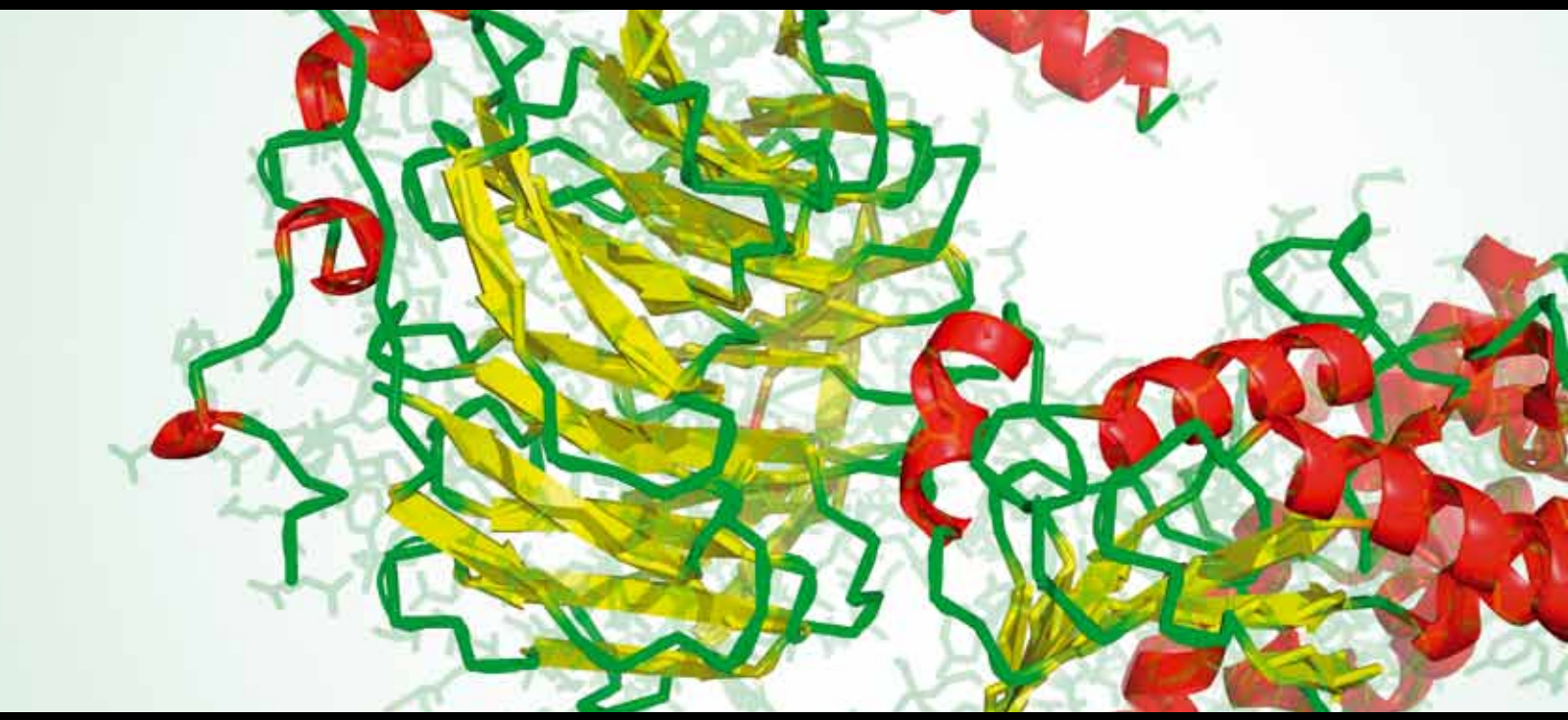
Journal of Signal Transduction

Signals Regulating Adhesion Dynamics

Guest Editors Donna J. Webb, Claire M. Brown, and Kris A. DeMali



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Editorial Board

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Editorial

Signals Regulating Adhesion Dynamics

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Adhesions are sites of contact between cells (cell-cell) and between cells and the extracellular matrix (cell-ECM) that are essential for numerous biological processes such as embryogenesis, wound healing, and the immune response. They are composed of many different molecules including adhesion receptors, signaling proteins (e.g., kinases, phosphatases, and adaptor proteins), and structural proteins (e.g., actin-binding proteins) [1–3]. Adhesions are dynamic structures whose molecular composition and structure can undergo rapid changes to allow cells to respond to external signals [1, 4]. Indeed, this dynamic nature of adhesions within localized regions of cells is critical for many complex processes such as cell migration.

For cell-ECM adhesions, their assembly is initiated by the binding of integrin adhesion receptors to the ECM. These new adhesions can either disassemble, allowing cells to migrate, or continue to mature by recruiting signaling and structural proteins to these sites. The dynamics and composition of adhesions are controlled by signaling networks that function to integrate molecular signals from outside and within cells. This special issue focuses on the molecular signals that regulate adhesion dynamics with an emphasis on cell-ECM adhesions.

Integrins are transmembrane adhesion receptors that provide a functional link between the ECM and the actin cytoskeleton. Integrin engagement of the ECM serves to transduce signals to the interior of cells which regulate cell behavior. RGD-dependent integrins are a subgroup of adhesion receptors that specifically recognize the RGD motif, which is a three-amino-acid sequence (Arg-Gly-Asp) that is found in some ECM proteins. In this special issue, Y. D. Benoit et al. review recent findings regarding the importance of RGD-dependent integrins in epithelial cell homeostasis. Integrins also play important roles in blood clotting and wound healing by regulating platelet adhesion and aggregation. G. F. Guidetti and M. Torti discuss recent insights into the role of Rap1 in regulating platelet adhesion. Rap1 is a member of the Rap family of small GTPases and is a downstream effector of integrin signaling. A-Kinase-Anchoring Proteins (AKAP) are emerging as pivotal scaffolding proteins that bring together key signaling molecules to modulate cell migration. S. Akakura and I. H. Gelman provide insight into the regulation of AKAP12 and how it contributes to cell adhesion. Moreover, they discuss the scaffolding function of AKAP12 and its contribution to cell migration, maintenance of cytoskeletal architecture, cell proliferation, and cytokinesis.

While integrin-containing matrix contacts were first identified in classic focal adhesions, they have subsequently been shown to exist in other structures including podosomes and invadopodia. Podosomes and invadopodia do not have the typical elongated shape of focal adhesions but form circular adhesive structures. Podosomes are found in phagocytic cells, while invadopodia are found in cancer cells, where as their name implies, they play a critical role in cancer cell invasion into the surrounding stroma. Focal adhesions, podosomes, and invadopodia share some of the same proteins, but they differ in their basic structure and function. P. P. Eleniste and A. Bruzzaniti compare and contrast the organization and function of focal adhesions, podosomes, and invadopodia. They highlight critical tyrosine kinases and signaling proteins that regulate the assembly and function of these adhesive structures.

Cytotoxic necrotizing factors (CNF) are a class of autotransporter toxins that are made by uropathogenic *E. coli* (CNF1-3) and *Y. pseudotuberculosis* (CNFγ). CNF toxins

deamidate and thus constitutively activate RhoA, Rac1, and Cdc42, which are critical regulators of adhesion dynamics. In this special issue, M. May et al. analyze the effects of CNF on cell-matrix adhesion. They show that CNF1 and CNFγ increase cell-matrix adhesion, resulting in reduced migration. They further indicate that the augmented cell-matrix adhesion is dependent on RhoA deamidation.

Adherens junctions are cell-cell adhesion sites that are important for the establishment and maintenance of apico-basal polarity of epithelial cells. C. Bertocchi et al. give an overview of the kinases and phosphatases that regulate the phosphorylation of adherens junction proteins. In addition, they discuss the phosphorylation events that control the assembly and disassembly of adherens junctions.

Overall, this special issue is a reflection of the diverse roles (e.g., epithelial cell homeostasis to the immune response to cancer) that adhesions play in biological processes and diseases. This issue provides an overview of the different types of adhesive structures, including focal adhesions, adherens junctions, and podosome as well as the molecular composition, regulation, and dynamics of these structures. The goal of this special issue is to provide the reader with an appreciation of the importance and function of adhesions and a synopsis of key signaling molecules that comprise adhesions.

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Claire M. Brown
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Review Article

RGD-Dependent Epithelial Cell-Matrix Interactions in the Human Intestinal Crypt

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Interactions between the extracellular matrix (ECM) and integrin receptors trigger structural and functional bonds between the cell microenvironment and the cytoskeleton. Such connections are essential for adhesion structure integrity and are key players in regulating transduction of specific intracellular signals, which in turn regulate the organization of the cell microenvironment and, consequently, cell function. The RGD peptide-dependent integrins represent a key subgroup of ECM receptors involved in the maintenance of epithelial homeostasis. Here we review recent findings on RGD-dependent ECM-integrin interactions and their roles in human intestinal epithelial crypt cells.

1. Introduction

Cell contacts with the extracellular matrix (ECM) provide both cohesive and functional properties in a variety of tissues, such as epithelia, nerves, muscle, and stroma, through specific interactions with cell membrane receptors [1, 2]. All ECMs are made up of collagen fibrils and/or networks, proteoglycans as well as specialized glycoproteins such as fibronectin and laminins that are archetypal of interstitial ECM and basement membrane (BM), respectively [3, 4]. Cells from multiple origins interact with ECM molecules using a variety of receptors, most of them being members of the integrin superfamily [2]. Integrins are noncovalent transmembrane α/β heterodimers. In mammals, over 24 distinct integrin heterodimers have been characterized to date, describing the association between 18α and 8β subunits [5–7]. The fact that integrin-mediated connections between the ECM and the cytoplasm regulate key cell functions such as adhesion, migration, proliferation, apoptosis, and differentiation is well recognized [8–11].

Epithelia express a wide variety of typical integrin receptors such as the $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta4$ integrins that serve as collagen and/or laminin receptors [12–15]. Although less well documented in epithelia, the RGD-dependent integrins are another group of receptors that appears to be involved in epithelial cell homeostasis [15–17]. RGD-dependent integrins include $\alpha5\beta1$-, $\alpha8\beta1$-, and αV-containing integrins and are named as such because they specifically recognize the RGD motif, a sequence of three amino acids (Arg-Gly-Asp) found in many ECM molecules such as fibronectin and osteopontin [5, 12, 14, 15]. Collectively, these interactions are termed the "RGD-dependent adhesion system" (Figure 1). Interestingly, RGD-dependent cell interactions represent a key role in hierarchical assembly and maturation of adhesion structures including focal complexes (FXs), focal adhesions (FAs), and fibrillar adhesions (FBs) [1, 2, 18].

Therefore, RGD adhesion can by divided into three distinct components, the extracellular component (e.g., fibronectin), the membrane receptor (e.g., the $\alpha5\beta1$ integrin), and the intracellular molecule (e.g., vinculin). Moreover, each component acts in concert with the others to organize and regulate RGD adhesion dynamics. In this paper, we will focus on the importance of the RGD-dependent adhesion system for human intestinal crypt cell homeostasis (Section 2). We chose to elaborate on recent findings from

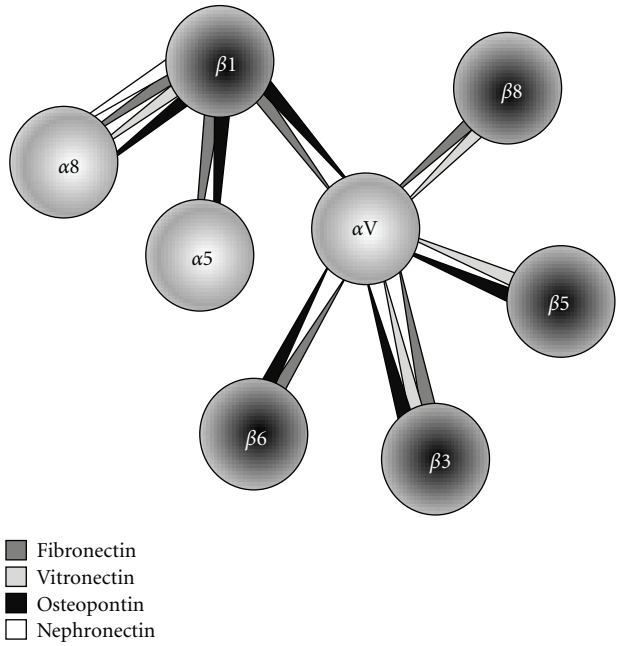

Figure 1: The RGD-dependent integrins. The RGD peptide (Arg-Gly-Asp) binding integrins represent a subclass of integrin receptors that specifically interact with the RGD motif found in several ECM elements. RGD integrins are formed by $\alpha 8/\alpha 5$ subunits coupled with the $\beta 1$ subunit and the αV subunit coupled with $\beta 3/\beta 5/\beta 6/\beta 8$ subunits. RGD-dependent α and β heterodimers are connected to each other with respect to their specific RGD containing ligands. The major RGD ligands are fibronectin (dark gray), vitronectin (light gray), osteopontin (black), and nephronectin (white).

our laboratory related to each of the RGD adhesion components, the $\alpha 8\beta 1$ integrin (receptor, Section 3), integrin-linked kinase (ILK) (intracellular molecule, Section 4), and type VI collagen (ECM, Section 5).

2. Cell-Matrix Interactions in the Human Intestinal Crypt

The small intestinal epithelium is a useful model to investigate the relationship between cell state and interaction with the ECM because of the well-defined architecture of its renewing unit, the crypt-villus axis. Indeed, proliferative cells, differentiating cells, and mature functional cells are topologically restricted to distinct compartments: the lower two-thirds of the crypt, upper third of the crypt, and villus, respectively. Gene expression in intestinal crypt cells must therefore be tightly regulated to efficiently control stemness, proliferation, migration, and differentiation in order to ensure the right equilibrium for the production of functional cells destined to renew the villus epithelium [19, 20]. There is strong evidence that cell-matrix interactions are involved in the regulation of these cell functions in the crypt [12, 21, 22]. For instance, differential spatial expression of laminins in the epithelial BM and their epithelial integrin receptors were observed along the crypt axis while in vitro studies have revealed functional relationships between laminin-binding integrins and specific intestinal cell functions such as proliferation, migration, and differentiation [23–30]. A schematic illustration of the human crypt-villus axis and the spatial expression of laminins, laminin receptors of the integrin family, and the two classic RGD components fibronectin and the $\alpha 5\beta 1$ integrin (as depicted by dark areas) is shown in Figure 2. Moreover, another example is the transient expression of the tenascin and osteopontin receptor $\alpha 9\beta 1$ integrin in the lower third of the crypt of the immature small intestine as well as in proliferative epithelial crypt cells [31] and its reexpression in colon adenocarcinoma cells [32].

The RGD archetype fibronectin is another ECM component that was found strongly expressed in the epithelial BM of the crypts in both human and small laboratory animals [33–36]. Synthesis and deposition of fibronectin by proliferating intestinal epithelial cells was confirmed in vitro [26, 34]. Furthermore, expression of the fibronectin receptors, $\alpha 5\beta 1$ and αV-containing integrins, was found to be associated with intestinal cell proliferation [21, 29, 37]. Taken together, these observations suggest that fibronectin may significantly contribute to the RGD system regulating intestinal crypt cell functions.

To investigate this hypothesis, we used a strategy combining expression studies in the intact human intestine and functional studies using HIEC cells, a human intestinal epithelial crypt cell model well-characterized for the expression of typical features of intestinal crypt cells [38–41]. As summarized in the next sections, this experimental approach has led to the identification and characterization of new components of the RGD-dependent adhesion system that emphasize the importance of this adhesion system in human intestinal crypt homeostasis.

3. Integrin $\alpha 8\beta 1$ as a Crucial Mediator of Crypt Cell-Matrix Interaction

3.1. Integrin $\alpha 8\beta 1$ Is a Novel Regulator of Epithelial Cell Adhesion. Initially characterized in the chicken nervous system [42, 43], integrin $\alpha 8\beta 1$ represents an important RGD-dependent receptor [44]. Ligand binding to integrin $\alpha 8\beta 1$ was shown to be important for RhoA GTPase activation and subsequent actin stress fiber assembly in vascular smooth muscle cells [45–47]. Integrin $\alpha 8\beta 1$ was also recently found to play an important role in microfilament organization which was central to RGD-dependent intestinal epithelial crypt cell adhesion [48]. $\alpha 8$ subunit knockdown experiments, carried out in HIEC cells, showed that this integrin is important for proper vinculin recruitment to adhesion structures [48] (Figure 3). Intestinal epithelial crypt cells in which $\alpha 8$ was knocked down exhibited lower numbers of vinculin-positive FAs compared to controls, while paxillin localization was not affected [48]. It is well known that RhoA/ROCK signalling enhances actin stress fiber assembly and increases cell adhesion [49–51]. RhoA activity was shown to promote scaffolding protein recruitment, including vinculin, to the developing adhesion structures [51, 52]. Thus, the increased RhoA activity displayed by $\alpha 8$ knockdown cells leads to the absence or reduced levels of vinculin observed within these cells [48, 53].

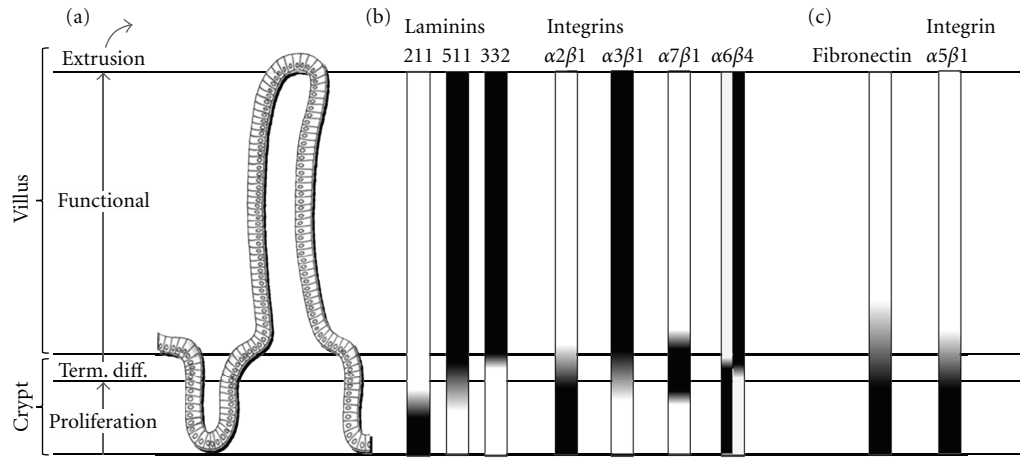

Figure 2: Distribution of laminins and laminin receptors of the integrin family as well as the RGD components fibronectin and the $\alpha5\beta1$ integrin receptor along the crypt-villus axis in the human small intestine. (a) Organization of the crypt-villus epithelial renewing unit. Villi are lined by functional epithelial cells responsible for digestion and absorption of nutrients. Stem cells located at the bottom of the gland generate transit amplifying cells that expand in the middle of the gland until they reach the upper gland region where they stop proliferating and undertake their terminal differentiation program before reaching the base of the villus. (b) Patterns of distribution of laminins at the epithelial BM as well as laminin receptors of the integrin family revealed differential expression of these molecules along the crypt-villus axis according to the cell state as shown by dark areas. (c) The RGD components fibronectin and its specific integrin receptor $\alpha5\beta1$ were found mostly confined to the crypt region (dark areas).

Based on the scheme of adhesion structures hierarchical assembly, vinculin recruitment occurs at later stages of FX formation, while paxillin is recruited at early stages [54]. Thus, observations made in intestinal epithelial crypt cells suggest that integrin $\alpha8\beta1$ is essential, at this particular stage of FX maturation into FA, via its role in RhoA activation [48] (Figure 3(a)). A similar function could also be predicted for the collagen-binding integrin $\alpha2\beta1$, considering the expression of this receptor in undifferentiated intestinal epithelium cells and its participation in RhoA activation [44, 55, 56].

Interestingly, ectopic expression of the enterocytic differentiation associated factor GATA-4 in intestinal epithelial crypt cells caused a depletion of $\alpha8$ subunit expression [39, 48]. In these same cells, reduced levels of $\alpha8\beta1$ were associated with a decrease in cell growth, marked by Cyclin D1 inhibition and accumulation of cells in the G1 phase [48]. Similarly, decreased RhoA activity was observed in differentiated and nonproliferative HT29 cells compared to their undifferentiated and proliferative counterparts [55]. Together with the role of integrin $\alpha8\beta1$ in RGD-dependent adhesion, these findings support the concept that cell-ECM interactions are crucial to maintaining a proliferative state in epithelial cells, which is anchorage and cell position dependent, reflecting its exclusive localization in the lower crypt of the intact intestine.

3.2. Integrin α8β1 Regulates Crypt Cell Migration. Due to the role of integrin $\alpha8\beta1$ in RGD-dependent cell adhesion and RhoA GTPase activity, this receptor was shown to exert a critical influence on intestinal epithelial crypt cell motility [48]. Alteration of RhoA activity was found to modulate migration in different systems [49, 57]. We recently reported that loss of RGD-$\alpha8\beta1$ interactions in intestinal epithelial cells caused increased cell migration [48]. From a physiological perspective, proliferating intestinal epithelial cells must be restricted to the lower two-thirds of the crypt to avoid premature terminal differentiation and loss of proliferative capacity [38, 40]. Therefore, without necessarily affecting the expression of differentiation master regulator genes, RGD-dependent adhesion plays a major role in regulating cell migration, which in turn is crucial for wound healing, cell differentiation, and tissue integrity [24, 58, 59].

