Ras-like GTPases of Rac and Rap subfamily in cell-cell and cell-matrix adhesions, complex processes which are essential for establishing and maintaining normal cell morphology, migration capacity and function.

In Chapter 2 we show that Rap signaling promotes cadherin-mediated cell-cell adhesion. Rap1 is a small GTPase of the Ras superfamily, whose role in cell-matrix adhesion is elaborately investigated. Namely, Rap1 is known as potent activator of integrins, stimulating thereby the dynamics of cell-matrix adhesions in various cellular systems (Bos, 2005). Intriguingly, while investigating the function of different small GTPases in cell-matrix and cell-cell adhesions, we observed that constitutively active Rap1 was able to revert mesenchymal Ras-transformed Madin-Darby canine kidney (MDCK) cells towards an epithelial morphology. For the first time, our data reveal a stimulatory role of Rap1 in the formation of cadherin-based cell-cell adhesions. We also show evidence for its role in maintenance of adherens junctions, as inhibition of Rap1 activity in OvCar3 ovarian carcinoma cells led to marked reduction in cadherin
levels at the site of cell-cell interaction. It is however not clear as how Rap1 conducts this effect on cadherin-based adhesions. Ras induces the scattering of MDCK cells at least in part by disrupting adherens junctions via MAP kinase-dependent pathway (Ridley et al, 1995; Tanimura et al, 1998). However, Rap1 apparently does not antagonize the signaling of oncogenic Ras, since activity of MAP kinase remains unaffected in Rap1-re-epithelialized Ras/MDCK cells. It is more likely that Ras and Rap signals connect independent from each other at the level of cadherin function, as it was similarly proposed for the function in integrin signaling (Bos et al, 2003). We examined Rap1 signaling in MDCK epithelial cells directly adhered to surfaces coated with canine E-cadherin extracellular domains, thus in the absence of integrin ligands. From these studies we conclusively established that Rap signaling is necessary for the formation of E-cadherin mediated homotypic interactions independently of the effects of Rap on integrin-mediated adhesion. Rap influences integrin-mediated adhesion by affecting integrin avidity (Sebzda et al, 2002; Bertoni et al, 2002; Katagiri et al, 2003) or affinity (Katagiri et al, 2000; Bertoni et al, 2002). It is plausible that Rap affects the cadherin-based adhesions in a similar fashion; however, cadherins do not undergo affinity changes, leaving the possibility that Rap1 influences the avidity (clustering) of the cadherins. Intriguingly, Rap1 was shown to be involved in integrin-mediated adhesions regardless of the integrin type, as long as their cytoplasmic tails couple to the actin cytoskeleton (van der Flier and Sonnenberg, 2001). Since cadherin-based junctions are also intimately connected to actin filaments, it is tempting to speculate that the common theme for Rap1 involvement in regulation of adhesion is the actin cytoskeleton-dependent receptor clustering. In conclusion, this chapter reveals a novel and intriguing role of Rap1 that next to its regulatory role in integrin-based cell-matrix adhesion now proves to be involved in cadherin-based cell-cell adhesion as well.

In Chapter 3 we describe the specific roles of Rac1 and Rac3 in cell adhesion and differentiation of neuronal cells. Previously, our group and others have shown that Rac1 is necessary for spreading and neurite outgrowth in neuroblastoma cells (Leeuwen et al., 1997; Sarner et al., 2000; Aoki et al., 2004) and primary hippocampal neurons (Schwamborn and Puschel, 2004). Apart from the fact Rac3 is enriched in brain (Haataja et al, 1997; Bolis et al, 2003; Corbetta et al, 2005), little
was known about the function of Rac3 in cell adhesion or neuronal cell differentiation. In Chapter 3 we show an exciting and unexpected finding that Rac3 opposes Rac1 in the establishment of the neuronal cell adhesion and differentiation. In murine neuroblastoma N1E-115 cells, frequently used as a model for neuronal differentiation, down-regulation of endogenous Rac1 by short hairpin RNA (shRNA) led to loss of cell-matrix adhesion and a round and contractile morphology, which corresponds to the well-established role of Rac1 in cell-matrix adhesion and neuritogenesis. Intriguingly, endogenous Rac3 depletion was followed by pronounced cell adhesion and formation of the neurite-like protrusions. Wild-type Rac3 expression resulted in a rounded, contractile morphology with poor cell-matrix adhesions, whereas expression of wild-type Rac1 induced cell spreading.

