Signaling behind bars: a role for bar domains

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GENERAL INTRODUCTION

Mechanisms of molecular communication within and between cells are characterized by stunning complexity based on a limited set of common principles. Extracellular signals including soluble ligands as well as cell- or extracellular matrix-associated proteins and lipids engage cell surface receptors, initiating a chain of intracellular signaling events that culminate in an appropriate response. The intracellular transport of such signals can occur through chains of sequential protein modifications such as phosphorylation, through random diffusion of conformationally altered molecules and through the use of physical means such as tightly controlled transport on membrane domains or vesicles. In order to better understand molecular interactions and mechanisms that govern signal transduction within the cell’s interior, classical biochemical methods need to be combined with modern approaches such as high resolution imaging and the use of selective removal of proteins by RNA interference.

Here, we focus on the regulation of signal transduction and cellular responses by two classes of proteins that play distinct, complementary roles in molecular cell biology. These are the family of BAR-domain proteins, non-enzymatic adapters that bind to lipids as well as proteins, bridging intercellular vesicular structures with the cellular signaling machinery. Secondly, we focus on the family of small GTPases, molecular switches that play key roles in cytoskeletal dynamics, cell morphology and cell adhesion and migration. Below, we will briefly introduce these protein families and provide additional background on their role in two exemplary processes, i.e. growth factor signaling and the control of cell-cell contact.

The BAR Domain Family

BAR-domain proteins are capable of sensing membrane curvature, e.g. generated by formation of clathrin coated pits (Doherty and McMahon, 2009). In addition, by binding as banana-shaped dimers (Peter et al., 2004) to negatively charged lipids, such as phosphoinositides and phosphatidyserine, they can further promote membrane curvature, which eventually leads to either invagination or protrusion depending on the type of BAR domain (Suetsugu et al., 2010). The first BAR domain was characterized almost two decades ago in Amphiphysin (David et al., 1994). Since then, many proteins were found to harbor a BAR domain. Interestingly, several different BAR-like domains were identified based on sequence homology and structural similarity, leading to the expansion of the BAR-domain protein superfamily. Currently, this family (Table 1) comprises proteins encoding one of six classes of BAR-domains: the classical BAR domain, or the N-BAR, BAR-PH, PX-BAR, F-BAR, and I-BAR domains (Frost et al., 2009; Qualmann et al., 2011; Suetsugu et al., 2010). As most BAR domain proteins can form oligomers, and contain one or more protein-binding scaffolding/adaptor domains (e.g. SH3 domains), they can form a molecular link between processes such as membrane dynamics and actin reorganization (Kessels and Qualmann, 2004; Kessels and Qualmann, 2006). As a result, BAR-domain containing proteins have emerged as important regulators of intracellular signaling pathways.
Membrane Binding and Deformation

Two major mechanisms allow BAR proteins to sense and bind to membranes resulting in the induction of membrane curvature. The first, also called the scaffold mechanism, involves the binding of BAR-domain proteins to membranes as intrinsically-curved crescent-shaped dimers (Qualmann et al., 2011). These dimers are lined with positively charged amino acids that allow binding to the negatively charged surface of membranes. The localization of these positive residues determines the direction of curvature induced by BAR proteins. In most cases, the concave surface is lined with these positive residues, which results in the induction of invaginations as is the case for the Arfaptin BAR- or the CIP4 F-BAR domain (Qualmann et al., 2011). In contrast, in the I-BAR domains, such as in IRSp53, these positively charged residues line the convex surface of the dimer thereby inducing outward protrusions such as filopodia (Mattila et al., 2007). Alternatively, proteins of the N-BAR subclass partially embed hydrophobic or amphipathic domains into the membrane (Qualmann et al., 2011). By inserting an amphipathic α-helix, the N-BAR proteins Endophilin and Amphiphysin (Gallop et al., 2006; Peter et al., 2004), can sense and induce membrane curvature, which is aided by the concave-surface located positively charged amino acids. In addition to the N-BAR sub-class, one member of the F-BAR subfamily was shown to insert a hydrophobic domain into the membrane to induce curvature. In conjunction with the positively charged amino-acids at the concave surface of the F-BAR dimer, PACSIN proteins use amphipathic “wedge” loops to sense and induce membrane curvature (Shimada et al., 2010; Wang et al., 2009), a mechanism similar to that used by amphipathic helices of the N-BAR sub-class.