3.3. Integrin α8β1 RGD-Dependent Interactions Act as a Check Point in the Intestinal Crypt Epithelium. Cell survival is tightly regulated by RGD-dependent ECM-integrin interactions [11]. Indeed, integrin receptors, such as $\alpha5\beta1$ and αV integrins, play a central role in controlling anoikis or apoptosis by loss of attachment [10, 11, 60]. Specifically, engagement of $\beta1$ integrins was found to be essential to intestinal epithelial cell survival through FAK signalling [60, 61].

As mentioned above, integrin $\alpha8\beta1$ is involved in efficient vinculin recruitment to developing adhesion structures [48, 53]. The presence of vinculin in cell-ECM adhesion structures affects cell survival signal transduction. As previously described, HIEC cells share a number of features with intestinal epithelial stem cells, including a proliferative and undifferentiated state as well as the expression of several putative stem cell markers [39]. Interestingly, silencing of vinculin expression in F9 embryonic teratocarcinoma cells, another cell model closely related to stem cells [62], has shown increased resistance to anoikis, while ectopic reexpression of vinculin restored sensitivity to anchorage-dependent survival [63]. A similar phenomenon was observed in nonadherent $\alpha8$ knockdown intestinal epithelial crypt cells [53]. In both studies, elevated levels of FAK phosphorylation

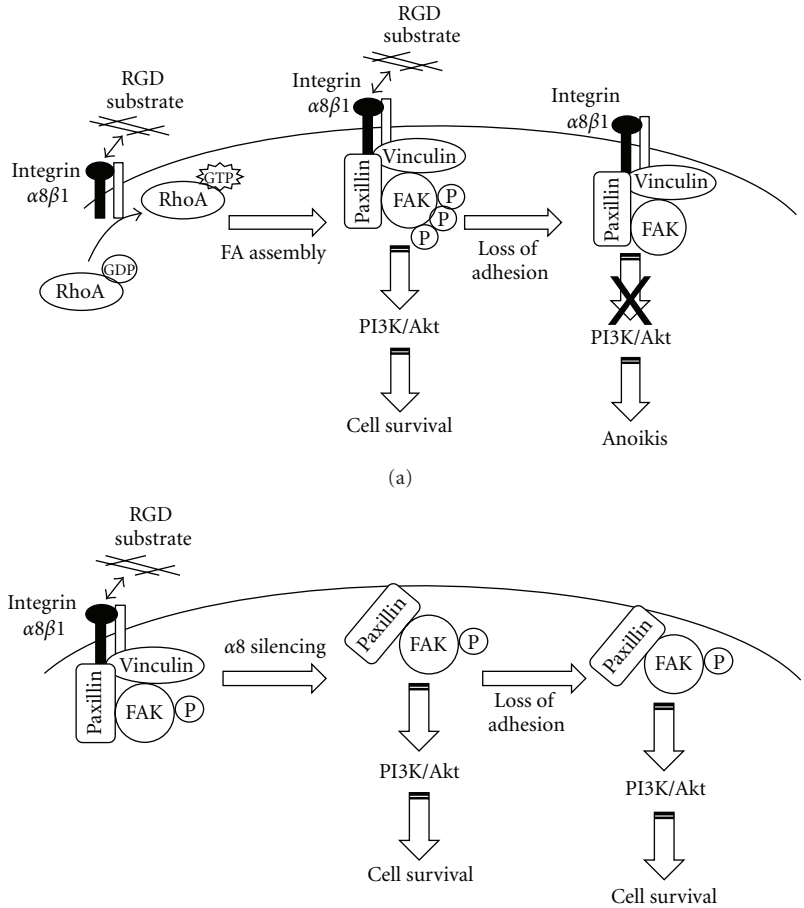

Figure 3: RGD-dependent adhesion influences anoikis sensitivity in undifferentiated epithelial cells. (a) Schematic representation of the proposed mechanism by which integrin $\alpha 8\beta 1$ and RGD-dependent adhesion regulates anoikis sensitivity, through differential interactions between vinculin, paxillin, and FAK in intestinal epithelial crypt cells. As described in Section 3, $\alpha 8\beta 1$ is essential to vinculin recruitment within maturing adhesion structures, while paxillin localization is not affected by $\alpha 8$ subunit silencing. (b) Following $\alpha 8$ subunit silencing, the absence of vinculin, combined with the presence of paxillin in the adhesion complexes, leads to an anchorage-independent activation of FAK and, consequently, anoikis resistance.

on tyrosine 397 were noted in nonadherent cell cultures. The absence of vinculin combined with the presence of paxillin in primitive adhesion structures prior to loss of adhesion could explain such a phenomenon (Figure 3). At the molecular level, it has been shown that paxillin exhibits partially overlapping binding sites for FAK and vinculin [64]. Thus, the vinculin tail domain appears to compete with FAK for paxillin binding. In the presence of vinculin, FAK activation would depend on ECM-integrin binding. However, absence of vinculin leads to a conformational change in adhesion structures which results in constitutive activation of FAK when bound to paxillin [63] where FAK activity no longer relies on ECM-integrin interactions [53, 63]. Additionally, nonadherent $\alpha 8\beta 1$-depleted intestinal epithelial cells showed increased activity of the PI3 K/Akt signalling pathway compared to nonadherent controls [53]. A summary of integrin $\alpha 8\beta 1$ contribution to anoikis regulation in epithelial intestinal crypt cells is presented in Figure 3.

Considering the proliferative and highly adaptive capacities of crypt cells, such as stem and transit amplifying cells, RGD-dependent $\alpha 8\beta 1$ interactions with ECM are suggested to act as a security switch that keeps the detachment of undifferentiated epithelial cells in check. It is worth noting that none of the five human colorectal cancer cell lines tested were found to express the integrin $\alpha 8$ subunit and that ectopic expression of this RGD-dependent receptor restored sensitivity of malignant cells to anoikis [53]. The mechanism by which colon cancer cells repress $\alpha 8$ expression to bypass this checkpoint remains unknown. However, in normal cells, this security step mediated by $\alpha 8\beta 1$ occupancy is potentially important to support homeostasis in the human intestinal crypt. New evidence from the literature has shown that colon cancer may originate from defective crypt stem cells [65]. Therefore, in light of the expression of $\alpha 8\beta 1$ in the region associated with intestinal stem cells, combined with its role in sensitizing epithelial cells to anoikis [53], it could

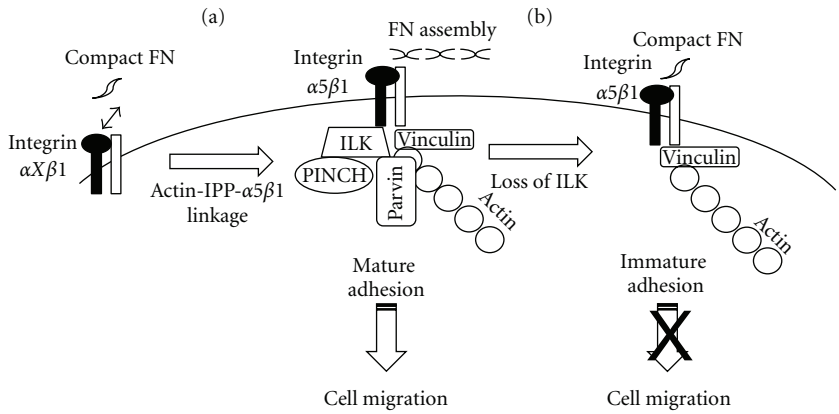

Figure 4: Regulation of the dynamic assembly of fibronectin by ILK. Schematic representation of the proposed mechanism by which ILK regulates FN assembly in HIEC. (a) Following cellular adhesion, the ILK/IPP complex is recruited to focal adhesions providing the link between the actin cytoskeleton and integrin $\alpha 5\beta 1$. IPP recruitment mediates the formation of mature focal adhesions that allow fibronectin deposition into the BM of HIEC. (b) Depletion of ILK in HIEC results in the disruption of the IPP complex which prevents actin cytoskeleton-$\alpha 5\beta 1$ linkage and focal adhesions cannot mature. Consequently, loss of the IPP complex reduces FN deposition and decreases migration of HIEC.

be speculated that $\alpha 8$ integrin silencing represents a key step in cancer initiation, in order to escape apoptosis upon modification of the malignant stem cell niche or location. In this context, $\alpha 8\beta 1$ could promote defective progenitor cell elimination, and consequently prevent the onset of ecto-cryptal proliferative structures. Such specific involvement of RGD-dependent integrins is not without precedent since altered expression of other heterodimers has been reported in colon cancer. For instance, integrin $\alpha V\beta 3$ expression has been found to be specifically decreased in anoikis-resistant Caco-2 cells [66].

4. Integrin-Linked Kinase (ILK) as an Integrator of Cell-Fibronectin Interaction

The intestinal epithelial cell mediates RGD interactions through expression of specific integrin receptors, as observed with $\alpha 8\beta 1$, as well as through the production and deposition of RGD ligands, such as fibronectin. The efficient deposition of fibronectin into the BM relies upon its recognition by RGD-dependent integrins, which mediate its unfolding to expose specific fibronectin structural domains, which in turn mediates the formation of insoluble fibronectin fibrils [67]. Fibronectin deposition is characterized by the formation of specialized cell-matrix contact structures containing integrins, cytosolic proteins, and actin referred to as fibrillar adhesion (FB) points [67].

The integrin-linked kinase (ILK) is a constituent of integrin containing adhesion sites where it mediates multiple cellular processes. ILK is a pseudokinase and scaffolding protein ubiquitously expressed in mammalian cells forming a trimeric complex with PINCH and parvins named the IPP complex [68–70]. ILK interacts with the cytoplasmic domain of integrin $\beta 1$ and $\beta 3$ subunits to create a physical link between integrins and the actin cytoskeleton [68, 71]. Interestingly, it has been suggested that ILK regulates fibronectin expression/deposition [72–74] and other studies have placed IPP complex members within FA points [75, 76]. In vivo, fibronectin expression is restricted to the BM underlying epithelial crypt cells and HIEC cells produce copious amount of fibronectin and generate numerous well-defined adhesion structures. The expression and roles of ILK were therefore investigated in human intestinal crypt cells.

We first focused on the localization of ILK-related components in the small intestine. As previously observed for fibronectin and integrin $\alpha 5\beta 1$ in the intact intestine [21, 29, 33], ILK, PINCH-1 α-parvin, and β-parvin were found to be predominantly expressed by the proliferative epithelial cells of the crypts [58]. In HIEC cells, ILK, PINCH-1, α-parvin, and β-parvin were all closely associated with FA points (Figure 4(a)). A siRNA strategy was used to knock down ILK expression in HIEC cells in order to further investigate the role of ILK in intestinal crypt cells [58]. Interestingly, ILK knockdown in HIEC was accompanied by severe disruption of the IPP complex including the loss of PINCH-1 and parvins as well as major alterations in fibronectin synthesis and functional matrix deposition (Figure 4(b)). Overexpression of ILK was previously shown to increase fibronectin deposition in rat intestinal cells [76] while ILK knockdown decreases fibronectin expression in mice and human colon cancer cells. Indeed, the fibronectin gene promoter contains response elements that have been shown to be potentially regulated by ILK-mediated signalling [68, 77]. However, in HIEC cells, although a reduction of fibronectin was observed at the transcript level, ILK knockdown had no net effect on fibronectin protein amounts found in the culture medium suggesting that it was mainly the ability to process and deposit soluble fibronectin that was altered by the loss of the IPP complex [58]. The exact mechanism by which ILK knockdown impairs fibronectin deposition remains to be elucidated. Expression levels of the fibronectin integrin receptors were not altered in HIEC ILK knockdown cells suggesting that the required receptors for fibrillogenesis [67] remain available for binding. However,

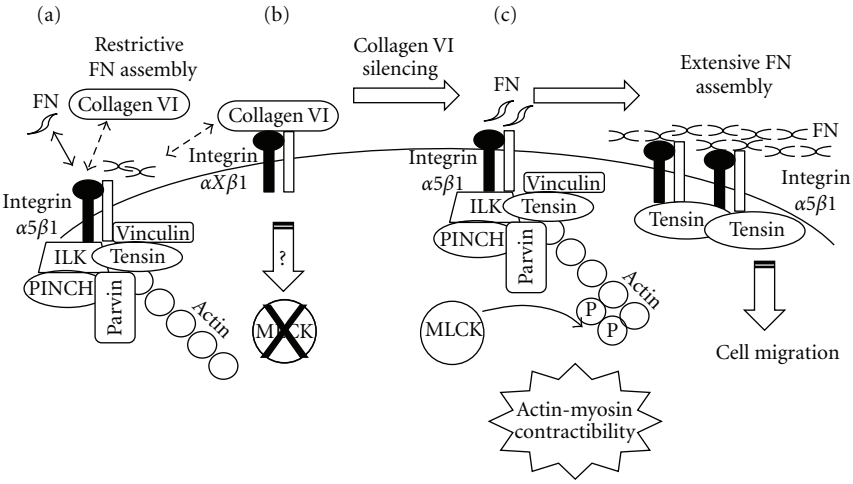

Figure 5: Regulation of dynamic assembly of fibronectin by type VI collagen. Schematic representation of the proposed mechanism by which collagen VI regulates FN fibrillogenesis. In HIEC, collagen VI is deposited into the ECM and interferes with fibronectin assembly by three distinct mechanisms. (a) First, in HIEC collagen VI competes with fibronectin for $\beta 1$ integrin binding in focal adhesions. (b) Second, collagen VI limits cellular accessibility of fibronectin through a direct interaction with FN preventing fibronectin association with other fibronectin molecules, a step required for the extensive formation of the fibronectin matrix. (c) The third mechanism involves the regulation of MLCK by collagen VI. When collagen VI is depleted from the ECM, the MLCK/MLC pathway is activated by an unknown mechanism and mediates fibrillar actin contractility that allows the recruitment of tensin to FBs generating extensive fibrillogenesis. The increase in fibronectin deposition followed the depletion of collagen VI is accompanied by an increase in migration.

because of the important scaffolding role of ILK and IPP complexes, the decrease of fibronectin deposition in these ILK-deficient cells may reflect a reduction in the cytoskeletal tension necessary for fibrillogenesis [78, 79]. Alternatively, alteration in signalization pathways may also be involved. Indeed, key signalling molecules such as Src, PI3 K, and ERK have been shown to modulate fibronectin deposition in various cell models [80–82] and ILK and the IPP complexes can regulate these signalling molecules [83–85].

In addition to an alteration in fibronectin deposition, ILK knockdown severely affected basic intestinal crypt cell functions such as cell spreading, migration restitution abilities as well as cell proliferation [58]. Alterations in these functions in ILK-knockdown HIEC cells were not surprising since these functions can be stimulated by fibronectin in intestinal epithelial cells [86–89]. Interestingly, exogenously deposited fibronectin was found to fully rescue the ILK-knockdown HIEC phenotype with regard to cell proliferation, spreading and migration [58].

Taken together, as summarized in Figure 4, these data reveal that ILK and, by extension, the IPP complexes, perform crucial roles in the control of human intestinal crypt cell homeostasis, especially as key mediator of fibronectin deposition in the BM, which in turn regulates cell proliferation, migration, and restitution.

5. BM Collagen VI as a Regulator of Crypt-Cell-Fibronectin Interaction

Type VI collagen is a ubiquitously expressed ECM component [90]. In interstitial ECM, collagen VI acts as an anchoring meshwork bridging collagen fibers to the surrounding matrix [91, 92]. Collagen VI has also been shown to directly interact with the BM-specific type IV collagen [93] supporting a key role for this collagen in connecting BM to ECM [94, 95]. However, we recently identified collagen VI as a *bona fide* component of the basal lamina in the intestinal BM and found that it is synthesized in significant amounts by crypt epithelial cells [59].

To investigate the function of type VI collagen in the intestinal epithelial crypt cell, we used a similar knockdown strategy with HIEC cells as described in the previous sections for integrin the $\alpha 8$ subunit and ILK. Surprisingly, abolition of collagen VI expression resulted in a striking increase in cell size and spreading accompanied by a significant increase in the number of stress fibers and tensin recruitment at the FB points [59]. The observations that removal of collagen VI emphasized features normally associated with fibronectin suggested that collagen VI regulates fibronectin assembly in epithelial cells. Interactions between collagen VI and fibronectin have been previously reported [93, 96]. Further investigation in collagen-VI-depleted HIEC cells revealed that fibronectin was increased at both protein and transcript levels and was subjected to extracellular rearrangement into long, parallel fibrils. Importantly, exogenous collagen VI, but not collagen I or IV, was able to fully rescue the knockdown phenotype indicating that the effect is specific for type VI collagen [59]. Considering that exposure of fibronectin-binding sites is critical for both cell binding and fibrillogenesis [67, 97], one may hypothesize that, under normal conditions, collagen VI acts by limiting cellular accessibility to fibronectin through competition for integrin receptors (Figure 5(a)) or by a direct interaction with fibronectin in the ECM (Figure 5(b)). Consistent with this possibility, collagen VI has been reported to be recognized by the RGD-binding $\alpha 5\beta 1$ and αV integrins [98–100]. Furthermore,

HIEC binding to collagen VI is integrin $\beta 1$ dependent and it was the FB complexes, specifically enriched in tensin and $\alpha 5\beta 1$ integrin [6, 54, 67], that were enhanced in collagen-VI-depleted HIEC [59].

To further investigate the mechanism underlying the generation of FB complexes in collagen-VI-depleted intestinal crypt cells, the regulation of actomyosin forces was analyzed. Actin contractility depends on the phosphorylation of the myosin light chain (MLC), which is mainly mediated by the kinases MLC (MLCK) and Rho (ROCK) acting on MLC and myosin phosphatase, respectively [101–104]. Interestingly, MLCK-dependent activation of MLC phosphorylation was observed in poor collagen VI/rich fibronectin ECM environments consistent with the observed generation of higher numbers of tensin-enriched FB complexes and extensive fibronectin fibrillar deposition [59] (Figure 5(c)).

As summarized in Figure 5, these data identified collagen VI as a major regulator of fibronectin synthesis and fibrillogenesis and suggest that collagen VI influences intestinal epithelial crypt cell behaviour by restraining cell-fibronectin interactions and their downstream events.

6. Conclusions

Described as a predominant epithelial BM component in the intestinal crypt more than 3 decades ago [33–36], fibronectin has been confirmed to play an important role in the RGD system regulating crypt epithelial cell functions. The recent findings summarized herein further emphasize the crucial importance of this RGD-adhesion system and its regulatory mechanisms. Indeed, intestinal epithelial cells can regulate RGD interactions through expression of specific integrin receptors, as exemplified by $\alpha 8\beta 1$, which exerts major regulatory influences on key cell functions such as cell proliferation, migration, and survival [48, 53]. Regulation of RGD interactions can also be accomplished by regulating production and deposition of their ligands, such as fibronectin, as illustrated by the finding that ILK/IPP complexes are key mediators of fibronectin deposition into the BM, which in turn regulates cell proliferation, migration, and restitution [58]. Finally, regulation of RGD-dependent cell interactions can also be achieved by interaction with other ECM molecules as shown with type VI collagen, a basement membrane component that regulates epithelial cell-fibronectin interactions. Taken together, these studies define new molecular elements and shed new light on the relative complexity of specific cell-matrix interactions in a well-defined environment such as the intestinal crypt and the critical impact these interactions have on cell function.

Authors' Contribution

Y. D. Benoit and J.-F. Groulx contributed equally to this work.

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Review Article

Focal Adhesion Kinases in Adhesion Structures and Disease

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Cell adhesion to the extracellular matrix (ECM) is essential for cell migration, proliferation, and embryonic development. Cells can contact the ECM through a wide range of matrix contact structures such as focal adhesions, podosomes, and invadopodia. Although they are different in structural design and basic function, they share common remodeling proteins such as integrins, talin, paxillin, and the tyrosine kinases FAK, Pyk2, and Src. In this paper, we compare and contrast the basic organization and role of focal adhesions, podosomes, and invadopodia in different cells. In addition, we discuss the role of the tyrosine kinases, FAK, Pyk2, and Src, which are critical for the function of the different adhesion structures. Finally, we discuss the essential role of these tyrosine kinases from the perspective of human diseases.

1. Introduction

The extracellular matrix (ECM) is an insoluble supra-structure comprised of a variety of matrix components including fibronectin, glycosaminoglycans, chrondronectin, osteonectin, collagens, laminin, proteoglycans, and growth factors [1–6]. The ECM provides the scaffold for cell attachment which is necessary for several diverse cellular activities, including cytoskeletal remodeling, polarization, differentiation, migration, and invasion [7–9]. Binding to the ECM is regulated by various signaling pathways that control the assembly and disassembly of three distinct, but functionally related actin and integrin-containing adhesion structures known as focal adhesions, podosomes, and invadopodia. In this review, we will discuss our current understanding of the similarities and differences between focal adhesions, podosomes, and invadopodia. We also will highlight several important tyrosine kinases and other signaling proteins that are known to control the formation and function of these adhesion structures, and we will discuss their role in pathophysiology.

2. Focal Adhesions

Focal adhesion formation and turnover has been used as a model system for understanding the mechanisms of cellular adhesion. Although focal adhesions, podosomes, and invadopodia share common signaling proteins, they are distinct in cellular architecture and function (summarized in Table 1). Focal adhesions, also known as "focal contacts," were identified over 30 years ago by electron microscopy and described as electron-dense plaques associated with actin filament bundles [10]. Focal adhesions can be considered to be large protein assembly complexes that spread mechanical forces from sites of cell adhesion to the cell body. In addition, focal adhesions regulate intracellular signaling pathways necessary for cell migration, growth, proliferation, embryogenesis, wound healing, and tissue repair [11–14]. Focal adhesions are comprised of a wide range of signaling proteins [15], such as the tyrosine kinases Pyk2 [16, 17], FAK [18, 19], Src [20, 21], Abl [22], and integrin-linked kinase [23]; the phosphatases PTP-PEST [24] and PTP1B [25]; the actin-binding proteins paxillin [26, 27], talin [23, 28–30], vinculin [23, 28–30] and tensin [31], the GTPases dynamin [32] and Cdc42/Rho [33, 34], as well as scaffolding proteins p130Cas [35] and Crk [27]. Many of these proteins have been shown to play predominantly a structural role or are involved in signal transduction [36].