Scarce data available thus far have shown either no or small functional difference between Rac1 and Rac3 (Joyce and Cox, 2003; Keller et al., 2005; Haataja et al., 1997; Mira et al., 2000). The structures of the three Rac proteins are remarkably similar, with the effector-binding regions (residues 25-46) and switch I and II regions (responsible for the nucleotide binding) fully conserved. As a matter of fact, they share 92% of overall homology, diverging predominantly in the carboxyl terminus (Haataja et al, 1997). In previous studies, the lack of any obvious difference in Rac1 and Rac3 function was blamed on the fully conserved effector binding region, binding probably the same downstream effectors, hence leading to the similar biological outcome (Haataja et al, 1997; Mira et al, 2000; Keller et al, 2005; Chan et al, 2005). However, these data were predominantly acquired by using constitutively active and dominant negative mutants of Rac1 and Rac3 to investigate their function. Indeed, also in our cell model, expression of constitutive active mutants of Rac1 and Rac3 induce a very similar spreading and aberrant migratory phenotype in N1E-115 cells (Chapter 3, supplementary material Fig. S2). Only when using exogenous expression of wild-type Rac1 and Rac3 proteins, which are activated by endogenous GEFs (Fukuhara et al., 2001), we found that Rac1 and Rac3 cause remarkably different phenotypes. Constitutive active and dominant negative Rac mutants are highly promiscuous in binding and sequestering downstream effectors and exchange factors, respectively (Feig, 1999), which makes them a very imprecise tool when determining the exact function of such a heavily homologous proteins. As a matter of
fact, all available data on Rac1, obtained in cell models that endogenously express both Rac1 and Rac3 by exclusively using constitutive active (CA) and dominant negative (DN) mutants, should be reexamined, since it is plausible that at least a portion of protein functions ascribed to Rac1, could actually be the result of for example CA Rac1 interaction with a bona fide Rac3 downstream effectors. In summary, when analyzing the specificity of such highly homologous proteins, it is imperative to use the tools that are able to discriminate between the two isoforms, like for instance shRNA or non-promiscuous activators (Chapter 3).

In addition to their functional difference, the intracellular localization of Rac1 and Rac3 is also divergent; we found that Rac1 is predominantly found at the plasma membrane, while Rac3 is foremost distributed in the perinuclear region (Chapter 3). Most Rho GTPases localizes in their active conformation to the plasma membrane, most likely at specialized signaling platforms like calveolae and rafts (Bivona and Philips, 2003; Pfeffer and Aivazian, 2004; Wennerberg et al., 2005), but also to specific structures like focal adhesions (Rosenberger and Kutsche, 2006). The correct subcellular localization of Rho GTPases depends on the type of lipid moiety that is posttranslationally added to the carboxy terminus (Wenneberger et al, 2005), but even more importantly on specific protein-protein interactions, like guanine nucleotide exchange factors (GEFs) (ten Klooster and Hordijk, 2007; García-Mata and Burridge, 2007). Apart from a few single amino acid differences, Rac1 and Rac3 protein sequence differ significantly only in two adjacent amino acid stretches: the CAAX box at the tip of carboxyl terminus, where an isoprenoid moiety is added to the carboxy tail (prenylation; Seabra, 1998; McTaggart, 2006), and the polybasic region (PBR) directly upstream to the CAAX box. The Rac1 CAAX motif is CLLL, and Rac1 is a known substrate for geranylgeranyl transferase, like the majority of the Rho GTPases (Didsbury et al, 1989; Menard et al, 1992). Intriguingly, the CAAX motif of Rac3 is CTVF, suggesting that Rac3 may be modified by either geranylgeranyl or farnasyl groups (Moores et al, 1991), making it tempting to speculate that the difference in prenylation is the cause of the functional difference between Rac1 and Rac3. However, our data show conclusively that only the polybasic region sequence, and not the CAAX box, is determinant for the difference in function and localization of Rac1 and Rac3 (Chapter 3). The polybasic region (PBR) is proposed to serve as an
additional binding site for effectors (van Hennik et al., 2003; Yamauchi et al., 2005) or GEFs (ten Klooster et al., 2006), so we suspect that the specific protein-protein interactions that depend on PBR are crucial for functional and location divergence between Rac1 and Rac3.

