Signaling behind bars: a role for bar domains

de Kreuk, B.J.

Citation for published version (APA):
de Kreuk, B. J. (2012). Signaling behind bars: a role for bar domains

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
SUMMARY AND CONCLUDING REMARKS
SUMMARY AND CONCLUDING REMARKS

Signal transduction, i.e. the translation of external cues to intracellular responses, is essential for proper functioning of cells and thereby of tissues and organs. Disturbed signal transduction caused by, for example, abnormal activation or inactivation of specific signaling pathways, can cause development of diseases such as chronic inflammation as well as cancer. In both types of disorder, deregulated cell motility as well as changes in cell-cell contact between endothelial or epithelial cells plays an important role.

Rho-family GTPases contribute to many steps of cancer development and inflammation (Millan and Ridley, 2005; Sahai and Marshall, 2002). As key regulators of the actin cytoskeleton, RhoGTPases are important mediators of cell polarity, adhesion, and motility (Jaffe and Hall, 2005; Le Clainche and Carlier, 2008; Ridley et al., 2003) and are therefore essential for both tumor cell migration as well as leukocyte extravasation (Nourshargh et al., 2010; Sahai and Marshall, 2002). In addition to regulating cell motility, RhoGTPases contribute to cancer and inflammation by acting on endothelial and epithelial cell-cell contacts. As important regulators of epithelial cell-cell adhesion (Citi et al., 2011), RhoGTPases have been implicated in epithelial-mesenchymal transition (EMT) (Guarino et al., 2007; Savagner, 2001). EMT is characterized by weakening of cell-cell contacts, which may allow detachment of cells from the epithelium leading to tumor cell invasion (Guarino, 2007). In line with this, abnormal activation and/or expression of RhoGTPases has been observed in many types of cancer (Sahai and Marshall, 2002). In addition to regulating epithelial cell-cell adhesion, RhoGTPases are key regulators of endothelial cell-cell contacts (Beckers et al., 2010; Wojciak-Stothard et al., 2001; Wojciak-Stothard and Ridley, 2002). By regulating endothelial cell-cell adhesion, RhoGTPases control endothelial barrier function which is important as increased permeability can cause excessive leukocyte extravasation. Furthermore, RhoGTPases contribute to leukocyte extravasation by governing endothelial actin dynamics required for formation of so-called docking structures upon binding of the leukocyte to the endothelium (Carman et al., 2003; Carman and Springer, 2004). These docking structures are required for efficient leukocyte diapedesis. Thus, RhoGTPase activation and signaling is tightly controlled in order to prevent diseases such as chronic inflammation and cancer. Therefore, understanding of the mechanisms that drive these different processes is of great importance.

In this thesis, we aimed to generate additional insights in modes of regulation that cells use to control RhoGTPase signaling. We describe several novel components such as the F-BAR protein PACSIN2 and HMHA1 that regulate RhoGTPase activity. In addition, we provide new insights into how Rac1, in conjunction with Nedd4 and Dvl1, regulates epithelial junction integrity. Finally, we identify a novel role for PACSIN2 in growth factor receptor activation and signaling.
Regulation of RhoGTPase activity and function

RhoGTPases act as molecular switches. Stimulation of cells by external cues allows guanine-nucleotide-exchange factors (GEFs) to catalyze the exchange of GDP for GTP thereby activating the GTPases (Rossman et al., 2005). Upon activation of RhoGTPases, specific downstream effectors, such as the p21-activated kinase (PAK) serine/threonine kinase for Rac1, or Rho-associated coiled-coil-containing protein kinase (ROCK) (Bishop and Hall, 2000) for RhoA, are in turn activated to further initiate downstream signaling. Inactivation of RhoGTPases is mediated by GTPase-activating proteins (GAPs) that stimulate the low intrinsic GTPase activity which controls hydrolysis of GTP to GDP (Bernards and Settleman, 2004). As human RhoGAPs and GEFs outnumber the 22 human members of the Rho-family GTPases (Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007) it could be speculated that various factors such as activation upon external stimulation, selective intracellular localization, and cell-specific specific signaling and expression of GEFs and GAPs are important determinants in the regulation of RhoGTPase (in)activation.