Several protein kinases are recruited to focal adhesions upon cell attachment. These protein platforms recruit adaptor proteins and lead to the activation of complex network of signaling cascades that regulate basic cellular functions

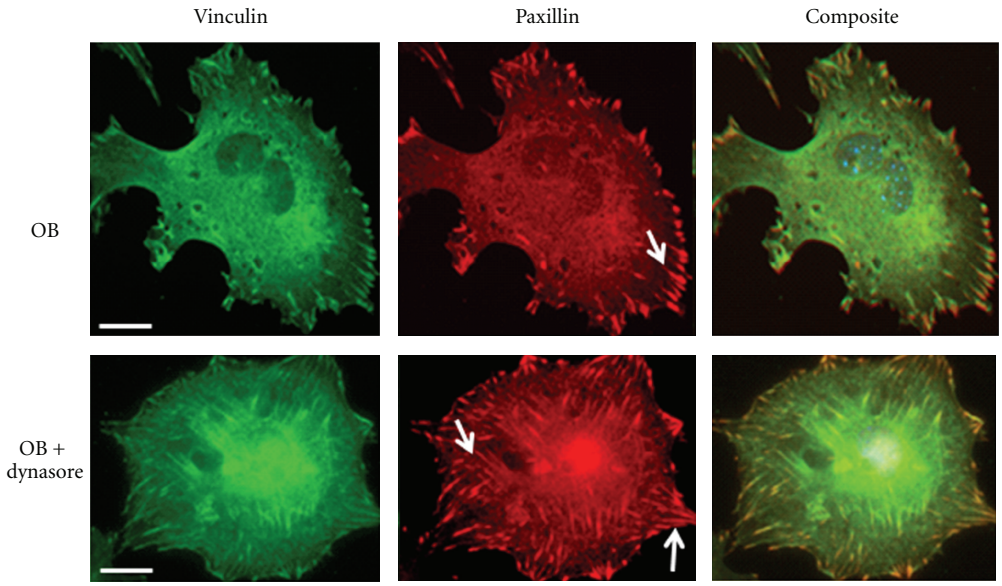

FIGURE 1: Inhibition of dynamin increases focal adhesions. Calvarial-derived osteoblasts (OBs) were treated with dynasore (90 μM) or vehicle for 1 hr and labeled for vinculin (green) or paxillin (red). Green and red channels were merged to form the composite image. Scale bar indicates 10 μm. Arrows show location of focal adhesions.

[16, 36]. An important tyrosine kinase found in focal adhesions is the focal adhesion kinase (FAK). FAK is a 125 kDa cytoplasmic tyrosine kinase that is activated upon integrin engagement and controls signaling pathways crucial for cell proliferation, migration, and survival [37]. The C-terminal domain of FAK is known as the focal adhesion targeting domain (FAT). As its name implies, the FAT domain is involved in directing FAK to focal adhesion complexes in a variety of cells [38]. In contrast, the N-terminal domain of FAK is known as the FERM domain (F for the 4.1 protein, Ezrin, Radixin, and Moesin). The central kinase domain of FAK, which itself is activated by phosphorylation, directs the phosphorylation of several signaling protein such as paxillin, Grb2 and p130Cas [39]. *In vitro* studies have shown that the FERM binds directly to the intracellular domain of the β1-integrin subunit and regulates FAK kinase activity [40]. It was also discovered that blocking β1-integrin function leads to FAK dephosphorylation, which in turn increases the sensitivity of malignant tumors to ionizing radiation and delays the growth of human head and neck squamous cell carcinoma cell lines [41].

FAK and the tyrosine kinase Src play a central regulatory role in focal adhesion turnover, and deletion of either of these kinases increases focal adhesion stability [42]. In addition, it has been shown that FAK and Src work in concert with the GTPase dynamin to regulate microtubule-induced focal adhesion disassembly [43]. In studies by Ezratty and colleagues, FAK−/− fibroblasts exhibited reduced dynamin accumulation around focal adhesions compared to controls [43], suggesting that FAK regulates dynamin localization and recruitment to focal adhesions. In addition, Wang and others demonstrated that Src phosphorylates dynamin at tyrosine residues, which promotes the translocation of dynamin to focal adhesions by FAK [32]. Disruption of the Src-FAK-dynamin complex blocked focal adhesion disassembly and fibroblast migration [32]. Using bone-forming osteoblasts as our model system, we also found that dynamin is expressed in osteoblasts and inhibition of its GTPase activity with the chemical inhibitor dynasore, increased the number of vinculin and paxillin-positive focal adhesions in osteoblasts, compared to control cells (Figure 1). Interestingly, we found that dynamin is also localized to actin-rich podosomes, in bone-resorbing osteoclasts [44, 45]. Moreover, dynamin knockdown with shRNA or overexpression of a GTPase-inactive dynamin mutant increased podosome stability and the thickness of the podosome belt and decreased osteoclast bone resorbing activity [44]. Together, these studies reveal that dynamin's GTPase activity is necessary for both focal adhesion turnover in osteoblasts as well as podosome turnover in osteoclasts. Furthermore, these findings suggest potential similarities in the mechanism of turnover of focal adhesions and podosomes, which is likely to be dependent on the complement of signaling and scaffolding proteins present in different cell types.

3. Podosomes

Podosomes are highly dynamic adhesion structures found in a wide variety of migratory cells including macrophages, osteoclasts, endothelial cells [46–50], transformed fibroblasts [51], and carcinoma cell lines [52]. They were first identified in the 1980s in v-Src-transformed fibroblasts [53, 54]. Podosomes and focal adhesions are both cell-matrix adhesion sites, but they differ in their structural design and turnover rates [55–59] (Table 1) despite sharing a large number of common signaling proteins, such as FAK, dynamin, talin, paxillin, Wasp, and vinculin [60]. Podosomes turnover occurs very rapidly with an apparent half-life of 2–12 min and involves the polymerization

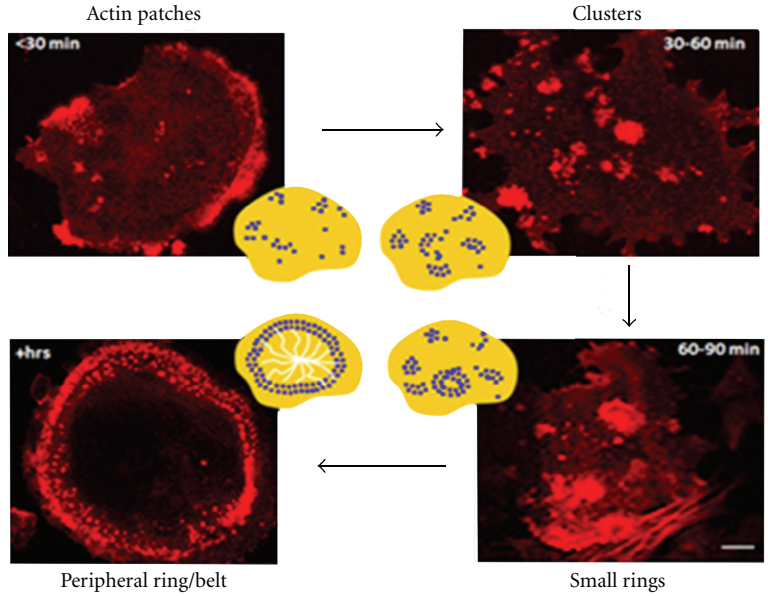

Figure 2: Dynamics of podosome organization in osteoclasts. Osteoclasts were generated from mouse bone marrow and plated on FBS-treated coverslips for various times. Cells were fixed and stained with rhodamine phalloidin. Actin patches are found soon after osteoclast attachment. Actin patches then reorganize into small rings and then into a peripheral podosome belt. The podosome belt is stabilized by the microtubule network. Scale bar is 10 μm.

Table 1: Common and unique features of focal adhesions, podosomes, and invadopodia. See text for details.

	Focal adhesion	Podosomes	Invadopodia
Appearance	dense plaques of F-actin	F-actin bundle core surrounded by actin cloud	F-actin bundle core surrounded by actin cloud
Size	width: 2–6 μm	width: 0.5–2 μm length: 0.5–2 μm	width: 0.5–2 μm length: >2 μm
Duration (half-life)	hours	minutes	hours
Cell expression	numerous nonmigrating fibroblastic cells	monocytic cells osteoclasts endothelial cells smooth muscle cells Src-transformed fibroblasts	carcinoma cells Src-transformed fibroblasts
Location	often found at leading edge of cell	ventral side of the cellular membrane	ventral side of the cellular membrane
Extracellular matrix degradation	no	yes	yes
Common signaling molecules	focal adhesion proteins GTPases actin regulators motor proteins tyrosine kinases phosphatases scaffolding molecules	focal adhesion proteins GTPases actin regulators motor proteins tyrosine kinases phosphatases scaffolding molecules	focal adhesion proteins GTPases actin regulators motor proteins tyrosine kinases phosphatases scaffolding molecules
Distinct Features	integrin receptors	Integrin receptors matrix-degrading enzymes	matrix-degrading enzymes

and depolymerization of the central F-actin core [50, 61]. Podosomes first appear as small actin dots which are then reorganized into small rings or rosettes with a diameter of 0.5–1 μm and a depth of 0.2–0.4 μm [62, 63] (Figure 2). The assembly of podosomes in macrophages and osteoclasts is dependent on an intact microtubule system [49, 64]. The central core of F-actin is surrounded by a ring of molecules that are involved in adhesion, matrix degradation, or migration. These proteins include the tyrosine kinases Pyk2 and Src [13], actin-associated proteins [51, 65], integrins [66], and their associated proteins [50], intermediate filaments [47], motor proteins [67] and metalloproteases [50, 68]. *In vitro* studies demonstrated that RhoA, Rac1, and Cdc42 are also involved in the

regulation of podosomes turnover [69, 70] and perhaps in recruiting podosomes to the leading edge of cells following microtubule-dependent cell polarization [64, 69, 71–73].

In contrast to focal adhesions, podosomes are found at sites of ECM degradation [51, 74]. The metalloproteases MT1-MMP and MMP-9 have been localized to podosomes, strongly supporting a role for podosomes in ECM degradation [56, 68, 75] in addition to adhesion [76]. This is well illustrated in osteoclasts, the primary bone-resorbing cells found in the body. In mature osteoclasts, podosomes are organized into a ring or belt-like structure at the cell periphery (referred to as the sealing zone) [50, 77]. This unique actin- and integrin-rich structure functions to dock osteoclasts to ECM proteins in bone and seals off the bone-resorbing compartment. This allows for the localized secretion of acidifying protons, chloride ions, and bone matrix-degrading metalloproteases [70].

In response to integrin engagement, and in the presence of intracellular calcium, Pyk2 is autophosphorylated at tyrosine residue Y402, which is essential for its catalytic activity [37, 78, 79] and for downstream signaling via p130Cas, Src, Cbl, integrins, gelsolin, and paxillin and the tyrosine phosphatase PTP-PEST [24, 80–82]. Pyk2 is expressed at high levels in the nervous system and in various hematopoietic cells [57, 83]. Pyk2 is expressed in osteoblasts [84, 85] and osteoclasts [45, 64]. Deletion of Pyk2 in osteoblasts affects differentiation, migration, and actin remodeling [84, 85]. In osteoclasts, Pyk2 is localized to the podosome belt and deletion of Pyk2 leads to a decrease in osteoclast bone resorption, which contributes to the osteopetrotic phenotype observed in Pyk2-deficient mice [64, 84]. Whereas deletion of Pyk2 in osteoblasts affects focal adhesion turnover (our unpublished findings), osteoclasts lacking Pyk2 exhibit structurally disorganized podosomes [64]. Src has also been shown to be indispensable for osteoclast function and is necessary for podosome assembly/disassembly [86, 87]. Osteoclasts lacking Src exhibit abnormal podosome rings, resulting in a dysfunctional sealing zone [88]. Leupaxin is a member of the focal adhesion-associated adaptor proteins and has been found to be associated with the podosome-belt (sealing zone) in osteoclasts [89, 90]. It was also demonstrated that leupaxin forms a signaling complex with Pyk2, c-Src, and PTP-PEST which regulates the migration of prostate cancer cells [91]. Finally, as discussed above, the GTPase dynamin regulates podosome assembly and dynamics in osteoclasts [44, 63, 92] in a process that involves Src [44]. These studies and others demonstrate that distinct signaling proteins work in concert to regulate podosome organization and turnover in osteoclasts, and perhaps in podosome-containing migratory cells.

The tyrosine kinase Pyk2 is a homolog of FAK and shares 45% overall sequence identity and 60% amino acid identity within the catalytic domain. Structurally, Pyk2 also contains an N-terminal FERM domain, a central catalytic core, several proline rich domains (PRDs), and a C-terminal FAT domain [64, 79, 93]. The FERM domain is involved in localizing Pyk2 to the plasma membrane and facilitates Pyk2 binding to phosphatidylinositol bisphosphate (PIP2) [94, 95]. Although structural similarities exist between FAK and Pyk2, these proteins appear to exhibit unique effects on adhesion structures in different cells. Recently, it was reported that deletion of FAK in osteoclasts leads to the formation of peripheral podosome belt, whereas deletion of Pyk2 resulted in small podosome rings [96]. In addition, deletion of FAK but not Pyk2, in lung carcinoma CL1-5 cells resulted in decreased formation of podosomes rosettes [96]. These findings suggest that FAK and Pyk2 may regulate different patterning of podosome organization in osteoclasts [96]. Although the mechanism is unknown, the recruitment of downstream effector proteins is likely to be important in the differential roles of these kinases.

4. Invadopodia

Invadopodia appear as dynamic protrusions of the plasma membrane, containing a central actin core surrounded by adhesion proteins, signaling molecules, and scaffolding proteins [59]. In addition, invadopodia are sites of ECM degradation and are often observed in highly migratory metastatic cancer cells [57]. Invadopodia share, overlapping features with podosomes, especially with regards to their intracellular localization, composition of proteins, and cell types in which they are found [55, 59, 62, 97–99] (see Table 1). However, differences between invadopodia and podosomes do exist. In particular, podosomes are short lived (minutes) and found in phagocytic cells such as osteoclasts, whereas invadopdia persist for hours and found primarily in cancer cells [97, 100]. Like podosomes, invadopodia are regulated by a multitude of signaling proteins such as the Src-family kinases, protein kinase C [55, 101–104], cdc42, N-WASP, and Arp2/3 [105, 106]. Dynamin has also been shown to participate in focal extracellular matrix degradation by invasive cells [107]. Although integrin signaling in the initiation of podosome formation is well established, the role of integrins in invadopodia is not yet clear [108].

The life cycle of invadopodia involves initiation, extension, ECM degradation, and disassembly. Each of these steps involves F-actin remodeling and the activation/deactivation of signaling proteins around the central actin core. The initiation of invadopodia is known to be stimulated by EGF, PDGF and reactive oxygen species (ROS) [76]. Following initiation by EGF receptor activation, Src and the tyrosine kinase Abl (Abelson) are recruited and activated [102, 105, 109]. This results in an increase in actin polymerization and cortactin phosphorylation within the elongating invadopodium [102, 105, 109]. Microscopic imaging has shown that cortactin accumulates in invadopodia prior to F-actin nucleation [101], matrix metalloprotease accumulation, and matrix degradation [55], suggesting that cortactin isan early player in this process. In addition to the filamentous actin network, microtubules and intermediate filaments also participate in the elongation and extension of invadopodia [110], with the resulting structure resembling the arrangement of actin filaments in podosomes. The growing protrusive membrane is supplied by vesicular trafficking to sites of invadopodia extension and is controlled

by the Golgi apparatus and by F-bar proteins such as CIP4 (cdc42 interacting protein) [107, 111] and the Ena/VASP family protein, Mena [112–114]. Membrane fusion and actin remodeling by dynamin have also been shown to be involved in invadopodia formation [115, 116]. Like podosomes, the formation and stabilization of invadopodia involves microtubule-dependent transport [108, 110]. The third step in the life cycle of invadopodia, and the major function of these structures, is ECM degradation. This function is shared with podosomes but is absent in focal adhesions. ECM degradation is facilitated by secretion of a variety of matrix metalloproteases and serine proteases [56, 112, 117, 118] and is thought to be regulated by cortactin, an actin regulating protein [55]. The secreted proteases act to degrade components of the ECM, thereby facilitating cellular migration and invasion [119, 120]. Finally, the disassembly of invadopodia involves depolymerization of the actin core [121] and has been shown to be regulated by ERK, paxillin, and the calcium-dependent cysteine protease, calpain, which degrades cortactin [121, 122].

The Src family kinases have been demonstrated to be critical for invadopodia formation and maturation. However, several lines of evidence support a role for Src in focal adhesion and podosome stability [123–125]. Similarly, as discussed above, FAK is important for focal adhesion turnover [126] but deletion of FAK has been also shown to increase invadopodia formation [6, 18] and suppress podosomes rosettes formation n fibroblasts [96]. Moreover, FAK has been shown to regulate a switch from phosphotyrosine-containing proteins at focal adhesions to invadopodia through the temporal regulation of active Src [6]. In the same study, it was shown that FAK-Src signaling also plays a significant role in cancer cell invasion [6, 116]. The apparent overlapping role of FAK and Src in different adhesion structures can be explained by the formation of dynamic proteins complexes between these molecules. For example, the major autophosphorylation site in FAK is Y397 (Y402 in Pyk2) which serves as an SH2-binding site, allowing Src to bind FAK (or Pyk2) [127]. The binding of Src leads to release of its own autoinhibitory catalytic domain, leading to the full activation of Src and to the activation of distinct downstream signaling cascades [128].

5. Adhesion Proteins and Human Disease

In the following section, and summarized in Table 2, we provide an overview of the role of key focal adhesion proteins and their potential link to human diseases.

5.1. Skeletal Disease. Bones provide structural rigidity to the skeleton and are constantly remodeled to maintain calcium and mineral homeostasis and repair skeletal damage. Bone architectural integrity relies in part on the rate of apoptosis of bone-forming osteoblasts. The activity of Pyk2 is also linked with a variety of metabolic conditions, including the regulation of bone mass. Deletion of Pyk2 leads to increased bone mass in mice [64, 84] due in part to defects in focal adhesion signaling in osteoblasts (our unpublished findings) as well as changes in podosome dynamics in osteoclasts [14, 87]. Src is also important for osteoclast function [129]. Deletion of Src impairs osteoclast bone resorbing activity and mice lacking Src exhibit severe osteopetrosis and exhibit defects in tooth eruption [129]. Studies have shown that disruption of the interaction of α-actinin with integrins at focal adhesions increases osteoblast apoptosis, which shifts the balance in favor of osteoclast activity, resulting in bone loss [130].

5.2. Role in Cancer. As discussed above, invadopodia formation is tightly linked with cancer metastasis. For example, recently, it was demonstrated that the transcription factor Twist1, a central regulator of the epithelial mesenchymal transition, promotes invadopodia formation through upregulation of platelet-derived growth factor receptor expression and activity, which play significant role in human breast cancer metastasis [120]. Invadopodia formation and therefore cancer invasion also involves the adaptor proteins TKS4 and TKS5 (tyrosine kinase substrate 4 and 5) [131]. It was also found that TKS5 colocalizes to invadopodia in different human cancer cells and that decreased TKS5 expression leads to decreased podosome formation and to reduced tumor metastasis [132]. Thus, TKS4 and TKS5 could potentially be used as therapeutic targets for the treatment of certain types of cancer. Other studies have demonstrated that loss of function of the *Fgd1* gene, which encodes a GTP-exchange factor, was associated with a rare inherited human developmental disease called faciogenital dysplasia. Fgd1 mutations in humans cause skeletal and neurological effects. However, Fgd1 was also shown to be involved in invadopodia biogenesis and ECM degradation [133], and to be expressed in human prostate and breast cancer cells, suggesting it may also be critical for cancer progression and tumorigenesis.

Several independent studies have demonstrated a critical role for FAK in tumor progression and invasion. Elevated FAK phosphorylation has been observed in several cancers, including breast, colon, thyroid, prostate, oral, neck, and ovarian cancer [134, 135]. Deletion of FAK from tumor cells or breast cancer cells resulted in decreased tumor progression [136, 137], while in endothelial-specific tamoxifen-inducible FAK knockout mice, tumor growth and angiogenesis were reduced [138], indicating that FAK may be important for tumorigenesis. In addition, quantitative real-time PCR has shown an elevation of FAK expression in malignant gastrointestinal stromal tumors [139]. Increased FAK expression was also detected in esophageal squamous cell carcinomas and was associated with cell differentiation, tumor invasiveness, and lymph node metastasis [140]. Also, it was found that FAK was overexpressed in esophageal squamous cell carcinoma which have led to cell differentiation, tumor invasiveness, and lymph node metastasis [140]. *In vitro* evidence also demonstrates that Src-FAK signaling is associated with elevated tumor cell metastases, invadopodia formation, and promotes cell invasion [141, 142]. The Src family of tyrosine kinases are important for embryonic stem cell self-renewal and are key regulators of signal transduction in various cells, including cancer

Table 2: Signaling proteins and their link to adhesion structures and disease. [+]Humans mutations are associated with disease. Mutations in dynamin are linked to centronuclear myopathy and Charcot-Marie-Tooth neuropathy in humans. *Bone mass regulation is based on knockout mice studies. Other disease indications are predicted based on animal studies and *in vitro* studies. n/d: not determined. See text for details.