Normally, serum-deprivation is a pre-requ irement to induce stronger cell-matrix adhesions and initiate neuritogenesis in N1E-115 cells (Fishman et al, 1981), since serum-born factors trigger actomyosin contractility, with the foremost example of lysophosphatidic acid (LPA) – RhoA - MLCK/Rho kinase pathway (Amano et al., 1996; Leeuwen et al., 1997; Kranenburg et al., 1997). However, Rac3 depletion induces neuritogenesis even in the presence of serum, suggesting that the endogenous Rac3 acts upstream or impinges on the LPA downstream pathway. Small GTPases cross-talk abundantly and share common pathways or counteract each other (Bar-Sagi D and Hall A, 2000; Ellenbroek and Collard, 2007), and since RhoA signaling antagonizes Rac1 function in neuronal and other cells (van Leeuwen 1997; Sander et al., 1999; a plausible hypothesis would be that Rac3 counteracts Rac1 effect on morphology by impinging on RhoA signaling pathway. A thorough analysis however has shown that this is not the case (Chapter 3), leaving the possibility that Rac3 role is positioned upstream of the serum-born regulators of the N1E-115 morphology.

A crucial step in induction of neurite outgrowth is the strengthening of the cell-matrix adhesions and the associated actomyosin relaxation (Nikolic, 2002). Starting from the hypothesis that Rac3 somehow negatively affects adhesion to the surface, we looked elaborately for possible molecules and/or agents that could counteract Rac3-induced cell contraction and loss of cell-matrix adhesion. Rac1 co-expression, of either wild-type or constitutive active mutant, or expression of its activator Tiam1 were not sufficient to induce spreading in Rac3-expressing N1E-115 cells (data not published). Similarly, incubation with differentiation-stimulating agents, like neurite growth factor, or plating the cells on substrates such as laminin-1 (van Leeuwen et al, 1997), were also not proficient in antagonizing the Rac3-induced morphology. In contrast, expression of constitutively active mutant of small GTPase Rap1 was able to increase cell-matrix adhesion and induce spreading in otherwise contractile and
round Rac3-expressing cells. Apparently, only direct stimulation of cell-matrix adhesions just upstream of the integrin clustering was efficient in neutralizing Rac3 effect, suggesting that Rac3 functionally impinged somewhere upstream in the integrin-mediated pathway to negatively regulate cell-matrix adhesions. Cell-matrix adhesion, and subsequently cell spreading and neurite outgrowth are highly dependent on functional integrin signaling (Ridley et al., 2003; Govek et al., 2005). Growth factor- and extracellular matrix-triggered signaling leads to integrin clustering and the formation of complex signaling structures like focal complexes (FC) or focal adhesions (FA) (Ridley et al., 2003; Brown and Turner, 2004). In neuronal cells, initiation of neurite outgrowth is accompanied with peripheral dot-like distribution of focal adhesion molecules like paxillin (Leventhal and Feldman, 1996; Yamauchi et al, 2006). Our data show that Rac3-expressing N1E-115 cells exhibit a diffuse distribution of paxillin, in contrast to the paxillin distribution at the tips of the cell periphery as in spreading control cells or Rac1-expressing cells (Chapter 3). This suggests that Rac3 acts upstream of paxillin distribution to negatively affect cell-matrix adhesions.

Having established that Rac3 counteracts the Rac1 effects on cell spreading and differentiation of neuronal cells, an intriguing issue aroused as of what downstream signaling does Rac3 use to induce a contractile morphology, diminish the strength of the cell-matrix adhesions and postpone the neuritogenesis. When we expressed Rac1 and Rac3 simultaneously, we observed that the Rac3-induced rounded morphology prevails (data not shown), suggesting that a competition for the same binding partner but with a different outcome might be the root of the opposing morphologies. Hence, the Rac1 downstream effectors that participate in spreading- and neuritogenesis-related Rac1-mediated signaling pathways are most likely the candidates for binding and negative regulation by Rac3. In the first place, Rac1 binds and activates PAK kinases (p21 activated kinases), thereby suppressing myosin light chain (MLC) phosphorylation that ultimately inhibits myosin contractility and leads to the cell spreading (Sander et al., 1999; Bokoch, 2003). Through PAK kinases, Rac1 also phosphorylates myosin II heavy chain (van Leeuwen et al, 1999), an event subsequently followed by cell relaxation. In addition, Rac1 promotes actin polymerization via LIM motif-containing protein kinases (LIMK)/cofilin pathway, a step
necessary for lamellipodia formation and neurite outgrowth (Kuhn et al., 2000). Notably, Rac1 promotes cell-matrix adhesion formation via Git1/βPIX/paxillin complex, with as a consequence leads to paxillin distribution to the cell periphery and focal adhesions (Turner et al., 1999; Manabe et al., 2002; Paris et al., 2003). Finally, Rac1 stimulates the transcription of genes related to neuronal differentiation by activating c-terminal Jun kinase (JNK) (Teramoto et al., 1996; Kita et al., 1998).