An important feature in the activation of RhoGTPases is the translocation between the cytosol and the plasma membrane. In contrast to inactive GTPases that mainly reside in the cytosol in complex with RhoGDI (Rho Guanine nucleotide Dissociation Inhibitor) (del Pozo et al., 2002; Olofsson, 1999), active RhoGTPases are localized at the plasma membrane. Furthermore, GTPase inactivation most likely occurs intracellularly, suggesting an important role for vesicular traffic in the regulation of RhoGTPase activity. In line with this, several studies have shown the importance of internalization for inactivation of the RhoGTPase Rac1. Loss of cell adhesion to the matrix induces internalization of Rac1 from cholesterol-rich membrane domains leading to the inactivation of Rac1 (del Pozo and Schwartz, 2007). Inhibition of internalization, e.g., by inhibition of Dynamin, results in increased Rac1 activity and aberrant downstream signaling (Schlunck et al., 2004). Thus, it is generally accepted that traffic plays a key role in regulating RhoGTPase activation and signaling.

Proteins of the BAR-domain superfamily are important regulators of membrane dynamics, including internalization and vesicular transport. So far, six subclasses of BAR-domains are represented in this family: the classical BAR domain, the N-BAR, BAR-PH, PX-BAR, F-BAR, and I-BAR domains (Qualmann et al., 2011). As a result of sensing membrane curvature, BAR-domains associate as banana-shaped dimers with membranes and by doing so can further promote curvature which leads to formation of invaginations or protrusions depending on the type of BAR domain (Frost et al., 2009). Most BAR domain proteins contain one or more protein-binding scaffolding/adapter domains and are often capable of forming dimers (Chitu and Stanley, 2007; Kessels and Qualmann, 2004) allowing linkage of membrane dynamics to signaling pathways governing actin dynamics. Consequently, many BAR-domain proteins have been implicated in the regulation of RhoGTPase signaling. This topic has been reviewed in Chapter 2. BAR-domain proteins involved in RhoGTPase signaling can be divided in two major classes. First we have identified a class of BAR-domain proteins that regulate RhoGTPase function by mediating the internalization or targeting of specific GTPases, such as PACSIN2 (described in more detail in Chapter 3) or by forming a physical link between RhoGTPases and their up- or downstream activators/effectors, such as Abba and IRSp53. In addition, we identified a second class of RhoGTPase-regulating BAR-domain proteins that harbor, in addition to the BAR domain, also a RhoGAP/GEF domain and thereby control RhoGTPase function by regulating GTP binding or hydrolysis. Well-known members of this subclass are the BAR-RhoGEF, Tuba (Cestra et al., 2005) and the BAR-RhoGAPs, GRAF1, Oligophrenin-1, and SH3BP1 (Cicchetti et al., 1995; Fauchereau et al., 2003; Hildebrand et al., 1996). A common theme for many of these BAR-GAPs is the notion that their BAR domain is required for targeting to specific sites due to its membrane-binding capacity as well as for auto-inhibition. Specific stimuli, leading to, e.g., translocation or protein-protein interactions, can release this auto-inhibition by the BAR domain allowing localized downregulation of GTPase activity.

In Chapter 4 we identify a novel member of this BAR-RhoGAP family called Human Minor Histocompatibility Antigen-1 (HMHA1). Sequence alignment revealed high homology of HMHA1 with well-known RhoGAPs including p50RhoGAP and GRAF1. Furthermore, we generated a homology model of the HMHA1 GAP domain in association with RhoA which indicates that HMHA1 fulfills the structural requirements to function as a RhoGAP. In vitro GAP assays with purified proteins confirmed that HMHA1 is a genuine RhoGAP. Interestingly, in contrast to the HMHA1 C1-GAPtail construct, which lacks the BAR domain, full-length HMHA1 shows little GAP activity towards RhoGTPases suggesting that the N-terminal BAR domain acts as an inhibitory module for GAP function. These observations place HMHA1 in a larger subfamily of RhoGAPs that comprise both a BAR- and a RhoGAP domain (De Kreuk and Hondrik, 2017) that are auto-inhibited by their N-terminal BAR domain (Eberth et al., 2009). As a regulator of RhoGTPase activation, HMHA1 controls actin dynamics. Cells expressing HMHA1 mutants that lack the N-terminal BAR domain, show altered F-Actin and focal adhesion distribution and are less adhesive. This correlates well with the notion that inactivation of RhoGTPases affects cell adhesion and migration. Although in vitro HMHA1 shows GAP activity towards Rac1, Cdc42, and RhoA, the in vivo targets remain to be investigated. Our data suggest that Rac1 and RhoA but not Cdc42 are in vivo targets of HMHA1 but further studies are needed to confirm this. In normal conditions, HMHA1 expression is restricted to the hematopoietic system (de Bueger et al., 1992) indicating that in other cell types different GAPs are involved in the regulation of RhoGTPases. This further underscores the notion that cell-type specific expression of RhoGAPs is an important aspect in the regulation of GTPase (in)activation.