Adhesion protein	Cell type	Adhesion structure	Disease indication
FAK	osteoblasts	focal adhesions	regulation of bone density*
	osteoclasts	podosomes	regulation of bone density*
	endothelial cells	podosomes?	angiogenesis
	lung carcinoma cells	podosomes	cancer metastasis
	various cancer cells	invadopodia	cancer metastasis
Pyk2	osteoblasts	focal adhesions	regulation of bone density*
	osteoclasts	podosomes	regulation of bone density*
	endothelial cells	podosomes?	angiogenesis
	various cancer cells	invadopodia	cancer metastasis
Src	osteoblasts	focal adhesions	regulation of bone density*
	osteoclasts	podosomes	regulation of bone density*
	various cancer cells	invadopodia	cancer metastasis
Dynamin	fibroblasts	focal adhesions	n/d
	osteoblasts	focal adhesions	regulation of bone density*
	osteoclasts	podosomes	regulation of bone density*
	neurons	n/d	neuropathy[+]
Twist1	epithelial cells	invadopodia	cancer metastasis
TKS4/5	human cancer cells	invadopodia	cancer metastasis
Leupaxin	osteoclasts	podosomes	regulation of bone density*
	cancer cells	invadopodia	cancer metastasis
Fgd1	osteoblasts	focal adhesions?	skeletal abnormalities[+]
	cancer cells	invadopodia	prostate and breast cancer metastasis

cells [143, 144]. Collectively, these findings provide strong evidence that overexpression of FAK and other proteins localized to invadopodia are important for invadopodia formation and tumor metastasis. Although there is a strong correlation between the expression of FAK and Src in invadopodia and the potential link of these kinases in cancer progression and invasion, it is not yet clear if Src-FAK signaling specifically in invadopodia is critical role for tumor growth. Nevertheless, FAK may be a useful biomarker for cancer cell metastasis and inhibitors to FAK or Src may be useful to limit disease progression [145]. To this end, the FAK inhibitor PND-1186 was found to dramatically decrease FAK activity in breast carcinoma cells, resulting in tumor cell apoptosis [146].

Lung cancer is considered to be one of the leading causes of mortality among the malignant tumors worldwide. It has been reported that small cell lung cancers (SCLCs) constitute 15–25% of all newly diagnosed primary lung cancers [147]. In the same study, it was shown that inhibition of Pyk2 by lentiviral RNAi or Src using a chemical inhibitor (PP2) reduced SCLC survival and proliferation in liquid culture and in soft agar [147]. In addition, it was demonstrated that Pyk2 also plays an important role in human non-small cell lung cancer (NSCLC) [148]. This was based on the detection of higher levels of Pyk2, as determined by Western blotting and immunohistochemistry, in NSCLC biopsies compared to nontumors [148]. In other studies, FAK signaling was shown to be important in the early stages of mammary adenocarcinoma lung metastasis [149]. It was further demonstrated that the dominant-negative FAK inhibitor, FRNK, blocked lung metastasis if added one day before tumor cell injection, but had no effect if given several days after tumor cell injection [149]. Furthermore, it was demonstrated that depletion of FAK, but not Pyk2, in lung carcinoma CL1-5 cells, decreased the formation of podosome rosette structures and decreased cell invasion [96]. Nevertheless, despite strong *in vitro* and *ex vivo* evidence linking FAK, Pyk2, and Src to various cancers, a direct link between kinase activity, effects on podosome/invadopodia formation, and cancer cell metastasis/function is currently lacking.

Several studies also suggest a link between the adhesion kinases and prostate cancer. For example, it has been shown that metastatic prostate cancer cells express elevated FAK mRNA levels and protein phosphorylation [150]. More recent studies also suggest that inhibition of Pyk2 and FAK may be an important therapeutic strategy to decrease prostate cancer progression [151]. Sun et al. used a mouse xenograft model injected with a chemical inhibitor of FAK and Pyk2 (PF-562,271) [151]. After two weeks of treatment with PF-562,271 (25 mg/kg), the mouse xenograft model showed a 62% decrease in tumor growth, compared to control mice [151]. Leupaxin was found to associate with Pyk2, c-Src, and PTP-PEST. *In vitro* studies also suggest that the migration of prostate cancer cells (PC-3) may

be regulated by protein complexes involving leupaxin, Pyk2, and the tyrosine phosphatase PTP-PEST, which dephosphorylates Pyk2 [91, 152]. Furthermore, it was shown that invasion of PC3 cells in a gelatin matrix is controlled by invadopodia and ECM degradation [153].

Astrocytomas represent the most common intracranial neoplasms accounting for 60% of all primary brain tumors. In separate studies, FAK and Pyk2 expressions have been shown to elevated in human brain astrocytomas [154–156]. In addition, a novel kinase inhibitor of FAK (TAE226) has been shown to increase tumor cell apoptosis in brain tumors [157]. Finally, others have demonstrated that administration of Src family kinase inhibitors, PP1 and Dasatinib, results in a dramatic increase in apoptosis of several pediatric brain tumor cell lines, compared to control cell lines was observed [158]. Collectively, the above findings suggest that inhibition of Pyk2 and FAK and other signaling molecules impair tumor migration by blocking the biogenesis of invadopodia which are important for ECM degradation.

5.3. Pulmonary and Other Diseases. Pyk2 was identified as a central regulator for angiogenesis of pulmonary vascular endothelial cells [159]. Additional studies show that Pyk2 is essential in regulating airway inflammation, Th2 cytokine secretion, and airway hyper-responsiveness in ovalbumin-sensitized mice during antigen challenge *in vivo* [160]. Inhibition of Pyk2 blocked broncho-alveolar lavage, eosinophilia, mucous gland hyperplasia, and airway hyper-responsiveness, conditions that are also characteristic of the asthmatic state in humans. In addition, deletion of Pyk2 leads to developmental defects, abnormal macrophage activity, obesity, and insulin resistance under a high-fat diet [161, 162]. Pyk2 activity in the heart may also protect against arrhythmia [163]. Although the mechanism by which Pyk2 regulates these physiological processes is still unknown, therapeutic strategies that target Pyk2 might be a novel approach for the treatment of a variety of metabolic and pathological diseases. Finally, it has been shown that dynamin mutations are associated with human centronuclear myopathy and Charcot-Marie-Tooth neuropathy [164–166]. These diseases are currently attributed to defects in dynamin-mediated endocytosis. However, it is interest to note that dynamin plays an important role in actin remodeling, which is linked to its function in membrane endocytosis [115, 116]. Therefore, it is possible that dynamin's role in actin remodeling and adhesion structure turnover [43, 44, 63, 92, 107] may also be involved in these pathologies, although this remains to be determined.

6. Summary and Perspectives

In summary, focal adhesions, podosomes, and invadopodia facilitate adhesion to the matrix and cellular migration. In addition to adhesion, podosomes and invadopodia have evolved the unique function of ECM degradation. The focal adhesion kinases, FAK and Pyk2, exhibit overlapping and unique roles in the biogenesis, stability, and disassembly of these different adhesion structures. There is currently a growing body of evidence linking these and other kinases to the biogenesis of different adhesion structures. In addition, a great deal of studies suggests a link between the expression levels of these kinases and several human diseases, especially cancer (see Table 2). Finally, emerging evidence suggests that disrupting the activity of the adhesion kinases not only disrupts the formation of the adhesion structures, but it may also be useful in the treatment of serious medical conditions such as cancer and osteoporosis. A greater understanding of the function of adhesion kinases and the adhesion structures they control will offer future avenues for therapeutic interventions against several human diseases.

Abbreviations

ECM: Extracellular matrix
OC: Osteoclast
OB: Osteoblast
FAK: Focal adhesion kinase
SCLC: Small cell lung cancer
NSCLC: Nonsmall cell lung cancer.

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Review Article

Regulation of Adherens Junction Dynamics by Phosphorylation Switches

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Adherens junctions connect the actin cytoskeleton of neighboring cells through transmembrane cadherin receptors and a network of adaptor proteins. The interactions between these adaptors and cadherin as well as the activity of actin regulators localized to adherens junctions are tightly controlled to facilitate cell junction assembly or disassembly in response to changes in external or internal forces and/or signaling. Phosphorylation of tyrosine, serine, or threonine residues acts as a switch on the majority of adherens junction proteins, turning "on" or "off" their interactions with other proteins and/or their enzymatic activity. Here, we provide an overview of the kinases and phosphatases regulating phosphorylation of adherens junction proteins and bring examples of phosphorylation events leading to the assembly or disassembly of adherens junctions, highlighting the important role of phosphorylation switches in regulating their dynamics.

1. Introduction

Adherens junctions (AJs) are cell-cell adhesion sites where calcium-dependent cadherin receptors bind with their extracellular domains to cadherins on opposing cells and with their cytoplasmic tails connect—via adaptors—to filamentous actin [1, 2]. By essentially providing a physical link between the actin cytoskeleton of neighboring cells AJs facilitate the integration of individual cells into a tissue. Additionally, AJs are instrumental in setting up and maintaining the apicobasal polarity of epithelial cells [3, 4], they function as mechanosensors [5] and serve as a nexus for signaling affecting important cell decisions, such as survival and differentiation [6].

During the development and lifetime of an organism, cells frequently change shape and position relative to their neighbors. Hence, the ability of cells to regulate their adhesive interactions plays a key role during tissue morphogenesis, repair, and renewal [3, 7, 8]. Defects in the adhesive characteristics of epithelial cells are pathological signs and loss of cell-cell adhesion can generate dedifferentiation and invasiveness of human carcinoma cells [9]. Thus, there is great interest in understanding the factors that affect assembly and disassembly of cell-cell adhesion at the molecular level.

When considering regulatory mechanisms controlling AJ proteins, we distinguish between three subsequent steps of regulation: synthesis, localization, and activation. First, a cell controls whether proteins are synthesized or not. Indeed, transcriptional regulation of E-cadherin, notably by the snail transcription factor, plays an important role in the breaking down of AJs accompanying epithelial to mesenchymal transition [10]. Once a protein is expressed the cell can determine its localization by controlling its transport. In fact, both exocytosis and endocytosis of E-cadherin are tightly controlled and the balance between the two processes has been shown to regulate AJ turnover both *in vitro* and *in vivo* [6]. Finally, a cell can control the activity and interactions of a protein at a given location by posttranslational modifications. These modifications include glycosylation, lipidation, ubiquitination, acetylation, proteolysis, and phosphorylation [11]. Phosphorylation of tyrosine (Y), serine (S), or threonine (T) residues, the topic of this review, is a rapid and reversible form of

regulation affecting the majority of AJ proteins [12–16]. In some cases, posttranslational modifications have secondary effects on transcription and/or protein transport [17, 18]. However, here we will focus on the more direct mechanisms in which AJs are regulated by phosphorylation. First, we will introduce the enzymes responsible for phosphorylation and dephosphorylation at AJs and discuss how they are recruited into AJ and activated. Next, we will describe the targets of phosphorylation within AJ and by examining the consequences of specific phosphorylation events will show how phosphorylation is involved both in assembly and disassembly of AJ, essentially driving the dynamics of this highly responsive structure. In the end, we will point out open questions and suggest methods to address them.

2. Recruitment of Protein Kinases and Phosphatases into AJ

So far, twelve S/T kinases and one S/T phosphatase have been implicated in regulating phosphorylation of AJ proteins, and they are all cytoplasmic (Table 1). Prominent kinases in this group include PKC-α, cAMP-dependent protein kinase, Casein Kinase 1, Pak1, and ROCK1. Nine tyrosine kinases and twelve tyrosine phosphatases have been shown to be active in AJ, roughly half of them cytoplasmic and half part of a transmembrane receptor (Table 1). Prominent tyrosine kinases include the cytoplasmic Src, Fyn, Fer, and Abl, and the receptors of epidermal and hepatocyte growth factors. Major phosphatases involved are the cytoplasmic PTP-1B, PTP-PEST, SHP-1, SHP-2, and receptor-type tyrosine-protein phosphatases Mu, U, and Kappa.

Some of these kinases and phosphatases have been localized to AJ by immunofluorescence (e.g., [19–21]) and others have been shown to associate with AJ by coimmunoprecipitation (e.g., [21–23]), but the exact mechanism of recruitment into AJ of most of them is largely unknown. A few were shown to bind directly with cadherin, such as CSK with VE-cadherin and PTP-1B with E- and N-cadherin [19, 24, 25]; others bind one of the catenins (adaptor proteins linking cadherin with actin), such as MET and PTPRF with β-catenin [26, 27] and ROCK1 with p120-catenin [21]; some interact with other AJ adaptor proteins, such as PRKCA with vinculin and ROCK1 with Shroom3 [28, 29].

While it is most likely every kinase and phosphatase can recognize at least one docking site within the AJ, it is not currently known which of the kinases and phosphatases reside in AJ permanently and which are transient components, homing in to phosphorylate or dephosphorylate AJ proteins only under specific conditions. Even permanent residents may not always be active, as most kinases and phosphatases need themselves to be activated.

3. Activation of Kinases and Phosphatases in AJ

Receptor tyrosine kinases are commonly activated by an external ligand, such as a growth factor or cytokine, which induces dimerization, cis-phosphorylation or autophosphorylation and activation of the catalytic domain [124, 125]. Receptor tyrosine phosphatases may be activated by homophilic association with their counterparts on neighboring cells [126], as well as by tyrosine phosphorylation [127]. Several S/T kinases are activated by binding of Rho GTPases, for example ROCK1 is activated by RhoA and PAK1 is activated by Rac1 and Cdc42 [128, 129]. S/T kinases are also regulated by tyrosine phosphorylation and tyrosine kinases and phosphatases are regulated by S/T phosphorylation, in a complex web of feedback and feedforward loops that is poorly understood (Figure 1). For example, Src phosphorylates PRKCD, which phosphorylates PTPN6, which in turn dephosphorylates SRC (feedback) [130–132]; PRKACA phosphorylates Src and Csk, and Csk also phosphorylates Src (feedforward) [133–135].

As will be discussed further below, some of the phosphorylation events serve to activate the kinases or phosphatases and others are inhibitory. One well-understood example of kinase activation is the mechanism of activation of Src. As reviewed in [136], the family of Src tyrosine kinases can be found in a nonactive "closed" conformation or in an "open" active conformation, depending on the phosphorylation status of a tyrosine residue at the C-terminus. When this residue is phosphorylated, it interacts with an SH2 domain in the middle of Src, blocking the catalytic site. Upon dephosphorylation of this specific tyrosine, the SH2 domain is released, and the protein unfolds, allowing autophosphorylation of another tyrosine residue situated within the enzyme's activation loop, rendering the kinase fully active [137]. It is important to point out that cadherin ligation and clustering may act as an activation signal for some kinases. Most notably, Src and Fer have been shown to be recruited to the membrane upon cadherin binding [138, 139], and EGFR signaling was shown to be stimulated by AJ formation independently of EGF ligand [140]. Furthermore, cadherin clustering has been found to indirectly induce activation of Rho GTPases [141], which in turn could activate S/T kinases.

4. Phosphorylation Targets within the AJ

The AJ can conceptually be divided into four layers (Table 2). The first, in the plane of the membrane, is where cadherins and other transmembrane proteins, such as nectin and AJAP1, reside. The next layer consists of membrane-bound adaptors, such as ERM proteins and MAGI1, and adaptors that directly bind transmembrane proteins, such as p120- and β-catenin (bind cadherin) and afadin (binds nectin). The following layer is composed of adaptor proteins, such as α-catenin and vinculin, which bind to the second layer adaptors and also bind F-actin. F-actin, along with actin-binding proteins, such as α-actinin, and actin regulators, such as cortactin, would be considered the last layer. Regulatory proteins, such as GAPs, GEFs, and GTPases, can be found throughout the AJ as reviewed in [14, 142].

There is evidence demonstrating both Y and S/T phosphorylation of proteins in all layers of the AJ (Table 2). As illustrated in Figure 1, often the same kinase will phosphorylate proteins from different layers. For example, Abl

Table 1: Kinases and phosphatases regulating phosphorylation of AJ proteins.

Gene symbol	Protein name	Phosphorylation type	Localization	Reference
Kinases				
SRC	Proto-oncogene tyrosine-protein kinase Src	Tyr	nonreceptor	[23, 30, 31]
CSK	c-src tyrosine kinase	Tyr	nonreceptor	[24, 32, 33]
FYN	Tyrosine-protein kinase Fyn	Tyr	nonreceptor	[34–36]
ABL1	Abl1	Tyr	nonreceptor	[37, 38]
SYK	Tyrosine protein kinase SYK	Tyr	nonreceptor	[39, 40]
PTK2B	Protein-tyrosine kinase 2-beta	Tyr	nonreceptor	[41–43]
FER	Tyrosine-protein kinase Fer	Tyr	nonreceptor	[44]
EGFR	Epidermal growth factor receptor	Tyr	Receptor	[45]
cMET/HGF	Hepatocyte growth factor receptor	Tyr	Receptor	[46]
PRKCA	Protein kinase C alpha type	Ser/Thr	nonreceptor	[47]
PRKACA	cAMP-dependent protein Kinase catalytic subunit alpha	Ser/Thr	nonreceptor	[48, 49]
ROCK1	Rho-associated, coiled-coil containing protein kinase 1	Ser/Thr	nonreceptor	[50]
PRKCD	Protein kinase C delta type	Ser/Thr	nonreceptor	[51, 52]
CSNK1E	Casein kinase I isoform epsilon	Ser/Thr	nonreceptor	[53]
CSNK2A1	Casein kinase 2	Ser/Thr	nonreceptor	[54]
PAK1	Serine/threonine-protein kinase PAK 1	Ser/Thr	nonreceptor	[55–57]
MAPK8	JNK	Ser/Thr	nonreceptor	[58]
PRKD1	Protein kinase D1	Ser/Thr	nonreceptor	[59]
PRKCI	Atypical protein kinase C-lambda/iota	Ser/Thr	nonreceptor	[60]
PRKCZ	Protein kinase C zeta type	Ser/Thr	nonreceptor	[60]
MARK2	MAP/microtubule affinity-Regulating kinase 2, Par-1	Ser/Thr	nonreceptor	[61]
Phosphatases				
PTPN1	Tyrosine-protein phosphatase non receptor type 1, PTP1B	Tyr	nonreceptor	[19, 62–64]
PTPN6	Tyrosine-protein phosphatase non receptor type 6, SHP1	Tyr	nonreceptor	[65]
PTPN11	Tyrosine-protein phosphatase non-receptor type 11, SHP2	Tyr	nonreceptor	[66]
PTPN12	Tyrosine-protein phosphatase non-receptor type 12, PTP-PEST	Tyr	nonreceptor	[67]
PTPN14	Tyrosine-protein phosphatase non-receptor type 14, PEZ	Tyr	nonreceptor	[68]
ACP1	Acid phosphatase of erythrocyte, LMW-PTP	Tyr	nonreceptor	[69, 70]
PTPRJ	Receptor-type tyrosine-protein phosphatase eta (R-PTP-eta), DEP1	Tyr	Receptor	[71]
PTPRM	Receptor-type tyrosine-protein phosphatase mu (RPTP mu)	Tyr	Receptor	[72–74]
PTPRT	Receptor-type tyrosine-protein phosphatase T (R-PTP-T)	Tyr	Receptor	[75]
PTPRU	Receptor-type tyrosine-protein phosphatase U (R-PTP-U)	Tyr	Receptor	[76, 77]
PTPRK	Receptor-type tyrosine-protein phosphatase kappa	Tyr	Receptor	[78, 79]
PTPRF	Receptor-type tyrosine-protein phosphatase F, LAR	Tyr	Receptor	[80–82]
PPP2CA	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	Ser/Thr	nonreceptor	[83–85]

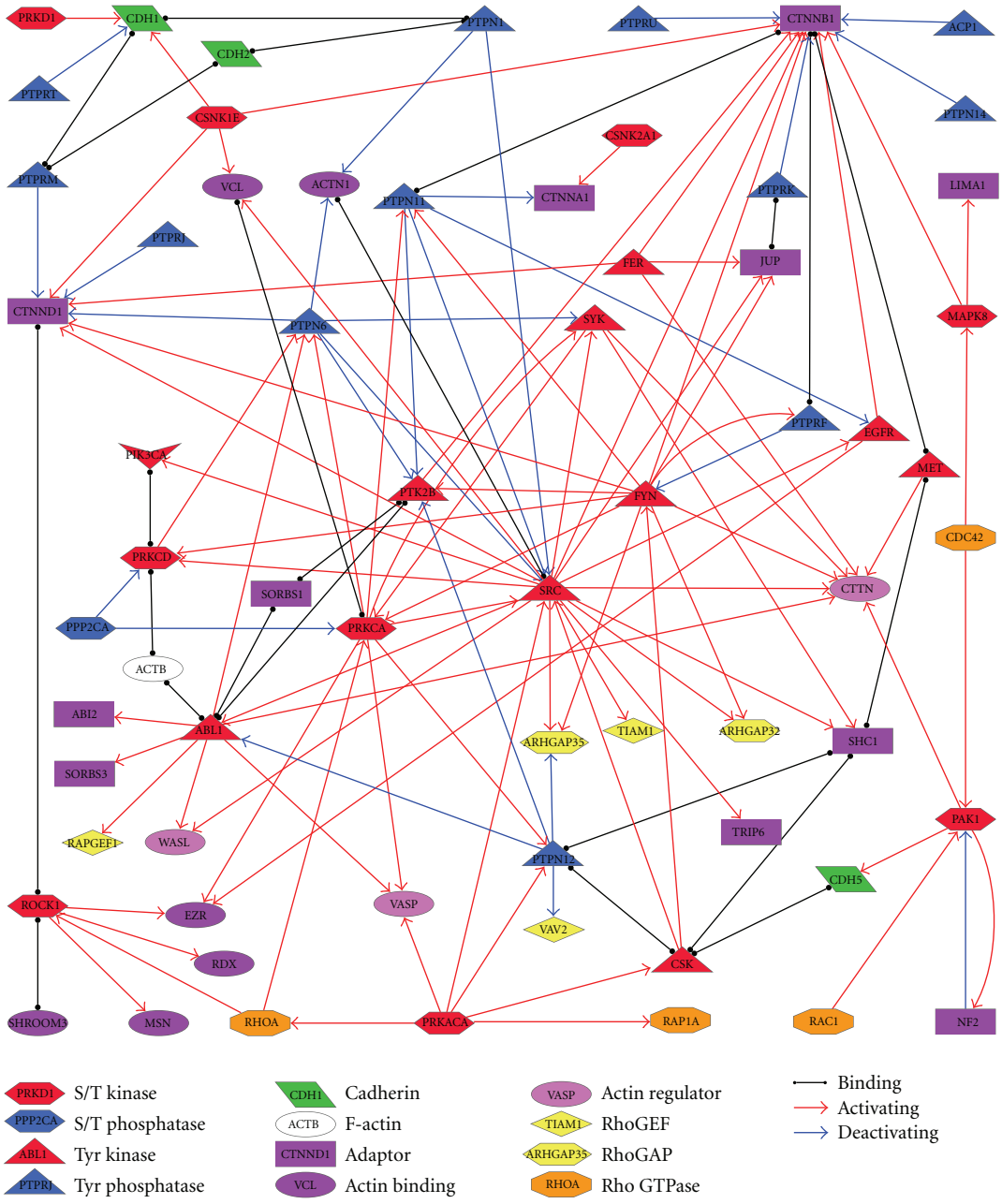

Figure 1: Network of phosphorylation enzymes and targets in the adherens junction.

phosphorylates actin regulators WASP and VASP [143, 144], as well as cadherin-bound adaptor δ-catenin [145] and second layer adaptors Abi2 and Vinexin (SORBS3) [146, 147].