Functions of BAR Domain Proteins

In general, BAR domain proteins can act as adaptor proteins linking various processes. Most BAR domain proteins comprise one or more protein-binding scaffolding/adaptor domains (e.g. SH3 domains) with which they associate to regulatory proteins including Rac1, N-WASP, Dynamin, and Synaptojanin (Chitu and Stanley, 2007; de Kreuk et al., 2011; Kessels and Qualmann, 2004). In addition to these protein-protein interaction domains, some BAR domain proteins use their membrane-binding BAR domain to mediate protein-protein interactions. This was shown for IRSp53 that utilizes its I-BAR domain for both Rac1 binding and formation of protrusions (by creating outward curvature) (Abou-Kheir et al., 2008; Miki et al., 2000). As mentioned above, BAR proteins are known to oligomerize (Kessels and Qualmann, 2004; Kessels and Qualmann, 2006) which allows for multiple interactions. Due to this feature, several BAR proteins were found to provide a link between membrane dynamics, e.g. through interactions with Dynamin, and the actin cytoskeleton, e.g. through interactions with N-WASP. During endocytosis, BAR proteins are translocated to the plasma membrane promoting formation of tubular invaginations mediated by their BAR domain followed by the targeting of Dynamin to these tubular invaginations to ensure proper vesicle scission. Subsequently, BAR proteins can target the actin-polymerization machinery to these sites, via interactions with N-WASP, to provide force needed for vesicle internalization. In line with their role in endocytosis and vesicle transport, BAR proteins are also implicated in traffic of growth factor receptors, regulating receptor activation and downstream signaling (see below). Finally, several BAR proteins were shown to regulate activity and function of GTPases. It was shown that the BAR protein Arfaptin is an effector of Arf family GTPases. Arfaptin interacts with Rac1 as well providing a link between the Arf and Rho family signalling pathways (Tarricone et al., 2001).
In general, it can be concluded that BAR domain proteins are important regulators of many processes by providing docking platforms for the targeting of proteins to specific sites or pathways, or by linking different processes to ensure proper signaling.

Rho-Family GTPases
RhoGTPases constitute a distinct subfamily within the superfamily of Ras-related small GTPases and are involved in the regulation of cell polarity and motility through their effects on the actin cytoskeleton and membrane traffic (Jaffe and Hall, 2005; Ridley, 2006). The subfamily of RhoGTPases consists of 22 members (Wennerberg and Der, 2004). Interestingly, although they share very high sequence homology, RhoGTPases induce unique biological effects (Bishop and Hall, 2000; Bosco et al., 2009). This is partially mediated by differential binding of effector proteins to activated GTPases. Although some effectors are known to bind to several activated RhoGTPases, often each GTPase interacts with a specific subset of effectors thereby regulating specific cellular responses (Bishop and Hall, 2000). Furthermore, the hypervariable C-terminal region of the RhoGTPases contributes to differential cellular signaling as well. In addition to its role in subcellular targeting and control of signaling (Michaelson et al., 2001; ten Klooster and Hordijk, 2007; van Henrik et al., 2003), the C-terminal hypervariable region of RhoGTPases was shown to mediate protein-protein interactions. Our group has extensively studied binding partners of the Rac1 C-terminus and found several proteins, such as PACSIN2, Caveolin1, and β-Pix, that interact specifically with the hypervariable region, independent of the activation status of Rac1 (de Kreuk et al., 2011; Nethe et al., 2010; ten Klooster et al., 2006). Thus, although RhoGTPases share high sequence homology, hardly any sequence homology is found in their C-terminal hypervariable domain (Fig. 1A) explaining the large variety in interaction partners and biological outcomes. However, this will not explain the wide variety of biological effects exerted by a single RhoGTPase. In this case, specific localized signaling is secured by so-called “spatio-temporal signaling modules” (Pertz, 2010) which allows RhoGTPases to interact with different regulators at different intracellular locations.

Regulation of RhoGTPase Activity
RhoGTPases act as molecular switches (Fig. 1B). They cycle between an inactive GDP-bound state and an active GTP-bound state (Hall, 1998). This transition is regulated by guanine-nucleotide-exchange factors (GEFs) that facilitate the exchange of GDP for GTP (Rossman et al., 2005) and by GTPase-activating proteins (GAPs) that stimulate the low intrinsic GTPase activity thereby hydrolizing GTP to GDP (Bernards and Settleman, 2004). An important feature in the activation cycle is the translocation of RhoGTPases between the cytosol and the plasma membrane. Whereas, inactive RhoGTPases reside in the cytosol, bound to RhoGDI (Rho guanine nucleotide dissociation inhibitor), most activated RhoGTPases are localized at the plasma membrane. This translocation suggests an important role for vesicular trafficking.