As described in Chapter 2, many BAR-family proteins have been described to regulate RhoGTPase activation and signaling. In Chapter 3 we identify the F-BAR protein PACSIN2 as a novel regulator of Rac1 activation and signaling. Although...
several BAR proteins, such as the I-BAR protein IRSp53 and the BAR protein Arfaptin (Miki et al., 2000; Tarricone et al., 2001), were previously described to regulate Rac1, no studies have focused on PACSIN proteins and their role in regulating RhoGTPases. In this study we show that PACSIN2 interacts with the hypervariable C-terminal region of Rac1. Thus, PACSIN2 is part of a larger group of proteins we identified earlier to interact with the Rac1 C-terminus and regulate its function. These include caveolin1, the Rac1 GEF β-Pix, and the nuclear proto-oncogene SET/IZP2 (Nethe et al., 2010; ten Klooster et al., 2006; ten Klooster et al., 2007). Although these proteins all interact with the same region in Rac1, they act independently of each other and reside in different intracellular compartments supporting the notion of compartmentalized, parallel signaling in so-called ‘spatio-temporal signaling modules’ (Pertz, 2010). In addition, we showed that PACSIN2 regulates Rac1 activity, and as a consequence cell spreading and migration, by promoting its internalization. Furthermore, PACSIN2-mediated downregulation of Rac1GTP levels could be prevented when Dynamin function was inhibited. These data are in line with other studies that showed the importance of Dynamin-mediated internalization in regulation of Rac1 activity (Schlunk et al., 2004). Further support comes from studies that show that PACSIN2 associates with Dynam (Chitu and Stanley, 2007; Kessels and Qualmann, 2004).

**RhoGTPases in the regulation of cell-cell contacts**

Via anchorage to junctional complexes, the actin cytoskeleton is involved in regulating junction assembly, disassembly, and maturation (Mege et al., 2006). RhoGTPases, and in particular Rac1 and RhoA, have been implicated in the regulation of cell-cell adhesion (Citi et al., 2011; Wojciak-Stothard and Ridley, 2002). Moreover, RhoGTPases are important regulators of epithelial-mesenchymal transition (EMT) which is caused by weakening of cell-cell contacts resulting in the dissociation of single cells from the epithelium (Guarino et al., 2007; Savagner, 2001). In line with this, aberrant expression of RhoGTPases has been observed in many cancers (Sahai and Marshall, 2002).

Although some studies found opposing effects of RhoGTPases on junction stability, it is clear that tightly balanced RhoGTPase signaling is key to junction stabilization and the integrity of the epithelium (Citi et al., 2011; Yamada and Nelson, 2007). Although the regulatory role of RhoGTPases in junction stability has been widely studied, we identified in Chapter 6 a novel mechanism through which Rac1 stabilizes epithelial cell-cell junctions. Upon Rac1 activation, the HECT E3 ligase Nedd4 is translocated to cell-cell junctions where it colocalizes with the scaffold protein Dvl1, which is a negative regulator of epithelial cell-cell contact (Elbert et al., 2006). Our data suggest that here, Nedd4 mediates the ubiquitylation of Dvl1 leading to degradation of Dvl1 and as a consequence junctional integrity is increased. When Nedd4 expression was reduced by RNA interference, Dvl1 levels increased and as a result, junctional integrity was impaired. As mentioned before, RhoGTPases regulate junction integrity by controlling actin dynamics. However, in this study we found no effects of either Nedd4 or Dvl1 on the actin cytoskeleton. Interestingly, in unpublished data we did see clear effects on the microtubule network in that depletion of Nedd4 increased the acetylation of microtubules. It has been well established that the microtubule network is important in the regulation of cell-cell junctions by allowing transport of specific proteins such as N-Cadherin to and from junctions (Mary et al., 2002). Also, to different affinities for microtubule-linked motor proteins, acetylated microtubules show altered directionality of protein traffic along microtubules (Reed et al., 2006). Interestingly, Dvl1 also increases acetylation of microtubules (Krylova et al., 2000) suggesting that decreased Dvl1 degradation in Nedd4-depleted cells results in increased microtubule acetylation. As microtubule acetylation affects the stability of, and traffic along microtubules, it could well be that the Rac1-Nedd4-Dvl1 axis mediates junctional integrity by controlling polarized traffic along the microtubule network. Indeed, experiments with TSA, a pharmacological inhibitor of histone deacetylases (HDACs), or Tubastatin, an inhibitor of HDAC6, increased microtubule acetylation and resulted in decreased junctional integrity similar to what we observed for Nedd4-depleted cells (unpublished data). In addition to the regulation of epithelial cell-cell junctions by actin dynamics, we thus revealed a novel microtubule-dependent mechanism through which Rac1 stabilizes cell-cell junctions.