Some AJ proteins have additional functions in the cell, and phosphorylation is also involved in regulating their non-AJ roles [148]. The most notable example is β-catenin, which plays an important role in the Wnt signaling pathway as a cotranscription factor of TCF/LEF [149]. Whether nonjunctional β-catenin will reach the nucleus or not depends on whether it is phosphorylated by GSK3 and Casein Kinse I in the "destruction complex" [150]. However, such phosphorylation events taking place outside the context of AJ are beyond the scope of this paper.

For the phosphorylation events occurring within AJ an important question is how do they affect the target proteins?

5. Consequences of Phosphorylation on AJ Proteins

A phosphorylated tyrosine, serine, or threonine residue can affect a protein in three major ways: it can increase the affinity for another protein, it can inhibit a protein-protein

Table 2: Targets of phosphorylation in AJ.

Gene symbol	Protein name	Phosphorylated residue	Reference
	Transmembrane		
CDH1	Ecadherin	S/Y	[54, 86, 87]
PVRL1	Nectin	Y	[88]
	Cadherin- or membrane bound		
CTNNB1	β-catenin	S/T/Y	[16, 89, 90]
EZR, RDX, MSN	ERM proteins (ezrin/radixin/moesin)	S/T/Y	[91–93]
CTNND1	p_{120}-catenin	S/T/Y	[94, 95]
JUP	Gamma-catenin	Y	[96]
PARD3	Partitioning defective 3 homolog	S/T/Y	[97, 98]
	Secondary adaptors		
CTNNA1	α-catenin	S/T/Y	[99, 100]
VCL	Vinculin	S/Y	[101, 102]
LIMA1	Eplin	S	[103]
VASP	Vasodilator-stimulated phosphoprotein	S/T/Y	[104–106]
SHC1	SHC-transforming protein 1	Y	[107, 108]
	Actin and actin regulators		
ACTN1	α-actinin	S/Y	[109, 110]
CTTN	Cortactin	S/T/Y	[111, 112]
ACTB	F-actin	S/Y	[113–115]
	GTPASE regulators		
PI3K	Phospho-inositide-3-kinase	Y	[116]
RAPGEF1	Rap guanine nucleotide exchange factor 1	Y	[117]
ARHGAP35	rho GAP p190A	Y	[118, 119]
ARHGAP32	p200RhoGAP	Y	[120, 121]
TIAM1	T-lymphoma invasion and metastasis-inducing protein 1	Y	[122]
VAV2	Vav 2 guanine nucleotide exchange factor	Y	[122, 123]

interaction, or it can activate enzymatic activity. In proteins with an intramolecular interaction, phosphorylation and dephosphorylation can elicit a conformational change in the protein. AJ components provide examples of each type of these outcomes, as detailed henceforth.

(1) Turn "on" Protein-Protein Interaction. Tyrosine phosphorylation can create a docking site for an SH2 or PTB domain of a partner protein. For example, tyrosine phosphorylation of cadherin creates docking sites for the SH2 domain of the adaptor SHC1 [151] and the PTB domain of the cell polarity protein Numb [152]. As mentioned earlier, SRC family kinases are inhibited by an intramolecular interaction between a central SH2 domain and a phosphorylated tyrosine at the C-terminus [137].

(2) Turn "off" Protein-Protein Interaction. Examples of interaction inhibition by phosphorylation are also found both between different proteins and intramolecularly: tyrosine phosphorylation of VE-cadherin at certain residues prevents the binding of p120-catenin and β-catenin [153]; phosphorylation of a threonine residue in the C-terminal actin binding domain of ERM proteins interferes with its interaction with the N-terminal FERM domain, helping to keep the protein in an active open conformation [154].

(3) Turn "on" Enzymatic Activity. Activation of the catalytic activity of tyrosine kinases and phosphatases by tyrosine phosphorylation has already been mentioned above [127]. Another important example is the activation of the motor activity of myosin by the phosphorylation of serine and threonine residues of myosin light chain [155].

We next address the question what are the ramifications of phosphorylation of AJ proteins on AJ structure and dynamics.

6. Global versus Specific Consequences of Phosphorylation on AJ Structure and Dynamics

Numerous experiments have been carried out over the years to address the role of Y and S/T phosphorylation in regulating AJ. Early experiments used broad-spectrum chemical inhibitors of kinases or phosphatases to conclude that phosphorylation negatively impacts cadherin function. For example, inhibition of S/T phosphatases by Okadaic acid or Calyculin-A was reported to lead to complete disassembly of AJ within an hour, and this disruption was attributed to an increase in S/T phosphorylation of β-catenin [89]. However, Calyculin-A has also been shown to increase actomyosin contractility in cells [156], suggesting that the disruption of

AJ in the above mentioned study may have been caused by mechanical tension at the junctions exceeding their adhesive strength. Inhibition of tyrosine phosphatases with sodium orthovanadate was reported to lead within minutes to a dramatic increase in phosphotyrosine signals at AJ, followed by the disassembly of AJ [157]. Consistent with the notion that excessive tyrosine phosphorylation in AJ causes their disassembly, cells expressing constitutively active Src kinase lost their AJ, and inhibition of tyrosine kinase activity by the drug tyrphostin was able to restore AJ in the Src-transformed cells [157]. These and similar experiments have led researchers in the late 90s of the previous century to the general conclusion that phosphorylation is a negative regulator of AJ.

However, in more recent years, there is accumulating evidence for a positive role of phosphorylation in AJ assembly, mainly coming out of loss-of-function experiments of specific kinases. For example, SRC and FYN were found to be essential for the formation of AJ in mouse keratinocytes [158]. Moreover, SRC activity was shown *in vitro* to be important for the recruitment of PI3K to AJ and the ability of cells to expand nascent cadherin-adhesive contacts [159]. Along the same lines, ABL1 tyrosine kinase activity was shown to be important for the maintenance of adherens junctions in epithelial cells [37], and S/T phosphorylation of E-cadherin by protein kinase D1 (PRKD1) was found to be associated with increased cellular adhesion and decreased cellular motility in prostate cancer [59].

Hence, the emerging view is that it is not possible to generalize the effect of phosphorylation on AJ. With some phosphorylation events leading to the switching "on" of a protein or interaction and other phosphorylation events, even on the same protein, serving as a switch "off", the effect of phosphorylation on AJ dynamics has to be examined on a residue-by-residue basis. After we delineate the effect of each individual phosphorylation event, we should be able to integrate this information into a single network of interconnected switches and perhaps then we can follow the global effects of a single phosphorylation switch, starting, for example, with hepatocyte growth factor stimulation [160].

7. Consequences of Specific Phosphorylation Events on AJ, Composition and Dynamics

We close this paper by giving a few examples of cases in which the consequences of specific phosphorylation events are known. The phosphorylation events presented occur on proteins from each layer of the AJ as well as one cell polarity protein.

(1) Cadherin. Serine phosphorylation of residues S840, S851 and S853 in the C-terminus of human E-cadherin (likely by CSNK1E or PRKD1) increases the binding affinity towards β-catenin, whereas phosphorylation of S846 is said to inhibit the same interaction [86]. Stronger binding of β-catenin to E-cadherin is conducive to a stronger AJ structure. Tyrosine phosphorylation of VE-cadherin at two critical tyrosines, Y658 and Y731, is sufficient to prevent the binding of p120- and β-catenin, respectively [161]. Phosphorylation by Src of three tyrosines in position 753–755 on human E-cadherin creates a docking site for the E3-ligase Hakai [162]. Ubiquitination of E-cadherin by Hakai leads to internalization of E-cadherin facilitating disassembly of the AJ [162, 163].

(2) P120-Catenin. Eight tyrosine residues in the N-terminus of p120-catenin can be phosphorylated by Src [94]. Upon phosphorylation, these sites serve as docking sites for the recruitment of interacting proteins carrying SH2 domains, such as the tyrosine phosphatase SHP-1 [164]. Under certain conditions tyrosine phosphorylation of p120-catenin was shown to increase its affinity to cadherin, while in other instances such an increase was not observed (reviewed in [95]). The affinity of p120-catenin to cadherin is significant for AJ dynamics because p120-catenin protects cadherin from being internalized [165].

(3) Zyxin. Phosphorylation of S142 of zyxin is thought to result in the release of an intramolecular head-tail interaction [166]. Opening of the protein expose its ACTA repeats that recruit VASP, whose actin polymerization activity (see below) is important for AJ assembly and maintenance. Since zyxin-mediated recruitment of VASP has a positive effect on AJ [167, 168], it is not surprising that expression of a zyxin phosphomimetic mutant results in ultrastable AJ [166].

(4) VASP (Vasodilator Stimulated Phosphoprotein). As its name suggests, VASP is often found phosphorylated in cells. Three phosphorylation sites on residues S157, S239, and T274 are phosphorylated by PKA and PKG, as well as PKC [169, 170] and dephosphorylated by unknown phosphatase/s. The phosphorylation of VASP was shown to reduce its affinity towards actin [171] and essentially turn off its actin bundling and anticapping/elongation activity [171, 172]. VASP-mediated actin elongation is important for the formation of AJ and for the maintenance of actin structures associated with AJ [173, 174]. Thus, the consequence of VASP phosphorylation is to negatively regulate AJ assembly and maintenance.

(5) PARD3. In Drosophila epithelial cells the par-3 ortholog Bazooka is confined to AJ as a result of phosphorylation by either apical or basal polarity complexes [4]. At the apical side of cells Bazooka is phosphorylated by aPKC, resulting in its release from the cortex [175, 176]. In the basolateral membrane Par1 kinase phosphorylates Bazooka on unique sites that also lead to its cortical release [4]. Recently, it was shown that the ratio between Par-1 and aPKC determines the position of Bazooka and AJ along the lateral side and a reduction in Par-1 kinase activity leads to a basal shift of AJ followed by folding of the epithelial sheet [177].

8. Conclusions and Outlook

From the examples presented above, it is clear that phosphorylation switches play a pivotal role in regulating AJ assembly and disassembly dynamics. At the same time it is also clear that our knowledge is only scratching the surface of the phosphorylation network regulating AJ. For the majority of known phosphorylation events in AJ, we know either of a kinase or of a phosphatase involved, but rarely do we know both. Furthermore, while traditional biochemistry techniques have facilitated the characterization of a handful of phosphorylation events on AJ proteins, phosphoproteomic data indicates that the majority of AJ proteins are phosphorylated on multiple serine/threonine and tyrosine residues [178]. Phosphoproteomics, which utilizes a variety of techniques to label cells, enrich for phosphorylated peptides and identify them using mass-spectrometry (reviewed in [179–181]), not only highlights the hole in our knowledge but also offers the means to fill it.

Phospho-proteomics offers an unbiased and comprehensive snapshot of phosphorylation events, and several different approaches can be taken to elucidate phosphorylation switches in AJ: during normal assembly and maturation, following a signal for disassembly, or when a certain kinase or phosphatase is activated or missing (e.g., [39, 182–184]). The phospho-proteomic data obtained, especially if it is dynamic, can be used for a systems level analysis of phosphorylation switches in AJ [185, 186], but it seems likely to us that before the network can be modeled in a meaningful way more in depth characterization of specific phosphorylation events will be necessary, using cell biological techniques.

While for the discovery and mapping of phosphorylation events in AJ, one wants to be as comprehensive as possible, when it comes to characterizing a particular switch the more specific the tools, the better. One example of a specific tool is phosphorylation site-specific antibodies, such as those recognizing individual phosphorylation events on β-catenin and p-120-catenin [187, 188]. Another example are site-specific phospho-mimetic or nonphosphorylatable mutations, such as those successfully applied to the study of the effects of phosphorylation on cortactin, VASP, VE-cadherin, zyxin, and paxillin [153, 166, 189–191].

Facing an ever-changing landscape of forces and signaling cues, a cell must respond rapidly by adjusting the strength of its AJs according to need. For this it relies on continuous turnover and assembly of core AJ components. Phosphorylation is particularly suitable for regulating the balance between assembly and disassembly as it is rapid and affects the AJ proteins directly. Feedback loops must guarantee a combination of phosphorylated residues at AJ that matches the requirements for a given condition. Experiments have shown that when it comes to phosphorylation both "all on" and "all off" treatments are deleterious to AJ. The challenge now is to elucidate the mechanisms by which the cell maintains a "just right" level of phosphorylation in AJ. While phosphorylation is probably the most prominent regulatory switch controlling cell adhesion, other switches, such as GTPases, lipids and proteases, do exist [192]. A future challenge, therefore, will be to integrate the phosphorylation switch network with the other regulatory switches to facilitate a true understanding of how different signaling pathways and force regulate AJ dynamics.

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Research Article

Increased Cell-Matrix Adhesion upon Constitutive Activation of Rho Proteins by Cytotoxic Necrotizing Factors from *E. coli* and *Y. pseudotuberculosis*

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Cytotoxic necrotizing factors (CNFs) encompass a class of autotransporter toxins produced by uropathogenic *E. coli* (CNF1) or *Y. pseudotuberculosis* (CNFy). CNF toxins deamidate and thereby constitutively activate RhoA, Rac1, and Cdc42. In this study, the effects of CNF1 on cell-matrix adhesion are analysed using functional cell-adhesion assays. CNF1 strongly increased cell-matrix binding of suspended Hela cells and decreased the susceptibly of cells to trypsin-induced cell detachment. Increased cell-matrix binding was also observed upon treatment of Hela cells with isomeric CNFy, that specifically deamidates RhoA. Increased cell-matrix binding thus appears to depend on RhoA deamidation. In contrast, increased cell spreading was specifically observed upon CNF1 treatment, suggesting that it rather depended on Rac1/Cdc42 deamidation. Increased cell-matrix adhesion is further presented to result in reduced cell migration of adherent cells. In contrast, migration of suspended cells was not affected upon treatment with CNF1 or CNFy. CNF1 and CNFy thus reduced cell migration specifically under the condition of pre-established cell-matrix adhesion.

1. Introduction

Cell-matrix adhesion involves several processes including integrin binding, cell spreading, and flattening against the substrate. Cultured cells, that spread out on ligand coated surfaces, rearrange their cytoskeleton and begin to move. Integrins thereby cluster together in "focal complexes" at the leading edge. These focal complexes grow into mature focal contacts, also called focal adhesions (FAs) [1]. Focal adhesions contain over 100 different proteins, including integrins, adapter proteins, and intracellular signaling proteins. Clustered integrins anchor actin filaments to the cell membrane and link them with the extracellular matrix (ECM) through adapter proteins such as talin and vinculin. The adapter protein paxillin links integrins to signaling proteins, forming a scaffold for Src kinases, the focal adhesion kinase (FAK), or the p21-activated kinase (PAK) [2–5].

The turnover of FAs in moving cells is driven by small GTPases of the Rho subfamily. FA formation and disassembly at the leading edge is driven by Rac1 and the localized suppression of Rho activity. Disassembly of FAs at the cell rear requires RhoA activity [6]. The activity of Rho proteins is regulated by the GTPase cycle. Rho proteins are active in the GTP-bound state and inactive in the GDP-bound state. In their active conformation Rho proteins interact with effector proteins to transmit downstream signaling. The cycling between these states is governed by guanine nucleotide exchange factors (GEF) and GTPase activating proteins (GAP), which catalyse the exchange of GDP to GTP or stimulate the intrinsic GTP hydrolase, respectively. A critical amino acid for GAP-induced as well as for intrinsic GTPase activity is Gln-63 in RhoA (Gln-61 in Rac1 and Cdc42). Gln-63/61 is deamidated by cytotoxic necrotizing factors (CNF), a class of autotransporter toxins produced by uropathogenic *E. coli* (CNF1-3) or *Y. pseudotuberculosis* (CNFy) [7, 8]. Deamidation results in inhibition of GAP-induced as well as of intrinsic GTPase activity, resulting in so called "constitutively active" Rho proteins. CNF1-induced

activation of Rho proteins leads to the assembly of F-actin in prominent stress fibers, membrane ruffles, and filopodia [9]. CNF1 triggers cell spreading of monocytes [10] and epithelial cells [11]. In CNF1-treated epithelial cells, cell spreading was accompanied by the formation of vinculin-positive FAs and the phosphorylation of FA proteins, including the focal adhesion kinase (FAK) and paxillin [12]. CNF1 exhibited antiapoptotic activity, which has been attributed to its capability of inducing cell spreading [13]. CNF1-treated cells finally acquire a multinucleated phenotype, which seems to be based on inhibited cytokinesis with ongoing cell cycle progression [14].

In this study, the effects of CNF1 on cell-matrix adhesion are analysed using functional cell adhesion assays. CNF1 strongly increases cell-matrix adhesion of suspended Hela cells and decreased the susceptibly of cells to trypsin-induced cell detachment. Increased cell-matrix adhesion is further presented to contribute to reduced cell migration. Increased cell-matrix binding is also observed upon treatment of Hela cells with isomeric CNFy, that specifically deamidates RhoA [15]. Increased cell-matrix binding appears to depend on RhoA deamidation.

2. Materials and Methods

2.1. Materials. The following reagents were obtained from commercial sources: DAPI (40.6-diamidino-2-phenylindole) (Serva), Hoechst 33342 (Cambrex), rhodamine-conjugated phalloidin (Sigma). The following reagents were obtained from commercial sources: RhoA (mAb-26C4), Rac1 (mAb-23A8) (Santa Cruz); β-actin (mAb AC-40) (Sigma); Cdc42 (mAb-44) (BD Transduction Laboratories); pS144/141-PAK1/2 (mAb EP656Y) (Abcam); vinculin (mAb hVIN-1) (Abcam); horseradish peroxidise-conjugated secondary antibodies (Rockland); anti-rabbit IgG Alexa Fluor 488 goat secondary antibody (Invitrogen).

CNF1 from *E. coli* and CNFy from *Y. pseudotuberculosis* were expressed as GST fusion proteins in *E. coli* and purified by affinity chromatography using glutathione-sepharose, as described earlier [15]. Toxin B (TcdB) from *Clostridium difficile* strain VPI10463 was purified as described [16].

2.2. Cell Culture and Transwell Migration Assay. HeLa cells were maintained in Dulbecco's minimal essential medium supplemented with 100 μg/mL penicillin, 100 U/mL streptomycin, 1 mM sodium pyruvate, and 10% FCS at 37°C in a humidified atmosphere containing 5% CO_2. The migration assay was performed in a Boyden chamber using 8 μm pore diameter 24-well transwell filter inserts (Corning). Suspended cells were directly placed on the membrane or allowed to adhere for 2 h. Cell migration was stimulated by FCS, present in the bottom solution for 5 h. Cells on the upper membrane surface were scraped with a cotton swab, and cells on the bottom surface stained with Hoechst 33342. After incubation at 37°C for 15 min, cells were analyzed by fluorescence microscopy using a Zeiss Axiovert 200 M (excitation 365 nm, emission 420 nm). Five adjacent microscope fields for each membrane were counted at ×20 magnification.

2.3. Cell-Matrix Binding Assays. Establishment of cell-matrix binding of suspended cells: HeLa cells were treated with the indicated toxin for 2 h and suspended by trypsin. Suspended cells were seeded onto cell culture dishes (polystyrol) coated with or without fibronectin. At the indicated time points, the medium containing nonadherent cells was removed, and attached cells were documented by phase contrast microscopy. Susceptibility towards trypsin-induced cell detachment: adherent HeLa cells were treated with the indicated toxin for 2 h. Cells were incubated with trypsin for the indicated time and the medium with suspended cells was removed. Adherent cells were documented by phase contrast microscopy. Five adjacent microscope fields for each membrane were counted at ×20 magnification.