![Figure 1: Regulation of RhoGTPases.](image-url)
traffic in the control of GTPase (in)activation, e.g. by targeting them to intracellular sites for GAP-mediated inactivation. Several studies have shown the importance of internalization in Rac1 inactivation. Active Rac1 resides in cholesterol-rich membrane domains and loss of cell adhesion to the matrix induces Rac1 internalization with Rac1 inactivation as a result (de Kreuk et al., 2011; Schlunck et al., 2004). As mentioned above, BAR-domain proteins are important regulators of membrane dynamics, regulating vesicle traffic and endocytosis. Logically, these proteins have been implicated in the control of RhoGTPase activity and function. In chapter 2 of this thesis, this will be further discussed.

It is generally accepted that RhoGTPase activity is controlled by GEFs that activate GTPases, and GAPs that inactivate GTPases. In recent years, accumulating evidence suggest an alternative mechanism of RhoGTPase inactivation, independent of GAP proteins. Via this mechanism, signaling is terminated as a result of RhoGTPase poly-ubiquitylation (Nethe and Hordijk, 2010; Schaefer et al., 2012). In contrast to GAP-mediated inactivation, which allows the GTPase to re-enter the activation pathway, poly-ubiquitylation of activated RhoGTPases results in their degradation and removal from the GTPases cycle.

**RhoGTPases as Regulators of Cell Adhesion and Migration**

Cell migration is an essential feature of physiological processes such as development and wound healing. The actin cytoskeleton regulates the capacity of cells to migrate by controlling cell polarity, organization of adhesive structures, and the generation of force (Ridley et al., 2003). RhoGTPases, and in particular Rac1, Cdc42, and RhoA, are important regulators of cytoskeletal remodeling and thereby regulate cell adhesion and migration. In order to migrate, cells acquire a polarized morphology. At the front, Arp2/3-mediated actin assembly drives formation of flat membrane protrusions, called lamellipodia, mediated by Rac1-driven actin polymerization as well as Cdc42-mediated formation of finger-like protrusions called filopodia (Jaffe and Hall, 2005; Le Clainche and Carlier, 2008). Upon formation of these membrane protrusions at the front, the cell forms mature adhesions that connect the actin cytoskeleton to the extracellular matrix. Whereas Rac1 and Cdc42 are generally considered to control actin remodeling at the front of the cell, RhoA acts primarily at the rear where it regulates actomyosin contractility and disassembly of adhesions (Jaffe and Hall, 2005; Le Clainche and Carlier, 2008), driving forward migration. Recent studies, however, have revealed that RhoA activation occurs in the leading edge as well (Pertz et al., 2006). This suggests that the regulation of the actin cytoskeleton by RhoGTPases is far more complex than initially thought. Furthermore, cytoskeletal remodeling is regulated in a temporal and spatial manner and does not rely on individual RhoGTPases but rather on a carefully balanced interplay between different GTPases. This idea is supported by the observation that altering signaling of one specific RhoGTPase affects the levels, activity and function of other GTPases as well (Bouler et al., 2010) further underscoring the complexity of RhoGTPase signaling.

**Control of Epithelial Cell-Cell Contact by RhoGTPases**

Cohesive sheets of epithelial cells which cover most, if not all, body surfaces are a fundamental feature of multicellular organisms. The epithelium forms a physical barrier that separates the internal milieu of the body from its external environment (Perez-Moreno et al., 2003; Watanabe et al., 2009). An important feature of the epithelium regulating this barrier function is the presence of tightly controlled cell-cell adhesions that are essential for tissue integrity. Adhesion between epithelial cells is generally mediated by three types of junctions: desmosomes, tight junctions, and adherens junctions (Perez-Moreno et al., 2003). Here, I will mainly focus on tight- and adherens junctions, as the role of RhoGTPase signaling in desmosomes seems minimal. One major role of tight junctions is to act as anatomical fences separating the apical from the basolateral domains of the plasma membrane (Diamond, 1977). On the other hand, adherens junctions play key roles in tissue sorting during development (Stepniak et al., 2009). Furthermore, both tight- and adherens junctions are important focal sites for anchorage of the actin cytoskeleton. In addition, these junctions can function as signaling hubs by their local concentration of downstream effectors (Citi et al., 2011). Tight junctions harbor several different proteins such as occludin, claudin, and ZO-1 (Citi et al., 2011). Adherens junctions are characterized by the presence of cadherins, which are calcium-dependent transmembrane receptors. Intracellularly, the catenins (α, β, γ, and p120-catenin), form a complex with the cadherins (Perez-Moreno et al., 2003), linking the transmembrane cadherin to the actin cytoskeleton.