Abnormal expression or activation of GAPs and GEFs, important regulators of GTPase activity (Bernards and Settleman, 2004; Rossman et al., 2005) is also frequently associated with cancer development and progression (Vigil et al., 2010). In Chapter 4 we identified HMHA1 as a novel RhoGAP regulating the actin cytoskeleton. Although in normal conditions, HMHA1 expression is restricted to the hematopoietic system (de Bueger et al., 1992), HMHA1 expression was observed in many epithelial tumors as well (Klein et al., 2002). Thus, aberrant expression of the RhoGAP HMHA1, and as a consequence disturbed RhoGTPase signaling, could be key to the transforming and invasive character of these epithelial tumor cells, although further studies are needed to firmly prove this.

Signaling via growth factor receptors is essential for many cellular processes such as proliferation, cell survival, and cell migration (Blume-Jensen and Hunter, 2001; van der Geer et al., 1994). Activation of growth factor receptors leads to activation of many downstream signaling pathways including activation of RhoGTPases (Itoh et al., 2008; Samson et al., 2010) and of the Erk and Akt pathways (van der Geer et al., 1994). Growth factor stimulation also triggers rapid internalization of the growth factor receptor. Although compartmentalized signaling from endosomes does occur, internalization generally results in the termination of signaling either by targeting the receptor for degradation or by recycling inactive receptors back to the plasma membrane (Sorkin and Goh, 2008; Wiley, 2003). Moreover, the amount of receptor on the cell surface is an important determinant for the magnitude of signaling output. Thus, it is clear that traffic of growth factor receptors is important in modulating growth factor signaling. Through their role in regulating membrane dynamics, the BAR-domain superfamily has often been implicated in regulating growth factor signaling.
In Chapter 5 we show that PACSIN2 is a novel regulator of Epidermal Growth Factor (EGF) receptor activation and signaling. Similar to BAR-family proteins such as endophilin and CIP4 (Hu et al., 2009; Soubeyran et al., 2002), PACSIN2 regulates traffic of growth factor receptors and thereby controls signaling output. We show that PACSIN2 depletion results in increased surface levels of the EGF receptor in resting cells. Upon EGF stimulation, we still observed increased surface expression of the EGF receptor compared to control cells. However, EGF-mediated internalization of the receptor still occurred normally suggesting that PACSIN2 does not regulate ligand-dependent internalization of the EGF receptor. Despite normal internalization, EGF stimulation increased receptor activation as well as downstream signaling in the absence of PACSIN2. In line with the notion that receptor surface levels are an important determinant for signaling output, it could well be that in PACSIN2-depleted cells, either through regulating ligand-independent internalization or by promoting recycling, the increased EGF receptor surface levels allow for increased signaling upon stimulation. Interestingly, it was previously shown that dominant active Rab5 (Q79L) GTPase caused ligand-independent internalization of the EGF receptor resulting in decreased surface EGF receptor levels. Subsequently, upon EGF stimulation, less receptor activation and downstream signaling was observed (Dinneen and Ceresa, 2004). These observations, together with our data that show that PACSIN2 localizes to Rab5-positive endosomes (de Kreuk et al., 2011) and that dominant-active Rab5Q79L accumulates PACSIN2 on these endosomes (unpublished data) suggest that PACSIN2 and Rac5 function in a similar pathway regulating traffic of the EGF receptor independent of the ligand. However, both for PACSIN2 and Rab5 a role in recycling of the (in)active receptor cannot be excluded. Therefore, further studies are needed to determine the exact mechanisms via which PACSIN2 regulates EGF receptor signaling. The regulatory role of PACSIN2 appears not specific for the EGF receptor as increased signaling, following loss of PACSIN2 expression, was also observed downstream of Hepatocyte Growth Factor (HGF) or Tumor Necrosis Factor-α (TNFα), suggesting a more general regulatory role for PACSIN2 in growth factor receptor signaling. As we have shown that PACSIN2 depletion results in increased Rac1 activity (Chapter 3) as well as increased EGF receptor activation and signaling (Chapter 5), it could well be that PACSIN2 links EGF signaling to RhoGTPase activation. However, although we found that the PACSIN2-Rac1 interaction was increased upon EGF stimulation (unpublished data) we could find no biochemical evidence for such a link, suggesting that PACSIN2 regulates both processes through independent mechanisms, but further studies are needed to confirm this.