2.4. Immunocytochemistry and Immunofluorescence. 4×10^5 HeLa cells were seeded onto cover slides and treated as indicated. Cells were fixed in 3.7% paraformaldehyde for 10 min at room temperature. The samples were washed with PBS and incubated with 5% BSA in PBS at room temperature for 1 h. Primary antibody was diluted in PBS and samples were incubated at room temperature for 1 h. Subsequently, cells were incubated with an Alexa Fluor 488 conjugated secondary antibody diluted to 1 : 1000 in PBS and DAPI (1 μg/mL) for 1 h at room temperature. Cover slides were fixed using prolong Antifade (Invitrogen) and analysed by fluorescence microscopy using Leica confocal microscope Inverted-2.

2.5. Rho Effector Pull-Down Assay. Effector pull-down assay were performed using the Rho binding domain of Rhotekin, encoding the N-terminal 90 amino acids of Rhotekin (C21), or the Rac/Cdc42 binding CRIB domain (amino acids 56–272 of PAK1). HeLa cells were lysed in binding buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, protease inhibitor complete (Roche), and 1% NP-40) at 4°C by sonification. The cell lysate was centrifuged at 13.200 ×g for 10 min, and the supernatant was incubated for 60 min with GST-C21(Rhotekin) (RhoA) and GST-PAK-CRIB (Rac1/Cdc42) immobilized to sepharose beads. Positive control was treated with 10 mM EDTA and 100 μM GTP[γS] at 30°C for 15 min to exchange the nucleotide. The complex of Rho protein and GTP[γS] was stabilized by the addition of 60 mM $MgCl_2$. Beads were washed with binding buffer. Bound Rho proteins were eluted by incubation in Laemmli lysis buffer at 95°C for 10 min and subjected to SDS-PAGE and Western blot analysis.

2.6. Western Blot Analysis. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat-dried milk for 60 min. Subsequently, the membrane was incubated with primary antibody at 4°C over night, and secondary antibody conjugated with horseradish peroxidase for 1 h at room

temperature. Blots were analysed by chemiluminescence reaction of ECL Femto using Kodak Image Station 440 CF.

2.7. Statistical Analysis. Statistical analysis was performed using Microsoft Excel and P values were analysed between two groups of data with a two-tailed student's t-test. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

3. Results

3.1. CNF1-Induced Activation of Rho Proteins and Formation of FAs. The morphology of Hela cells was analysed upon treatment with either CNF1 or CNFy for 24 h. CNF1, that deamidates RhoA, Rac1, and Cdc4 induced pronounced formation of actin stress fibres, membrane ruffles and lamellipodia and filopodia, as visualized in cells stained with rhodamine-phalloidin (Figure 1(a)). CNFy, that deamidates RhoA, induced the formation of pronounced actin stress fibres, but the formation of membrane ruffles or filopodia was less pronounced, indicating that CNFy activated RhoA in HeLa cells (Figure 1(a)) [15]. RhoA deamidation results in inhibited contractile ring formation in cytokinesis; cells treated with either CNF1 or CNFy undergo cell cycling but omit cytokinesis [14]. Therefore, CNF1-/CNFy-treated cells were binucleated and exhibited an increased cell size (Figure 1(a)).

RhoA deamidation was tracked by a reduced electrophoretic mobility on SDS-PAGE [17]. RhoA exhibited reduced electrophoretic mobility upon 2 h of treatment with CNF1 (Figure 1(b)), indicating RhoA deamidation. Deamidated RhoA was present in CNF1-treated cells over a time period of 12 h (Figure 1(b)). CNFy comparably induced RhoA deamidation upon treatment for 2 h (data not shown). Deamidated Rac1 has been reported to be rapidly degraded in a proteasome-dependent manner [18, 19]. Rho protein expression was analysed in CNF1-treated HeLa cells by Western blot. The protein level of Rac1 was reduced, whereas the levels of RhoA or Cdc42 were almost constant in HeLa cells (Figure 1(b)), corroborating former observations [19].

To provide direct evidence on the activation of Rho proteins, the relative cellular concentrations of activated Rho proteins were determined using effector pull-down assay exploiting the Rho-binding domain of Rhotekin for RhoA and the PAK-CRIB domain for Rac1 and Cdc42. Upon CNF1 treatment, the cellular levels of RhoA-GTP, Rac1-GTP, and Cdc42-GTP were increased (Figure 1(c)). In contrast, the cellular level of selectively RhoA-GTP was increased in CNFy-treated Hela cells (Figure 1(c)). HeLa cells were further treated with Toxin B from *C. difficile* strain VPI10463 (TcdB), a broad-spectrum inhibitor of Rho proteins [20, 21]. In TcdB-treated cells, the levels of active RhoA-GTP, Rac1-GTP, or Cdc42-GTP were significantly reduced (Figure 1(c)), indicating that glucosylation of Rho proteins resulted in their inactivation. CNF1 thus activated RhoA, Rac, and Cdc42, while CNFy specifically activated RhoA.

3.2. Increased Cell-Matrix Binding upon Treatment with CNF1 and CNFy. Following the hypothesis, that CNF1-induced activation of Rho proteins results in increased cell-matrix binding, the capability of suspended cells of establishing cell-matrix binding and cell spreading on fibronectin-coated polystyrol was analysed over time (Figure 2(a)). Suspended cells that bound to the matrix were initially rounded and then begin to spread out. Cells treated with either CNF1 or CNFy more efficaciously bound to the matrix compared to nontreated cells (Figures 2(a) and 2(b)). At later time points ($\geq$30 min), cell-matrix binding of CNF-treated and untreated cells was comparable (Figure 2(b)). Cell-matrix binding was completely blocked upon inhibition of Rho proteins by TcdB (Figure 2(b)), confirming the critical role of Rho proteins in cell-matrix binding. Both CNF1 and CNFy thus increased the efficacy of cell-matrix binding. Next, the effects of CNF1 and CNFy on cell spreading were analysed. CNF1-treated cells more rapidly spread out compared to CNFy-treated or nontreated cells on fibronectin-coated polystyrol (Figure 2(c)), as quantified in terms of spread per total cells. Cell spreading was further analysed on a polystyrol matrix. Cell spreading on polystyrol was clearly delayed compared to spreading on fibronectin-coated polystyrol (Figures 2(c) and 2(d)). CNF1 increased cell spreading also on the polystyrol matrix, while cell spreading of CNFy-treated cells was comparable to nontreated cells (Figure 2(d)). Cell spreading was specifically triggered by CNF1 (not CNFy), which correlated with the capability of CNF1 of activating Rac1 (Figure 1(c)).

The consequences of the CNF-induced activation of Rho proteins to cell-matrix adhesion were further analysed in terms of the susceptibility of cells to trypsin-induced cell detachment. Upon treatment with CNF1 or CNFy (less pronounced), trypsin-induced cell detachment was delayed (Figures 3(a) and 3(b)). In contrast, inhibition of Rho proteins by TcdB strongly increased the susceptibility towards trypsin-induced cell detachment, showing that Rho proteins were required for the establishment of cell-matrix adhesions (Figures 2(a)–2(d)). Treatment with CNF1 or CNFy triggered cell-matrix binding and increased the persistence of pre-established cell-matrix adhesion.

3.3. Formation of Focal Adhesions Induced by CNF1. The transition of cell-matrix adhesions from the initial punctate focal complexes into the mature elongated form, the focal adhesions (FAs), has been attributed to the activity of Rho proteins [22, 23]. To check if CNF1 induced FA formation, HeLa cells were stained for actin and vinculin, with the latter being an established FA marker. Elongated structures with a longitudinal length of $\geq 1.5\,\mu$m were considered as FAs. The number of FAs per cells strongly increased upon treatment with CNF1 (Figures 4(a) and 4(b)). Thereby, CNF1-induced formation of stress fibres and FAs was completely abolished upon treatment with the ROCK inhibitor Y-27632. RhoA-ROCK signalling was critical for FA formation in nontreated as well as in CNF1-treated cells. Furthermore, ROCK inhibition by Y27632 induced cell spreading in CNF1-treated cells (Figure 4(a)).

Pronounced cell spreading upon initial cell-matrix binding was exclusively observed in CNF1- (not in CNFy-)

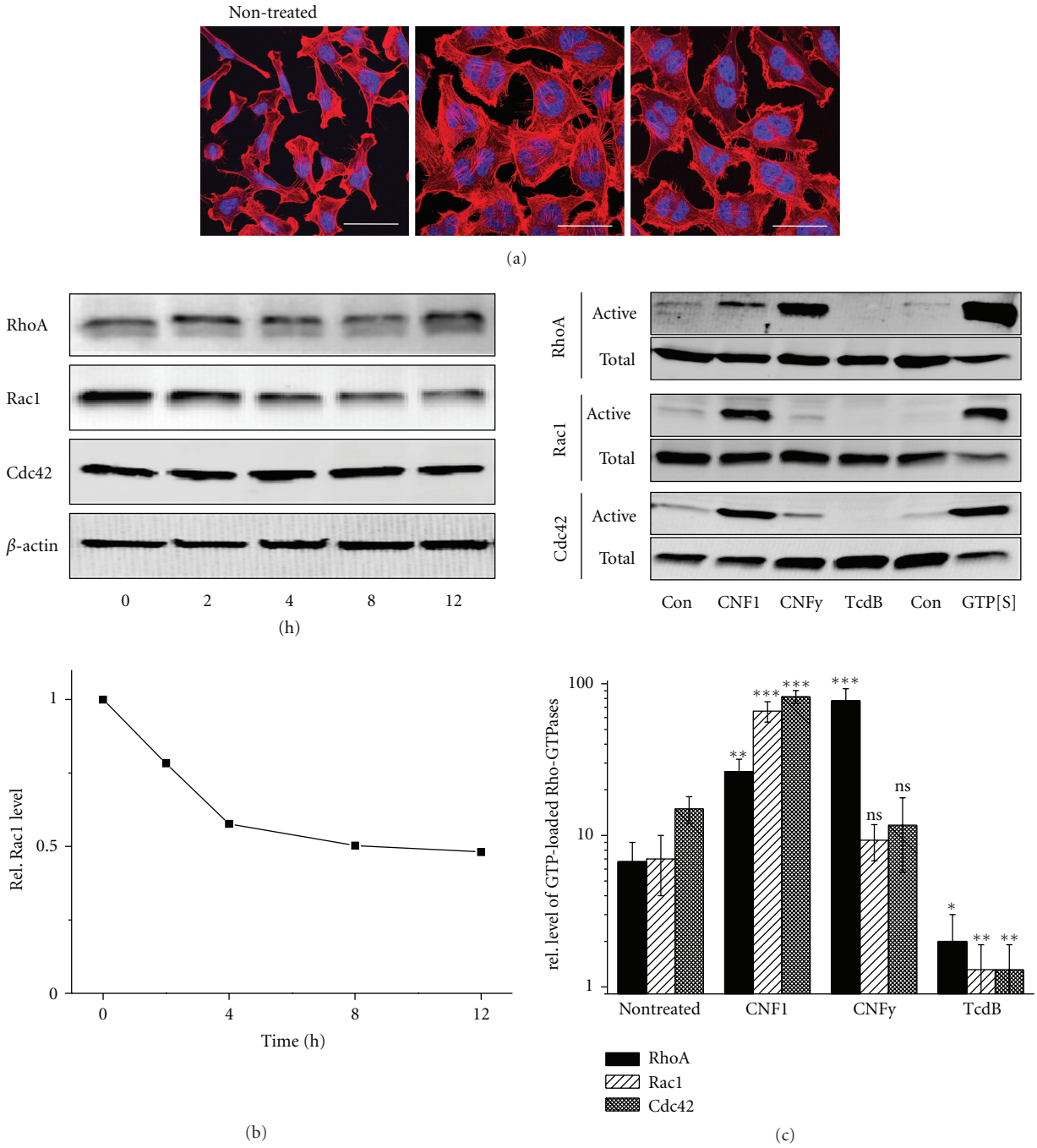

Figure 1: Formation of actin filaments upon activation of Rho proteins by CNF toxins. (a) HeLa cells were treated with CNF1 and CNFy for 24 h. The actin cytoskeleton and nuclei of CNF-treated HeLa cells was stained by rhodamine-phalloidin and DAPI, respectively. (b) HeLa cells were treated with CNF1 for 12 h. Reduced electrophoretic mobility of RhoA and the cellular levels of RhoA, Rac1, and Cdc42 were determined by Western blot analysis in time-dependent manner. Beta-actin was used as loading control. A representative Western out of three blots was presented. Signal intensities of Rac1 were quantified, and normalized to the level of beta-actin. (c) HeLa cells were treated with the indicated toxins for 5 h and the cellular levels of active GTP-bound RhoA, Rac1, and Cdc42 were determined by a pull-down assay using either GST-RBD (Rho-binding domain of Rhotekin) for RhoA and GST-PAK-CRIB (p21-binding domain of Pak1) for Rac1 and Cdc42. Total and precipitated Rho proteins were detected by immuno-blot analysis. A sample of lysates from nontreated cells was subjected to nucleotide exchange with the nonhydrolysable GTPγS. Signal intensities ($N = 3$) were quantified, and normalized to the level of total Rho proteins. Results displayed are the mean ± SD of three independent experiments. P values <0.01 (**) and <0.001 (***) were considered as statistically significant as compared with nontreated cells.

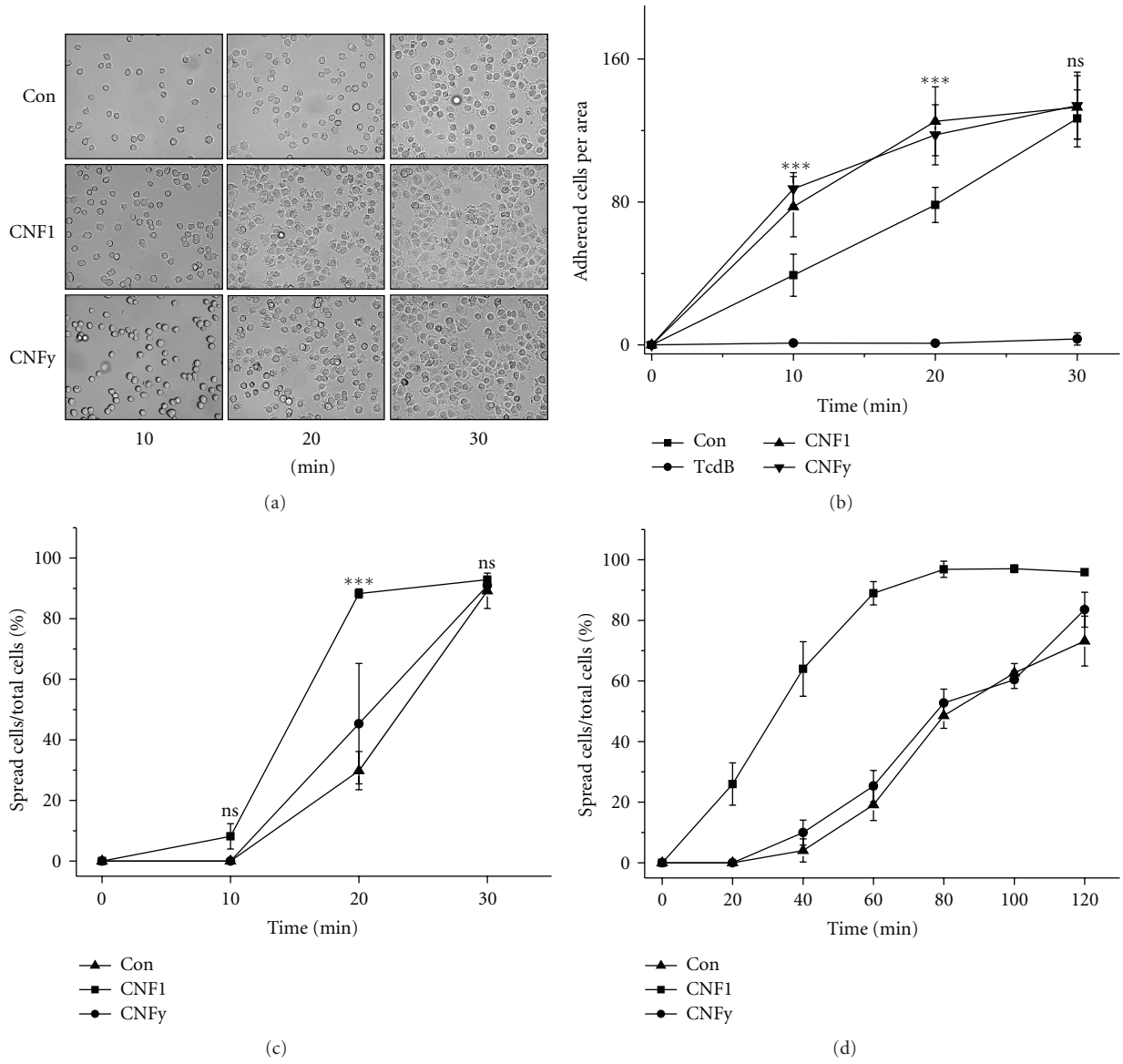

Figure 2: Increased cell attachment upon the activation of Rho proteins by CNF1 and CNFy. HeLa cells were treated with CNF1, CNFy, TcdB or buffer for 2 h and suspended by trypsin. Suspended cells were seeded onto a cell culture dish coated with fibronectin (a–c) or left uncoated (d). At the indicated time points, the medium containing nonadherent cells were removed. (a) Attached cells were documented by phase contrast microscopy. (b) The number of adherent cells per area was determined. Results displayed are the mean ± SD of three independent experiments (each experiment $n = 400$ cells). P values <0.001 (***) were considered as statistically significant as compared with nontreated cells. Cell spreading on fibronectin-coated polystyrol (c) or on noncoated polystyrol (d) was analysed in terms of the increasing number of spread per total cells. Results displayed are the mean ± SD of three independent experiments (each experiment $n = 100$ cells). P values <0.001 (***) were considered as statistically significant as compared with nontreated cells.

treated cells, suggesting that cell spreading depended on Rac1/Cdc42 activation rather than on RhoA activation. Next, the activity of the Rac1/Cdc42 effector protein PAK, another FA component [5], was analysed in FAs exploiting a phosphospecific antibody recognizing pS144/141-PAK1/2, the activated form of PAK1/2 (Figure 4(b)). CNF1 induced a pronounced increase of active PAK-positive FAs compared to nontreated or CNFy-treated cells. (Figure 4(b)). This observation showed that CNF1 (not CNFy) activated PAK1/2, reflecting specific activation of Rac1/Cdc42 by CNF1. Cell spreading thus correlated with the pronounced increase of PAK-positive FAs in CNF1-treated cells.