As shown in Chapter 4, we investigated this pool of binding partners of Rac proteins in an active or inactive state in N1E-115 cells, and established that the majority of the interacting partners bind equally well to either Rac1 or Rac3. As discussed above, Rac1 and Rac3 share high degree of homology, notably, the effector binding region of the two proteins is virtually identical, which on its own clarifies that the potential for binding the interacting partners and the intensity of the bindings are expected to be quite similar between the two Rac proteins. Intriguingly, we found that binding to Git1 (G-protein-coupled receptor (GPCR)-kinase-interacting protein 1), harbors an important difference. Rac1/Git1 interaction is mediated by the exchange factor βPix (PAK-interacting exchange factor; our data and Brown et al., 2002), whereas Rac3/Git1 interaction does not involve βPix (or any other Pix isoform, Chapter 4). βPix is an activator (GEF) for Rac1, and previous studies have shown that Rac1/βPix interaction depends on specifically the PBR region in carboxy terminus of Rac1 (ten Klooster et al., 2006). As discussed in Chapter 3, this non-conserved triplet of amino acids just prior to the CAAX box is responsible for intracellular localization of Rac1 and Rac3, and the distinct effects they have on the neuronal cell morphology. We found that Rac3 does not bind βPix, and established that the lack of this interaction does not prevent Rac3 from interacting with Git1. We suspect that, considering the high level of homology between Rac1 and Rac3, it is likely that also Rac3/Git1 interaction occurs via a GEF, but distinct to the Pix family, possibly a GEF specific to Rac3. For this the very recently described Rac3-specific GEF, P-Rex1, could be a candidate. Waters and collaborators showed that P-Rex1 expression inhibited NGF-stimulated differentiation in neuroblastoma cells (PC12) and that this effect was dependent on the Rac-GEF activity of P-Rex1. Moreover, depletion of P-Rex1 induced spontaneous formation of neurite-like structures (Waters et al, 2008). Since both gain and loss of function of P-Rex1 in neuroblastoma cells strongly
resemble Rac3-related phenotypes, it is possible that P-Rex1 is the specific activator of Rac3 in neuronal cells. However, further studies will be required to resolve this hypothesis.

Further investigation of Rac3 effect on Git1 signaling revealed two important facts: (1) Rac3 attenuates Git1/paxillin interaction and (2) the activity of a substrate for the GAP activity of Git1, Arf6, is strongly diminished by Rac3 (Chapter 4). Git1 is a ubiquitous multidomain protein involved in a variety of cellular processes. This protein traffics between three cellular compartments (cytoplasmic complexes, focal adhesions and the cell periphery) by interacting with a number of proteins including the GTPases Arf6, Rac1 and Cdc42, and PAK, βPIX, MEK1 kinase, phospholipase Cγ (PLCγ) and paxillin (Hoefen and Berk, 2006). Previous studies have shown that the participation of active Rac1 in the βPix/Git1/Pak complex stimulates cell adhesion and spreading. Namely, Rac1 participation in the complex promotes an open conformation of Git1, which is required for the binding of paxillin to the carboxyl terminus of Git1. As a consequence, the whole complex translocates to focal adhesions (FAs) where it stimulates the FAs formation and turnover (Brown et al., 2002; Di Cesare et al., 2000; Matafora et al., 2001; Totaro et al., 2007). Since we show here that (a) paxillin is not distributed to the periphery of the cell in Rac3-expressing N1E-115 cells (Chapter 3), (b) Rac3 interacts with Git1 but without intermingling of βPix, and (c) that Rac3 strongly attenuates Git1/paxillin interaction, it is highly plausible that Rac3 participation in the Git1 complex prevents Git1 from adopting the open conformation, in contrast to Rac1. The consequence would be a masked Git1 carboxy terminus, which becomes unavailable for paxillin binding, and that would subsequently lead to defect in paxillin distribution and ultimately cause loss of cell-matrix adhesion. The subsequent experiments in Chapter 4, where we found that expression of a paxillin-binding mutant of Git1 with a constitutively open conformation induces spreading and stretching in otherwise rounded and poorly adherent Rac3-expressing cells, provide support for this hypothesis.