The actin cytoskeleton which is anchored to the junctional complexes, has a profound role in junction assembly, disassembly, and maturation (Mege et al., 2006). As RhoGTPases are important regulators of cytoskeletal remodeling, their role in epithelial junction remodeling has been extensively studied. By promoting the formation of lamellipodia, Rac1 was implicated in the initiation of epithelial cell-cell contacts (Citi et al., 2011; Yamada and Nelson, 2007). In contrast to Rac1, the contribution of which in junction remodeling is broadly accepted, the role of RhoA in cell-cell contact is less clear. Some studies indicate that RhoA signaling is important for junction disassembly, e.g. it was shown that during epithelial-mesenchymal transition, RhoA activity is important for cadherin-contacts disruption (Bhowmick et al., 2001). Similarly, Schlegel and coworkers showed that increased RhoA activity caused epithelial barrier breakdown. However, they also showed that RhoA activity was needed to maintain barrier function (Schlegel et al., 2011) indicating a dual function for RhoA in barrier regulation. Several other studies showed that RhoA is involved in stabilization of junctions as well (Baum and Georgiou, 2011; Braga et al., 1997). An elegant study from Yamada and Nelson,
which involved high-resolution live-cell imaging of RhoGTPase biosensors, showed the differential roles of Rac1 and RhoA in junction remodeling. Whereas Rac1 has a key role in initiating adhesive contacts, RhoA regulates expansion and completion of cell-cell contacts (Yamada and Nelson, 2007). Recently we identified a novel mechanism through which Rac1 regulates junctional integrity which is described in Chapter 6. Rac1 activity targets the E3 ligase Nedd4 to epithelial junctions thereby stabilizing the junctions. Here, Nedd4 regulates the ubiquitylation and subsequent degradation of the scaffold protein Dvl1, which was shown to be a negative regulator of epithelial cell-cell contacts (Elbert et al., 2006; Nethe et al., 2012).

Thus, RhoGTPases are key regulators of epithelial junction remodeling and a tightly controlled balance between the different RhoGTPases and their activities is required to maintain and modulate epithelial barrier integrity.

Growth Factor Signaling

Growth factor signaling, including through receptor tyrosine kinases, is key to fundamental processes such as proliferation, migration, and cell survival (Blume-Jensen and Hunter, 2001; van der Geer et al., 1994). Logically, in several human diseases such as cancer, aberrant expression and activation of growth factor receptors has been observed. Internalization and intracellular trafficking is central to growth factor signaling. One of the best studied growth factor receptor-mediated pathways is that induced by Epidermal Growth Factor (EGF). EGF binding to the extracellular domain of the EGF receptor leads to receptor autophosphorylation within the intracellular regions and activation. Subsequently, several downstream signaling pathways, such as the Erk and Akt pathways are activated (Gan et al., 2010). The level of EGF receptors on the cell surface is an important determinant for the outcome of downstream signaling, further underscoring the importance of endocytic traffic in the regulation of growth factor signaling.

Endocytic Trafficking of the EGF Receptor

In resting cells, the EGF receptor is constitutively internalized in the absence of ligand. Upon internalization, the EGF receptor traverses the endosomal compartment after which most of the receptor recycles to the plasma membrane (Fig. 2; left panel). As the EGF receptor internalization rate is much slower than its recycling rate, the majority of the EGF receptors are localized to the surface (Herbst et al., 1994). Upon ligand binding, the EGF receptor is rapidly internalized and targeted to early endosomes (Fig. 2; right panel). In addition to signaling from the plasma membrane, compartmentalized EGF receptor signaling from early endosomes occurs which is important for specific signaling pathways such as the Erk pathway (Sigismund et al., 2008). To limit continuous signaling, the EGF receptor is subsequently targeted either to lysosomes for degradation or it recycles (in its inactive conformation) back to the plasma membrane (Sorkin and Goh, 2008; Wiley, 2003). In both cases, signaling is terminated.