3.4. Reduction of Directional Cell Migration upon Activation of Rho Proteins by CNF1 and CNFy. Finally, the hypothesis was followed that increased cell-matrix adhesion affects the migration of CNF-treated cells. HeLa cells were treated with the CNF toxins for 2 h and then allowed to adhere on a transwell membrane. Cell migration was stimulated using a serum gradient (Boyden chamber transwell migration

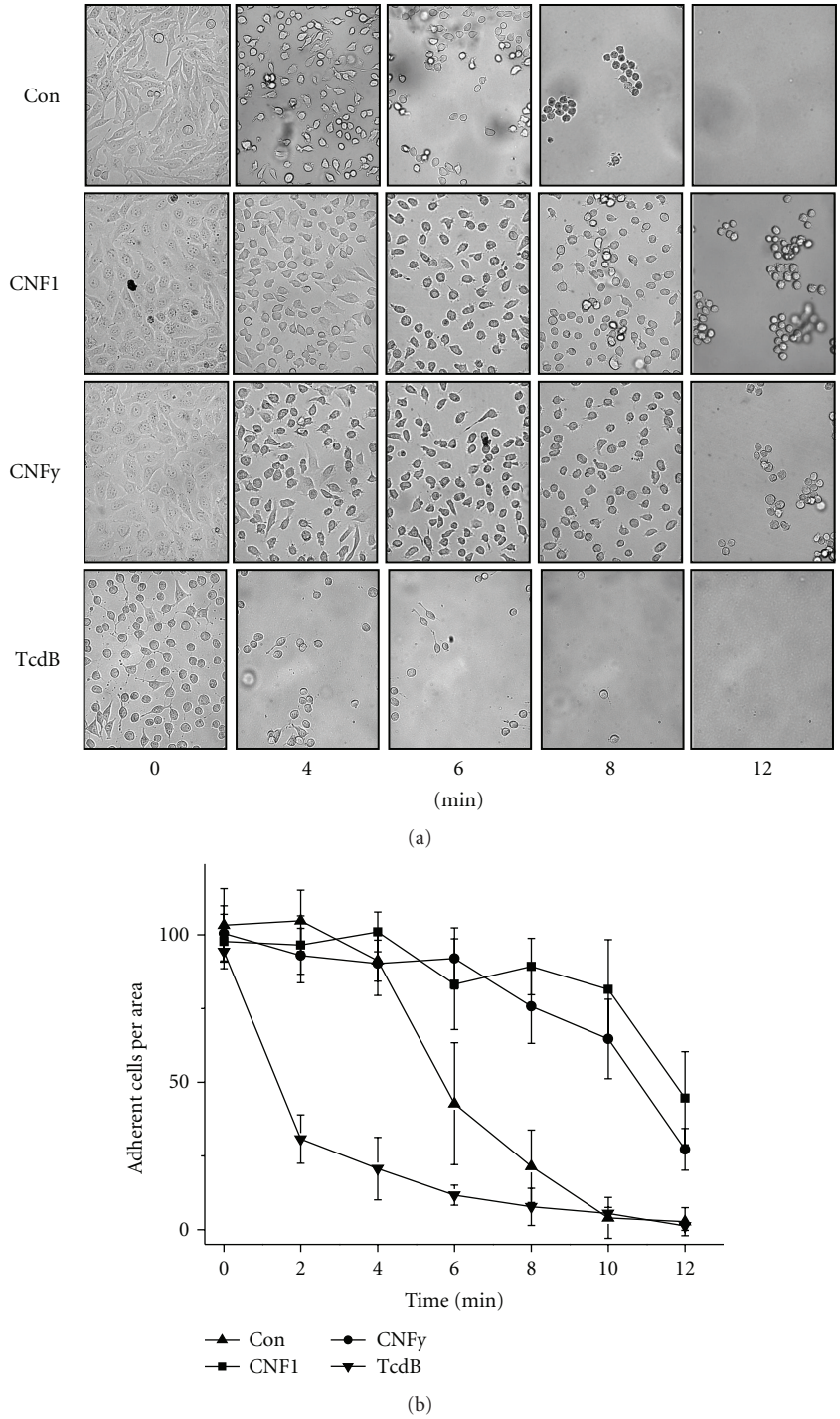

Figure 3: Reduced susceptibility of CNF1/CNFy-treated cells to trypsin-induced cell detachment. Adherent HeLa cells were treated with CNF1, CNFy, or TcdB for 2 h. Cells were incubated with trypsin for the indicated time and the medium with suspended cells was removed. (a) Adherent cells were documented by phase-contrast microscopy. (b) The number of adherent cells per area was determined in a time-dependent experiment. Results displayed are the mean ± SD of three independent experiments (each experiment $n = 100$ cells).

experiment). Under these conditions, about 50% of non-treated cells migrated through the membrane within a time period of 5 h (data not shown). TcdB treatment completely blocked migration of adherent cells (Figure 5). Cells either treated with CNF1 or CNFy migrated to some extent. The efficacy of migration was reduced compared to nontreated cells (Figure 5). Furthermore, the migration of suspended cells was analysed. Therefore, adherent cells were pretreated with the toxins for 2 h, suspended by trypsination, placed onto the Boyden chamber membrane, and directly allowed

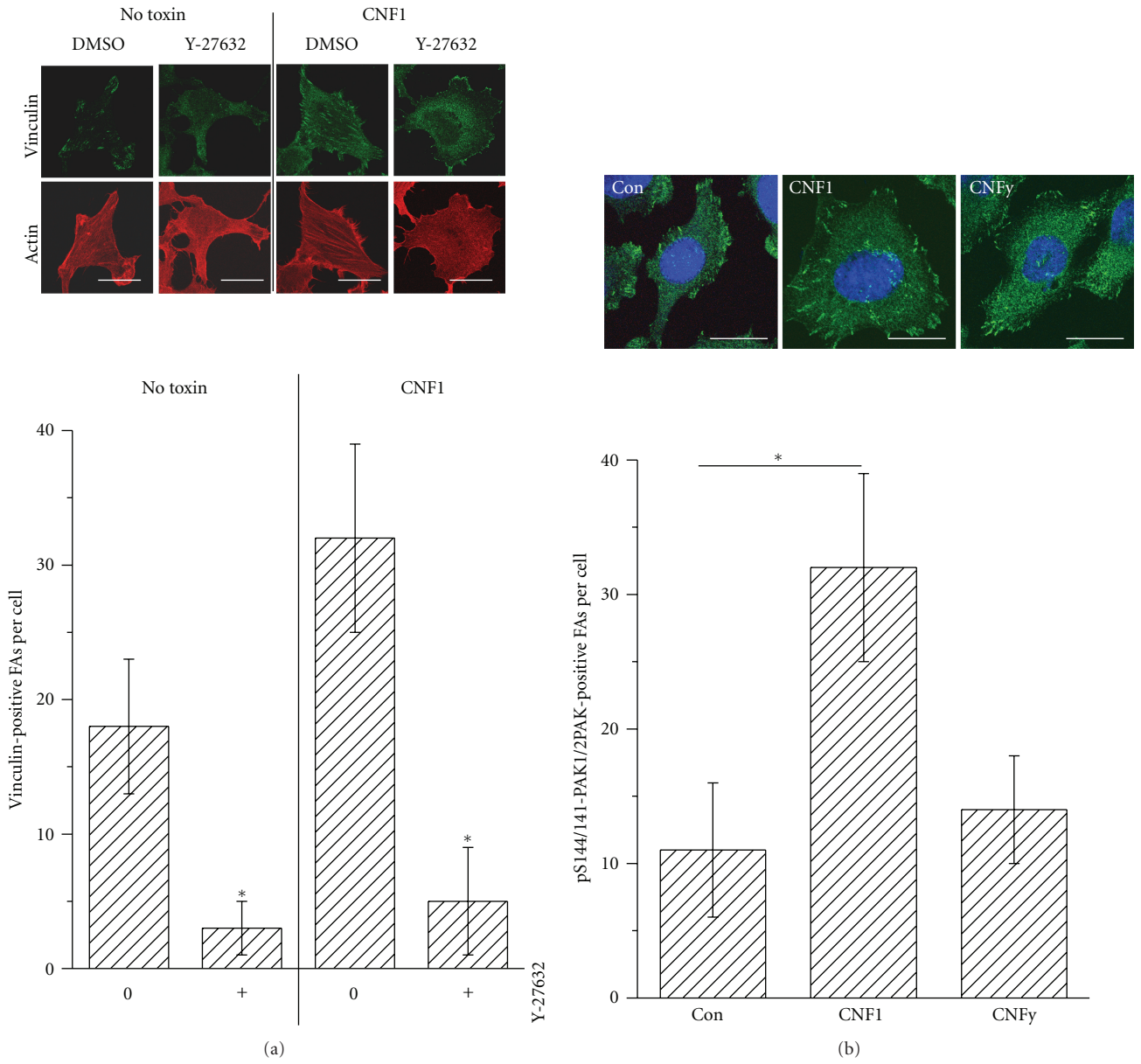

Figure 4: CNF1/CNFy-induced formation of focal adhesions. Focal adhesions in HeLa cells treated with the indicated CNF toxins or buffer were stained for the FA marker vinculin (a) or pS144/141-PAK1/2 (b) using immunocytochemistry. The number of vinculin-positive (a) or pS144/141-PAK1/2-positive FAs (b) per total cells was analysed in three independent experiments (each experiment $n = 30$ cells). P values <0.05 (*) were considered as statistically significant as compared with nontreated cells.

to migrate. Under this condition, about 50% of nontreated cells migrated through the membrane within a time period of 5 h (data not shown). Cell migration of adherent and suspended cell through the membrane was thus comparable. The migration of suspended cells was completely blocked upon TcdB treatment (Figure 5). Interestingly, treatment of suspended cells with either CNF1 or CNFy did not affect cell migration (Figure 5). Suspended cells pretreated with CNF1 or CNFy migrated with an efficacy comparable to that of nontreated cells (Figure 5). Deamidation of Rho proteins thus reduced cell migration under the condition of pre-established cell-matrix adhesion.

4. Discussion

In this study, the effects of Rho-modifying toxins on cell adhesion are analysed to dissect distinct roles of Rho proteins in cell adhesion. In particular, the application of the RhoA-specific CNFy is exploited to dissect the role of RhoA activation to the observed effects. The initial step of cell adhesion is cell-matrix binding. Cell-matrix binding is strongly increased by both CNF1 and CNFy to comparable extent, showing that increased cell-matrix binding of CNF1-/CNFy-treated depends on RhoA deamidation. Cell matrix-binding of TcdB-treated cells is completely prevented,

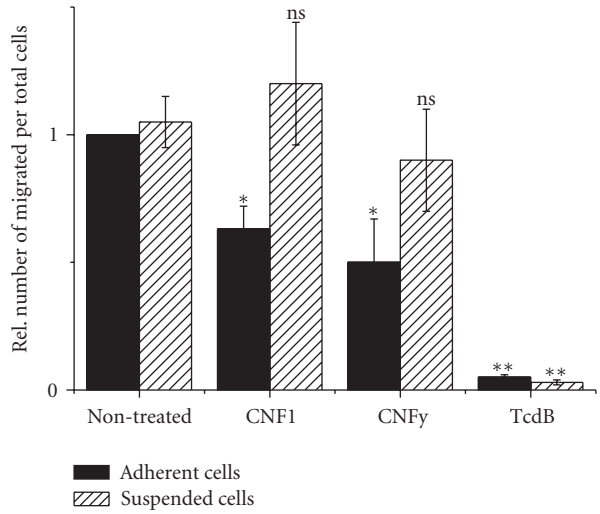

Figure 5: Reduced cell migration of adherent cells upon treatment with CNF1 and CNFy. Migration of HeLa cells was analysed in a Boyden chamber experiment. Cell migration was induced by a serum gradient of 10% bovine calf serum for 5 h. Cell migration was quantified as the number of cells that passed bottom membrane. Suspended cells: HeLa cells were pretreated with the indicated toxins for 2 h. Cells were suspended by trypsin, directly seeded onto the membrane and the serum gradient was applied for 5 h. Adherent cells: HeLa cells were seeded on the membrane and allowed to attach. Cells were then treated with the indicated toxins for 2 h. Afterwards the serum gradient was applied for 5 h. Cells on the upper membrane surface were scraped with a cotton swab, and cells on the bottom surface stained with Hoechst 33342. After incubation at 37°C for 15 min, cells were analyzed by fluorescence microscopy using a Zeiss Axiovert 200 M (excitation 365 nm, emission 420 nm). Five adjacent microscope fields for each membrane were counted at ×20 magnification. Results displayed are the mean ± SD of three independent experiments. P values <0.05 (*) and <0.01 (**) were considered as statistically significant as compared with nontreated cells.

showing that the activity of Rho proteins is required for cell-matrix binding. Cell-matrix binding is followed by cell spreading. Cell spreading is specifically triggered by CNF1 (not CNFy), suggesting that cell spreading rather depended on Rac1/Cdc42-dependent rather than RhoA-dependent signaling pathways. Furthermore, the RhoA effector protein ROCK seems to negatively regulate cell spreading, as cell spreading of CNF1-treated cells is increased upon inhibition of the ROCK by Y-27632.

Cell-matrix binding and cell spreading include the formation of focal contacts, which maturate into elongated focal adhesions (FAs) [22, 23]. Activation of Rho proteins by CNF1 strongly increased the number of vinculin-positive FAs per cells. The FAs formation depends on RhoA/ROCK signalling in CNF1- and nontreated Hela cells, as ROCK inhibition by Y-27632 completely blocks FAs formation. The latter observation is consistent with the view that the elongated form of FAs depends on the presence of stress fibres, which formation is inhibited upon Y-27632 treatment [4]. The increased number of FAs in CNF1-treated cells (compared to nontreated cells) likely results from CNF1-mediated activation of Rho proteins, which reportedly drives the maturation of focal contacts into focal adhesions [22, 23]. The Rac1/Cdc42 effector protein PAK1/2 is also regarded as a component of FAs [24]. In CNF1- (not CNFy-) treated the number of pS144/141-PAK1/2-positive FAs, that is, FAs with activated PAK1/2, is strongly increased. This observation reflects the fact that specifically CNF1 (not CNFy) activates Rac1/Cdc42. Interestingly, active PAK has been suggested to promote FA disassembly and cell detachment [3, 24]. Although active PAK is present at the FAs of CNF1-treated cells, these cells exhibited a reduced susceptibility of cells to trypsin-induced cell detachment compared to CNFy-treated and nontreated cells. The activation of RhoA-dependent and other Rac1/Cdc42-dependent pathways that positive regulates cell attachment (including those regulating cell spreading) obviously overbalances cell detachment driven by active PAK1/2 in CNF1-treated cells. CNFy-treated cells exhibit a reduced susceptibility of cells to trypsin-induced cell detachment compared to nontreated cells, showing that the activation of RhoA-dependent pathways is sufficient for increased cell adhesion.

The consequences of increased cell adhesion of CNF1-/CNFy-treated cells on cell migration is differentially analysed in adherent and suspended cells. Suspended cells treated with either CNF1 or CNFy migrated to an extent comparable to that of nontreated cells. Thus the presence of deamidated Rho proteins *per se* did not inhibit cell migration. In contrary, microinjection of Rac1-Q61L is a well-established method to trigger cell migration [25]. This may explain, why migration of CNF1-treated suspended cells is slightly (not significantly) increased. In contrast, the migration of suspended cells was completely blocked upon TcdB pre-treatment. This observation illustrates that activity of Rho proteins and cell-matrix adhesion are both prerequisites for cell migration [4]. The matrix adhesion of suspended TcdB-treated cells is completely blocked, based on the fact that the activity of Rho proteins is required for cell-matrix adhesion. The analysis of cell migration of adherent cells revealed that migration of CNF1-/CNFy-treated cells is reduced. Against the background that the deamidation of Rho protein *per se* does not reduce cell migration, this reduction most likely reflects that increased cell adhesion impairs cell migration. Cell migration includes FA disassembly and loss of cell adhesion at the cell rear [6]. This process mainly depends on RhoA and correlates with the observation of this study that both CNF1 and CNFy reduce cell migration. RhoA deamidation may thereby prevent FA disassembly and loss of cell adhesion at the cell rear of adherent cells, which remains to be analysed in detail. In conclusion, CNF1 and CNFy both reduce cell migration specifically under the condition of pre-established cell-matrix adhesion. This observations makes CNF toxins an interesting tool, as it allows the dissection of the role of Rho proteins in cell adhesion (CNF sensitive) from the role of Rho proteins in other processes (CNF insensitive) of the migrating cells.

In adherent epithelial cells, integrin-mediated cell survival is promoted through several pathways including the phosphatidylinositol 3-kinase (PI3K)-Akt pathway [26].

Epithelial detachment of epithelial cells results in decreased survival signalling. The detached cells undergo a type of apoptotic cell death, also referred to as anoikis [27]. Against this background, the capability of CNF1 of inducing increased cell adhesion is of particular interest. CNF1 has been presented to preserve epithelial cells from apoptosis induced by various stimuli [13, 28, 29]. Two distinct mechanisms thereby may be responsible for the antiapoptotic activity of CNF1: (i) activation of Rac1/Cdc42 suppresses apoptosis in a PI3K/Akt-dependent manner [27, 29, 30]; (ii) increased cell adhesion preserves cells from detachment and subsequent anoikis [13, 28]. Given that increased cell adhesion is sufficient for preserving the detachment of epithelial cells, CNFy should exert antiapoptotic activity as well, an aspect of the activity of CNFy that remains to be investigated.

Abbreviations

CNF: Cytotoxic necrotizing factor,
FA: Focal adhesion,
PAK: p21-activated kinase,
TcdB: Toxin B from *C. difficile* strain VPI10463.

Conflict of Interestes

The authors declare that they have no conflict of interests.

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Review Article

Pivotal Role of AKAP12 in the Regulation of Cellular Adhesion Dynamics: Control of Cytoskeletal Architecture, Cell Migration, and Mitogenic Signaling

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Cellular dynamics are controlled by key signaling molecules such as cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). AKAP12/SSeCKS/Gravin (AKAP12) is a scaffold protein for PKA and PKC which controls actin-cytoskeleton reorganization in a spatiotemporal manner. AKAP12 also acts as a tumor suppressor which regulates cell-cycle progression and inhibits Src-mediated oncogenic signaling and cytoskeletal pathways. Reexpression of AKAP12 causes cell flattening, reorganization of the actin cytoskeleton, and the production of normalized focal adhesion structures. Downregulation of AKAP12 induces the formation of thickened, longitudinal stress fibers and the proliferation of adhesion complexes. AKAP12-null mouse embryonic fibroblasts exhibit hyperactivation of PKC, premature cellular senescence, and defects in cytokinesis, relating to the loss of PKC scaffolding activity by AKAP12. AKAP12-null mice exhibit increased cell senescence and increased susceptibility to carcinogen-induced oncogenesis. The paper describes the regulatory and scaffolding functions of AKAP12 and how it regulates cell adhesion, signaling, and oncogenic suppression.

1. Introduction

The actin cytoskeleton plays an essential role in numerous aspects of cell biology such as cell adhesion, cell morphology, cytokinesis, and migration. Cell migration machinery is regulated by signaling intermediates that can be activated by diverse stimuli and that can exert control over a large number of downstream target molecules, all with temporal and spatial specificity [1, 2]. PKA, PKC, and Ca^{2+}-binding proteins are examples of cellular regulators that mediate diverse effects on cytoskeletal dynamics, cell adhesion, and cell migration [3, 4]. Control of the subcellular localization of PKA and PKC activities in a temporal manner by A-Kinase-Anchoring Proteins (AKAP) has emerged as a pivotal mechanism to control cell migration [2]. For instance, AKAP12/SSeCKS/Gravin (AKAP12) is thought to control a number of cellular events by scaffolding key signaling molecules such as cyclin D1, calmodulin, PKA, and PKC (Figure 1) [5].

SSeCKS (rodent AKAP12), the Src-Suppressed-C-Kinase-Substrate, was originally identified in a screen for genes severely downregulated by v-Src [6], but subsequently we and others showed that it is also downregulated by oncogenic forms of Ras, Myc and Jun [7, 8] and in SV40-transformed fibroblasts [9]. The gene encoding the human SSeCKS orthologue, Gravin, is localized on chromosome 6q24-25.2, a deletion hotspot in advanced prostate, ovarian, and breast cancer [10], implicating a role for the loss of AKAP12 in cancer progression. Importantly, AKAP12 orthologues have been identified in all vertebrate species, and, in humans and rodents, two major AKAP12 transcripts, α and β, are expressed ubiquitously in the embryo and the adult as 305- or 290-kDa products (rodents: 290-kDa or 280-kDa), respectively. AKAP12 transcript levels increase in confluent cultures of untransformed cells irrespective of the effects of serum growth factors [7, 10–12], yet, in subconfluent cultures, they are unaffected by either serum deprivation or inhibition of DNA synthesis [13]. Although

the coding sequence of AKAP12 contains several PEST motifs linked to protein instability, AKAP12 is a long-lived protein under certain conditions [14]. Newly synthesized AKAP12 in confluent cultures is not well phosphorylated, whereas serum addition to subconfluent cultures results in a rapid serine and tyrosine phosphorylation concurrent with G1 to S progression [13, 15].

AKAP12 is a major in vitro and in vivo substrate of PKC [9, 16]. Both AKAP12 and PKC isozymes (including conventional and novel) contain phosphatidylserine (PS) binding sites, and although PS enhances AKAP12/PKC binding [9, 17], recent data identify two PS-independent PKC binding motifs in AKAP12 [18]. Phosphorylation of AKAP12 in vitro with PKC decreases PS-mediated PKC binding [16], though it has not been shown that this phosphorylation inhibits PS binding itself. Interestingly, PKC-induced phosphorylation of AKAP12 causes it to translocate from plasma membrane and cytoskeletal sites to the perinucleus in fibroblasts, mesangial and epithelial cells [10, 11, 16, 19], suggesting that this event may play a role in the PKC-mediated reorganization of the actin cytoskeleton.

The functions of AKAP12 are based upon its ability to scaffold key signaling proteins in a spatiotemporal manner and specific scaffolding functions have been described for the control of (i) cell migration, (ii) maintenance of cytoskeletal architecture, (iii) cell proliferation, and (iv) cytokinesis.

2. Role of AKAP12 on Cellular Architecture, Adhesion, and Migration

One of major scaffolding roles for AKAP12 is as a critical regulator of cell migration. For instance, AKAP12 reexpression is sufficient to inhibit *src*-induced anchorage-independence and Matrigel invasiveness as well as to induce formation of normalized stress fibers and vinculin-associated adhesion plaques typical of those found in untransformed cells [20]. In addition, reexpression of AKAP12 in the rat metastatic prostate cancer cell line, MAT-LyLu, suppresses colony formation in soft agar, decreases refractility, and increases cell-cell interactions [10]. Importantly, AKAP12 attenuates specialized motility, such as chemotaxis and invasiveness, rather than generic cell motility. For instance, AKAP12 reexpression in MAT-LyLu cells has no effect on short- and long-term motility in monolayer wound-healing assays [10]. In contrast, AKAP12 inhibits chemotaxis via the attenuation of a PKC-Raf/MEK/ERK pathway [21]. In addition, upregulated AKAP12 facilitates HGF-induced, c-Met-dependent cell motility through the upregulation of PKA activity and PKA-induced genes, presumably through AKAP12's scaffolding function [22]. Interestingly, AKAP12 phosphorylation by PKC decreases AKAP12-PKC scaffolding but no change in AKAP12-PKA binding [23] or agonist-induced PKA activation [17]. These findings suggest that AKAP12 controls mutually exclusive activation between PKA and PKC. It is likely that the differential PKA-PKC control relates to an overlap between PKC binding and phosphorylation sites mapping to the N-terminus of AKAP12, whereas the PKA binding site maps to the C-terminus of AKAP12 lacking phosphorylation sites (Figure 1). The C-terminal domain of AKAP12 is required for AKAP12 to target PKA to the cell periphery [24]. However, PKC activation by phorbol esters causes translocation of the AKAP12/PKA complex to the perinucleus [23, 24]. Taken altogether, these data strengthen the notion that AKAP12 promotes the differential activation of PKA and PKC in processes such as cell motility.

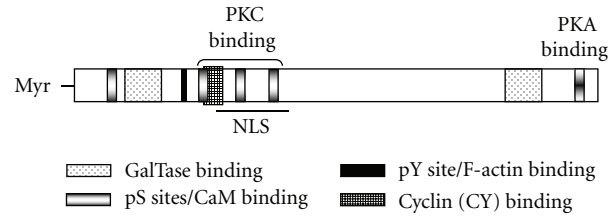

Figure 1: AKAP12 binds to key signaling molecules. AKAP12 contains various demonstrated protein binding domains as well as PKC phosphorylation sites (pS) and a tyrosine phosphorylation site (pY). NLS, nuclear localization signals (at least 4 T_{ag} motifs); CaM, calmodulin; GalTase, β1,4-galactosyltransferase; Myr, N-terminal myristoylation.