As mentioned above, the second large effect of Rac3 on Git1 signaling is a strong attenuation of Arf6 activity (Chapter 4). Arf subfamily proteins of the small Ras-like GTPases are ubiquitously expressed in eukaryotic cells, and six highly conserved
members of the family have been identified in mammalian cells (D'Souza-Schorey and Chavrier, 2006). Git1 contains a GTP-activating protein domain that stimulates GTP hydrolysis and thus inactivation of the small GTPase Arf6 (Albertinazzi et al, 2003; Meyer et al, 2006). This versatile regulatory protein is the least conserved member of the Arf subfamily, and it localizes to the plasma membrane and endosomal compartments, where it regulates endocytic membrane trafficking and actin remodeling, affecting in this way cell adhesion, migration, invasion of the extracellular matrix and neurite outgrowth (D'Souza-Schorey and Chavrier, 2006). Activation of Arf6 stimulates lamellipodia formation and cell spreading in HeLa and other cell types, while inhibition of Arf6 results in cell contractility and attenuation of cell-matrix adhesion (Donaldson, 2003). Since we found that restoration of Arf6 activity independently by three different tools is able to re-induce spreading and cell-matrix adhesion in otherwise contractile and rounded Rac3-expressing N1E-115 cells (Chapter 4), it is conceivable that Rac3-GIT1-mediated effect on Arf6 activity is causal in Rac3-induced cell rounding. Rac3 could inhibit Arf6 activity by somehow increasing the GAPing activity of Git1 while participating in the Git1 complex. A support for this hypothesis is provided by evidence that the Git1 point mutant that abolishes GAP activity but does not affect any other properties of the molecule (Git1R39K) counteracts the characteristic cell rounding of Rac3/N1E-115 cells (Chapter 4).

Our model proposes that the binding of Rac1 to the βPix/Git1 complex and its subsequent activation by βPix initiates Pak interaction with this complex and stimulates an open conformation of Git1. By contrast, Rac3 interaction with Git1 attenuates the accessibility of Git1 to paxillin. Thus the binding of paxillin to Git1 and subsequent targeting of the Pak/Rac1/βPix/Git1/ paxillin complex to the focal adhesions at the cell periphery, peripheral actin rearrangements and cell spreading facilitated by Rac1, are abolished in the case of Rac3 coupling to Git1. The latter stimulates a closed conformation of Git1 and, as a consequence, paxillin is not redistributed to the focal adhesions. Simultaneously, Rac3 binding possibly stimulates the Git1 ArfGAP activity towards Arf6, resulting in strongly reduced Arf6 activity. Due to this dual effect of Rac3 on Git1 signaling, the expression of Rac3 results in cell rounding and impaired differentiation of neuronal N1E-115 cells.
In summary, we found that endogenous mammalian Rac1 and Rac3 are both required for normal morphology of neuronal cells and that, in spite of high sequence homology, these two proteins exert different and opposing functions. Our data furthermore implicate that Rac3 and Rac1 oppose each other by differently affecting Git1 function. We propose that Rac1 and Rac3 each stimulate a different aspect of Git1 signaling in neuronal cells, leading to strikingly different morphological outcomes.

Recently, a Rac3 knockout mouse has been generated that showed no apparent developmental defects (Corbetta et al., 2005). However, Rac3-null mice appeared to be superior to their wild-type littermates in motor coordination and motor learning, indicating a possible function of Rac3 in development of the functional nervous system. Since Rac3 depletion induces pronounced neurite-like outgrowth and promotes the differentiation process (this thesis), it is tempting to speculate that Rac3 depletion in the circuitry in regions that facilitate the motor learning and coordination leads to enhanced neuritogenesis and arborization, facilitating stronger and more effective circuitry. Rac3 gene expression is highest postnatally 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 specific 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. Since our data implicate Rac3 as a suppressor of neurite outgrowth, it would be highly interesting to investigate whether Rac3 plays a role in maintenance of the neural stem cell plasticity, with possible implications for therapy for injuries of the peripheral nervous system (Tohill and Terenghi, 2004).

Taken all together, the data presented in this thesis reveal novel and intriguing aspects of small GTPases functions, and contribute to our understanding of complexity, specificity and cross-talk of these small but powerful regulating proteins in cell adhesion and related processes.
References