In conclusion, in this thesis we describe several novel components of growth factor receptor and RhoGTPase activation and signaling. We have demonstrated that the F-BAR protein PACSIN2 is an important regulator of Rac1 output and, as a consequence, cell spreading and migration. This study further established the importance of traffic in the regulation of RhoGTPase function. In addition, we identified HMHA1 as a novel hematopoietic cell-restricted RhoGAP that regulates the actin cytoskeleton as well as cell spreading. Because expression of the HMHA1 has been observed in epithelial tumor cells as well, future studies should define the mechanisms through which HMHA1 regulates the transformation and invasiveness of these tumor cells. Furthermore, owing to its role as a minor histocompatibility antigen, T cells are generated against epitopes of HMHA1. As the healthy epithelium does not express HMHA1, it might prove an excellent target for tumor therapy. In addition, we describe a novel mechanism through which Rac1 regulates epithelial junction stability independent of the actin cytoskeleton. Although many studies focused on the regulatory role of RhoGTPase in junction remodeling in the context of actin dynamics, our study revealed that additional mechanisms through which RhoGTPases affect cell-cell junctions exist, further underscoring the complexity of RhoGTPase signaling in junction remodeling. Finally, although many pathways that regulate growth factor receptor traffic have already been described, we demonstrate in this thesis a novel component, PACSIN2, that acts as an important regulator of growth factor receptor activation and signaling.
REFERENCE LIST

Beckers, C. M., van Hinsbergh, W. V. and van Nieuw Amerongen, G. P. (2010). Driving Rho GTPase ac-


Bernards, A. and Settlement, J. (2004). GAP control-


mology (PCH) proteins: coordinators of membrane-cytoskeletal interactions. Trends Cell Biol 17, 145-

154.


Dinneen, J. L. and Ceresa, B. P. (2004). Continual ex-


Elbert, M., Cohen, D. and Musch, A. (2005). BAR1 promotes cell-cell adhesion and inhibits dishevelled-


Miki, H., Yamaguchi, H., Suetsugi, S. and Tak-


chem J. 385, 329-337.


Olofsson, B. (1999). Rho guanine dissociation inhibi-
tors: pivotal molecules in cellular signalling. Cell Sig-
al. 11, 207-217.

Pertz, O. (2010). Spatial-temporal Rho GTPase sign-
ing - where are we now? J Cell Biol 123, 1841-1850.


Savagner, P. (2001). Leaving the neighborhood: mo-

lecular mechanisms involved during epithelial-mes-

Schulze, D. G., Klussendorf, W. B., Rusk, N., Syn-


Soubeyran, P., Kownatz, K., Szymkiewicz, I., Lang-

Tarricone, C., Xiao, B., Justin, N., Walker, P. A., Ritz-


Tcherkezian, J. and Lamarche-Vanne, N. (2007). Cur-

ent knowledge of the RhoGAP family of pro-


ten Krooper, J. P., Leeuwen, I., Scheres, N., An-


Wojcik-StoJad, B., Potapov, S., Eichholtz, T. and


Wojcik-StoJad, B. and Ridley, A. J. (2002). Rho GTPases and the regulation of endothelial permea-