To avoid aberrant signaling, transport of the EGF receptor, as well as of other growth factor receptors, through the different endocytic compartments is under tight control. Through their role in regulating membrane dynamics, the BAR-domain family of proteins has been implicated in growth factor receptor transport trafficking. Some BAR domain proteins such as endophilin are involved in the internalization of the EGF receptor, controlling the initial steps of trafficking (Soubeyran et al., 2002). Other BAR-family proteins, such as CIP4 and the sorting nexins (SNX) 1 and 5 regulate late events in EGF receptor traffic (Hu et al., 2009; Kurten et al., 1996; Liu et al., 2006). Instead of regulating EGF receptor internalization, they are involved in the sorting of the EGF receptor. Once internalized and targeted to early endosomes, these proteins regulate the fate of the EGF receptor by either targeting the receptor to lysosomes for degradation or to the recycling compartment.

Lack of control at any of these locations, e.g. through improper functioning of BAR proteins, could lead to delayed receptor degradation with increased and prolonged signaling as a result. In turn, this could lead to abnormal cell proliferation or migration which could possibly promote development of diseases such as cancer.
SCOPE OF THE THESIS

In this thesis, I wish to discuss the regulatory role of BAR-domain proteins in cellular control. In particular, I focused on the role of BAR-domain proteins in regulating RhoGTPase signaling and growth factor signaling. In addition, I focused on the Rac1-mediated regulation of epithelial cell-cell contacts.

In Chapter 2 we provide an overview of BAR-domain proteins involved in the regulation of RhoGTPases. BAR-domains are important modules functioning at the interface between the actin cytoskeleton and membrane dynamics. In this chapter, we discuss how membrane dynamics (e.g., endocytosis and vesicle traffic), regulated by BAR-domains, affects GTPase activation and function.

In Chapter 3 we identify the F-BAR domain protein PACSIN2 as an important negative regulator of Rac1 activation and signaling. We show that PACSIN2 regulates internalization of the small GTPase Rac1 thereby targeting Rac1 to intracellular sites for GAP-mediated inactivation. As a result, loss of PACSIN2 results in increased cell spreading and migration. This study identified a novel role for PACSIN2 in regulating Rac1 activation and signaling.

In Chapter 4 we characterize a novel role for the BAR-domain containing human minor histocompatibility antigen-1 (HMHA1) as a RhoGAP. Previously, HMHA1 was only studied in the context of its role in forming a histocompatibility barrier in transplantation. We show that HMHA1 regulates RhoGTPase activity and as a consequence affects the actin cytoskeleton and cell spreading and migration. In addition, biochemical studies suggest that HMHA1 is a genuine RhoGAP. This study identified a previously unknown role for HMHA1 in regulating RhoGTPase activation and function.

In Chapter 5 we identify a novel role for the F-BAR protein PACSIN2 in growth factor receptor activation and signaling. We demonstrate that PACSIN2 negatively regulates Epidermal Growth Factor (EGF) receptor activation and signaling by controlling receptor surface expression. Loss of PACSIN2 increases surface levels of the EGF receptor and, as a consequence, increases receptor activation and downstream signaling in response to EGF. Interestingly, we show that the role of PACSIN2 is not specific for the EGF receptor as signaling downstream of HGF in epithelial cells, but also in primary endothelial cells downstream of TNFα, is increased in PACSIN2 depleted cells as well.

In Chapter 6 we report a novel pathway by which Rac1 stimulates maturation of cell-cell contacts in epithelial cells. Rac1 triggers translocation of the HECT E3 ligase Nedd4 to cell-cell junctions. We show that Rac1 activity triggers Nedd4-mediated ubiquitylation of the scaffold protein Dvl1, a negative regulator of epithelial cell-cell contacts, and this could be prevented when Rac1 is inhibited. We also show that loss of Nedd4 results in decreased degradation of Dvl1 and as a consequence decreased junctional integrity. We demonstrate that Nedd4-mediated ubiquitylation is important as Nedd4 mutants, incapable of ubiquitylating, failed to induce Dvl1 degradation. Moreover, Dvl1 mutants, insensitive to Nedd4-mediated ubiquitylation, decrease junctional integrity to an even greater extent than wild-type Dvl1. This study identified a novel pathway in which Rac1, in conjunction with Nedd4 and Dvl1, promotes maturation of cell-cell contacts.


tains two motifs that control targeting and signaling specificity. J. Biol. Chem. 278, 39166-39175.