AKAP12 is involved in FAK-mediated signaling. AKAP12 induces integrin-independent tyrosine phosphorylation of FAK in suspension cultures [25]. AKAP12 reexpression also enhances integrin-mediated adhesion and superinduces FAK autophosphorylation levels, likely by physically disengaging Src from FAK complexes, which, in turn, leads to less focal adhesion turnover (Bing Su, Lingqiu Gao, Fanjie Meng, Li-wu Guo, Julian Rothschild, Irwin H. Gelman, Adhesion-mediated cytoskeletal remodeling is controlled by the direct scaffolding of Src from FAK complexes to lipid rafts by SSeCKS/AKAP12. *Oncogene*, in press). In addition, mitogen-induced, FAK-dependent tyrosine phosphorylation of AKAP12 modulates its binding to the actin-based cytoskeleton, suggesting a role for AKAP12 in mitogen-induced cytoskeletal reorganization [15]. Recently we discovered that AKAP12 negatively regulates FAK expression [26], implicating a role for AKAP12 in the control of FAK levels. Although further work is required to address how AKAP12 controls FAK expression, it is likely that AKAP12 affects both FAK-mediated adhesion and motility pathways.

AKAP12 is also a regulator of cytoskeletal architecture. The ablation of AKAP12 expression in glomerular mesangial cells leads to the thickening and polarization of F-actin stress fibers, an increase in the number of transverse focal adhesion plaques, and an increase of phosphotyrosine staining in focal complexes [11]. Consistent with this report, AKAP12-null mouse embryonic fibroblasts (KO-MEF) exhibit robust stress fiber formation as well as increased numbers of focal adhesion plaques ([27], Figure 2). Ablation of AKAP12 converts the stellate morphology of human mesangial cells and rodent fibroblasts to a spindle morphology. In actively dividing cells, AKAP12 associates with a cortical cytoskeleton and is enriched in lamellipodia [16, 25]. Overexpression of AKAP12 in untransformed NIH3T3 cells causes G1-arrest marked by severe cell flattening, elaboration of an AKAP12-associated cytoskeleton, a transient loss of actin

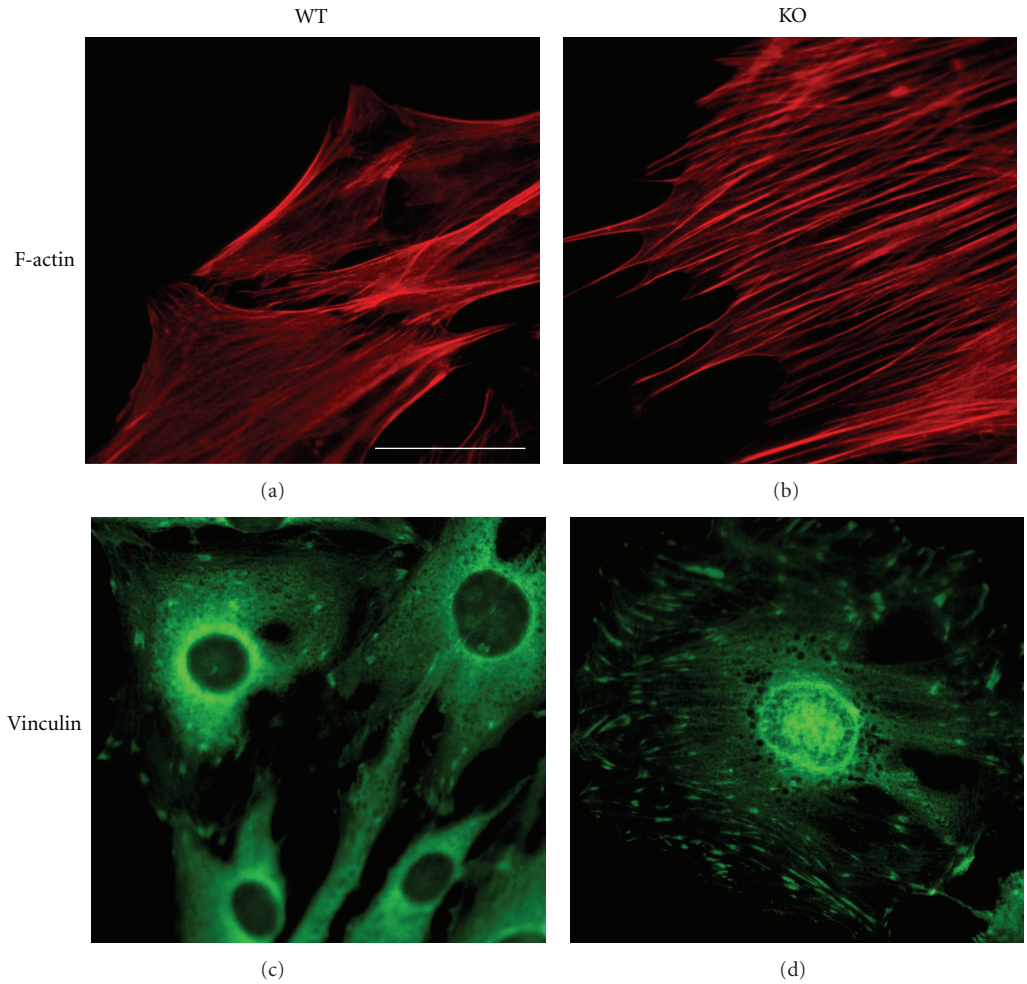

Figure 2: AKAP12-null cells exhibit robust stress fiber formation as well as upregulated focal adhesion complexes. Wild type and AKAP12-null mouse embryonic fibroblasts were stained with either rhodamine-phalloidin (F-actin, (a) and (b)) or antivinculin antibody ((c) and (d)) followed by Alexa488 antibody. Scale bar, 10 μm.

stress fibers and vinculin-associated adhesion plaques, and the production of filopodia and lamellipodia-like projections [25, 28]. Ectopic expression of AKAP12 diminishes stress fiber formation likely through a direct binding to F-actin [25] (Figure 1), suggesting that AKAP12 directly affects the formation or maintenance of stress fibers. These observations suggest that AKAP12 plays a critical role in the dynamic reorganization of the actin cytoskeleton during the processes such as cell migration or maintenance of cell architecture.

3. Role of AKAP12 on Cell Cycle Regulation and Cytokinesis

Another major scaffolding role for AKAP12 is as a negative regulator of G1 to S progression [6]. It is likely that AKAP12 controls cell cycle progression in NIH3T3 cells by regulating cyclin D1 in two ways: (i) directly scaffolding cyclin D1 via cyclin D1 binding (CY) motifs, thereby facilitating contact-inhibition by sequestering cyclin D1 pools in the cytoplasm, and (ii) decreasing cyclin D1 expression by suppressing serum-inducible ERK2 activity [28]. The CY domains overlap with major PKC phosphorylation sites on AKAP12 (Figure 1). The notion that AKAP12 scaffolding activity for cyclin D1 is antagonized after it is phosphorylated by PKC comes from the findings that (i) activation of PKC by phorbol esters induces cyclin D1 translocation to the nucleus, and (ii) phosphorylation of AKAP12 by PKC in vitro antagonizes its binding to cyclin D1 and cyclin E [29].

Intriguingly, AKAP12 changes its localization during the cell cycle. Consistent with previous reports [16, 23, 24], live cell imaging analysis demonstrates that localization of AKAP12 of resting cells is mainly in the cytoplasm, with some portion localizing to specific compartments such as plasma membrane and perinucleus (S. Akakura and I. H. Gelman, unpublished observation). Although further work is required to elucidate the role of AKAP12 in cell cycle regulation, it is conceivable that AKAP12 localization and the timing of expression are critical for cell cycle progression.

Evidence is accumulating that AKAP12 is involved in cytokinesis regulation [30–32]. We [27] and others [30, 32] suggested that the loss of AKAP12 leads to cytokinesis defects. A recent systems biology analysis implicates

the existence of a mitotic protein complex containing AKAP12 and other molecules such as Polo-like kinase 4, APC, dynein and profilin (http://www.mitocheck.org/cgi-bin/mtc?query=MCG_0000007). Choi et al. demonstrated that AKAP12 localizes on the anaphase abscission furrow [30]. The furrow contains the actin-myosin ring whose PKC-Rho GTPase-dependent contraction helps complete daughter chromosome separation [33], and, thus, it is conceivable that AKAP12 regulates cytokinesis via its ability to scaffold PKC and F-actin, and to attenuate Rho GTPase activity [34]. Since AKAP12 controls the activity of PKC, and PKCε mediates the completion of cytokinesis [35], it is conceivable that AKAP12 normally scaffolds PKC and regulates actomyosin ring formation through controlling PKCε-RhoA signaling during specific mitotic stages and structures.

4. AKAP12 as a Tumor Suppressor and Metastasis Suppressor

Despite the fact that AKAP12 is widely expressed throughout embryogenesis [14], AKAP12-null mice (KO-mice) are viable though they exhibit spontaneous prostatic hyperplasia [36]. Dysplastic foci were observed less frequently but were associated with the loss of E-cadherin staining and the loss of basal cell markers [36], suggesting that the loss of AKAP12 causes a cancer-prone condition. In fact, prostates of KO mice exhibit senescence associated β-galactosidase expression [27], which can be used as a premalignant marker [37], implicating that the loss of AKAP12 causes a precancerous condition. KO-MEF also exhibit premature senescence marked by senescence-associated β-galactosidase expression. These cells are readily transformed by single oncogenes such as Src or Ras, suggesting that the loss of AKAP12's tumor suppressor function renders the cell transformation-prone. Importantly, AKAP12 deficiency causes hyperactivation of PKC isozymes, leading to Rb-dependent senescence involving PKCα and δ but not PKCε. KO-MEF are also more susceptible to immortalization in culture [27]. Immortalized KO-MEF have decreased levels of senescence associated β-galactosidase staining and of the cyclin-kinase inhibitor, p16, indicating that the cells override Rb-dependent senescence. Moreover, expression levels of Cyclin-dependent kinase 4 and LATS/Warts (a mitotic kinase), which are downregulated in KO-MEF, are upregulated in immortalized KO-MEF. These data suggest that AKAP12 facilitates pathways for continued proliferation through G1 and G2 phase arrest points found in senescent cells.

It is likely that AKAP12 controls senescence through a direct scaffolding of PKC isozymes because reexpression of full-length AKAP12, but not AKAP12 deleted of its PKC-binding domains, suppresses senescence [27]. This suggests that AKAP12-null cells would be a unique tool to study the biological effects of PKC isozyme hyperactivation.

Rhim et al. reported that the protein levels of AKAP12 are higher in senescent human diploid fibroblasts and in aging rat and human keratinocytes [38]. Given our finding that KO-MEF express higher levels of p47phox, a component of NADPH oxidase, it is reasonable to speculate that AKAP12 suppresses the production of reactive oxygen species (ROS), ultimately serving as a protection against premature aging and spontaneous oncogenesis. Further work is required to address how AKAP12 regulates the aging process.

Evidence is accumulating that AKAP12 expression is downregulated in many cancer types, either associated with gene deletion or epigenetic downregulation due to promoter hypermethylation or changes in chromatinization. For instance, the expression level of AKAP12 is downregulated in breast cancer [39], leukemia [40], ovarian cancer [41], colorectal cancer [42], and hepatocellular carcinoma [43]. Many microarray-based studies demonstrate significant reduction in relative AKAP12 mRNA levels in many cancer types that cited in Entrez GEO (Gene Expression Omnibus) or Oncomine (http://www.oncomine.org/) linking AKAP12 expression with tumor suppression. In addition, we showed recently that AKAP12-null mice have increased susceptibility to papilloma and squamous cell carcinoma formation induced by DMBA and TPA, well-known skin carcinogens [26]. Interestingly, dermal layers in AKAP12-null mice are hyperplastic, and they show significant upregulation of FAK, a known promoter of carcinogen-induced squamous cell carcinoma [44].

AKAP12 has been shown to function as a metastasis suppressor possibly by inhibiting the expression of VEGF at distal sites [45], and by inhibiting oncogenic invasiveness [21]. Reexpression of AKAP12 in MAT-LyLu cells causes a small decrease in primary subcutaneous tumor growth yet severely suppresses the formation of macroscopic lung metastasis [10]. In addition, multiple Oncomine studies show significant decreases in AKAP12 expression in metastases compared to levels in primary tumors, suggesting a role for AKAP12 in suppressing metastasis. Taken altogether, these data suggest that AKAP12 is especially potent in regulating the metastatic process, a function likely relating to its ability to downregulate angiogenesis-controlling genes, such as VEGF [46], and invasion-controlling genes, such as MMP-2 [21].

5. Other Roles for AKAP12

There is mounting evidence that AKAP12 scaffolding of PKC plays a role in regulation of mesangial cell differentiation and proliferation, and thus, glomerular function [11]. Nelson et al. reported that AKAP12 mediates the control of the actin-based cytoskeletal architecture in mesangial cells by PKC [11]. Recently, Burnworth et al. reported that SSeCKS controls the localization and activity of cyclin D1 in glomerular parietal epithelial cells and influences response to proliferative injury in the glomerulus [47]. This paper demonstrates severely increased proliferative injury levels of glomerular parietal epithelial cells, leading to proteinurea in AKAP12-null versus wild type mice. Thus, AKAP12 plays a critical role in architectural maintenance of glomerular parietal epithelial cells, and AKAP12 deficiency increases the susceptibility to injury-induced glomerulonephritis [47].

AKAP12 is involved in the β_2-adrenergic receptor-mediated signaling [48]. Agonist stimulation of the β_2-adrenergic receptor leads to activation of kinases that are

associated with AKAP12. PKA-mediated phosphorylation of AKAP12 stabilizes the interaction between AKAP12 and the receptor, while PKC-mediated phosphorylation of AKAP12 causes it to translocate from the β_2-adrenergic receptor. Prolonged agonist stimulation leads to degradation of the receptor and induces desensitization [48, 49].

AKAP12 is also critical in the regulation of blood-brain barrier (BBB) [45, 50]. AKAP12 attenuates neovascularization as well as barrier formation through the downregulation of proangiogenic genes such as HIF1α or VEGF [46, 50]. AKAP12 is upregulated during normoxic transition of the mouse embryo at birth, and AKAP12 is responsible for suppressing brain angiogenesis through a JNK-dependent downregulation of VEGF and for inducing postnatal formation of the BBB by promoting tighter astrocyte/endothelial cell junctions [50]. Recently, Kwon et al. reported that AKAP12 is essential for the integrity of the endothelium by maintaining the expression of PAK2 and AF6, cell-cell adhesion regulators, during vascular development [51]. Although AKAP12-null mice do not exhibit blood vessel issues [36], deficiency of AKAP12 causes hemorrhage in embryos of zebrafish and overexpression of PAK2 and AF6 is sufficient to rescue the abnormal hemorrhage in AKAP12-depleted zebrafish embryos. Taken altogether, AKAP12 is essential for cellular architecture and is required for the integrity of cell-cell junction.

6. Conclusion

AKAP12 regulates cell cycle progression, cell motility, and cell morphology through its multiple scaffolding domains. Suppression of oncogenic proliferation, chemotaxis, and cellular senescence all involve attenuation of PKC activation through direct spatiotemporal scaffolding functions of AKAP12.

Several major issues regarding AKAP12 remain to be elucidated. First, relating to its subcellular localization, it is still not clear whether AKAP12 plays a role in the nucleus, even though it contains at least six nuclear localization signals. Second, the molecular mechanisms by which AKAP12 differentially regulates the crosstalk between PKA and PKC signaling pathways remain unclear. Lastly, other AKAP12 binding partners are likely to be found that either regulate AKAP12 functions or that are regulated by AKAP12 scaffolding.

Further studies are required to elucidate how the regulation of PKC and other molecules through scaffolding proteins such as AKAP12 maintains the integrity of cellular signaling and cytoskeletal control. Results from those studies would strongly suggest that targeting PKC-regulators such as AKAP12 should have therapeutic benefit for cancer patients.

Abbreviations

AKAP12: A-kinase-anchoring protein 12
SSeCKS: Src-suppressed C-kinase substrate
PKA: Cyclic AMP-dependent protein kinase
PKC: Protein kinase C
MEF: Mouse embryonic fibroblasts.

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Review Article

The Small GTPase Rap1b: A Bidirectional Regulator of Platelet Adhesion Receptors

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Integrins and other families of cell adhesion receptors are responsible for platelet adhesion and aggregation, which are essential steps for physiological haemostasis, as well as for the development of thrombosis. The modulation of platelet adhesive properties is the result of a complex pattern of inside-out and outside-in signaling pathways, in which the members of the Rap family of small GTPases are bidirectionally involved. This paper focuses on the regulation of the main Rap GTPase expressed in circulating platelets, Rap1b, downstream of adhesion receptors, and summarizes the most recent achievements in the investigation of the function of this protein as regulator of platelet adhesion and thrombus formation.

1. Introduction

The adhesion of circulating blood platelets to the subendothelial matrix exposed upon vessel wall injury represents the initial event of the haemostatic process required to limit hemorrhage. Platelets express several membrane receptors specific for all the major adhesive ligands of the vascular extracellular matrix [1]. Among these, collagen is probably the most important subendothelial matrix component involved in thrombus formation, and platelet adhesion to collagen is associated with a complex pattern of activatory signaling pathways. Integrin $\alpha_2\beta_1$ and glycoprotein VI (GPVI) are the two main platelet receptors for collagen and, in the rheological conditions of low shear rates, typically present in large veins and venules, are sufficient to mediate firm platelet adhesion. At high shear rates, characteristic of small arteries and stenotic vessels, platelets are unable to efficiently interact to exposed collagen fibers, and in these conditions adhesion is preceded by platelet tethering and rolling on the site of injury. This process is mediated by the membrane GPIb-IX-V complex, a platelet-specific receptor for the multimeric glycoprotein von Willebrand factor (VWF). At high shear stress, circulating VWF rapidly interacts with exposed collagen fibers and undergoes a conformational change that allows the interaction with the GPIb-XI-V complex, decelerating platelets and favoring the subsequent stable adhesion mediated by other platelet receptors [2].

The interaction of platelet adhesion receptors with subendothelial matrix components stimulates an intricate pattern of signal transduction pathways, that trigger spreading, secretion of soluble proaggregating molecules, thromboxane A_2 (TxA_2) synthesis and release, and phosphatydilserine exposure. These events recruit and activate additional circulating platelets to initiate a process of cell aggregation, that generates a rapidly growing thrombus at the site of damaged vessel wall.

The stability of platelet adhesion and of the subsequent thrombus formation is reinforced by autocrine stimulation by the released soluble agonists, in particular ADP and TxA_2, and by thrombin produced through the coagulation cascade. These agonists stimulate specific G-protein-coupled receptors (GPCRs) expressed on platelet surface and typically lead to the activation of phospholipase C (PLC) β isoforms, that release diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 mediates Ca^{2+} release from intracellular stores, whereas DAG stimulates several effectors containing the DAG-regulated C1 domain, such as classical and novel protein kinase C isoforms [3]. Concomitantly, other signaling molecules, including phosphatidylinositol 3 kinases (PI3Ks),

protein tyrosine and serine/threonin kinases, and Ras-like small GTPases, are activated and contribute to promote full platelet activation. These events eventually promote platelet aggregation and thrombus formation, which are supported by the stimulation of integrin $\alpha_{IIb}\beta_3$, that is converted into a high-affinity state for fibrinogen, whose binding mediates interaction of adjacent cells promoting thrombus growth [2, 4, 5].

Several signaling pathways evoked upon platelet adhesion require the intervention of one or more Ras-like small GTPases, that operate as molecular switches by cycling between an inactive state bound to GDP and an active state bound to GTP through the action of specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) [6]. Platelets express several Ras-like GTPase, including Ras [7, 8], Ral [9], Rho [10], Rac [9] and, Cdc42 [11], but are particularly rich of members of the Rap subfamily of GTPases [12]. This paper will focus on platelet Rap GTPases to highlight recent insights into the mechanism of activation and recruitment upon stimulation of the major platelet adhesion receptors.

2. Rap GTPases in Platelets

It is now well established that Rap GTPases are involved in several cell functions, including growth, proliferation, cell-cell contact, and adhesion [13]. The Rap family consists of five members: two Rap1 proteins (Rap1a and Rap1b) that share 90% sequence homology and three Rap2 proteins (Rap2a, Rap2b, and Rap2c) which are about 70% homologous to Rap1 [14–16]. An important difference between the two subfamilies is that Rap2 proteins typically display a lower sensitivity to GAPs, promoting a prolonged Rap2 signaling compared to that of Rap1. Although regulated by the same set of GEFs and GAPs, Rap1 and Rap2 can be involved in distinct processes, as shown in cell types different from platelets [17, 18], suggesting that they may operate through different effectors. The biochemical basis of these different effects has not been thoroughly investigated; however it might also be related to the differences in posttranslational modifications and subcellular localization of Rap1 and Rap2 isoforms [19].

Rap1b is the most abundant GTPase in platelets and represents up to the 0.1% of total proteins, but platelets also contain Rap2b, which, however, is about ten times less abundant than Rap1b. Very low levels of Rap1a and Rap2a have been detected in platelets, whereas Rap2c is not present [12, 16, 20]. Probably because of these different levels of expression, only a limited number of studies have addressed the biochemistry and function of Rap2b in platelets [19, 21–24], while the majority of the investigations have focused on Rap1b.

In platelets, the most abundant and functionally relevant RapGEF is certainly the Ca^{2+}- and DAG-regulated CalDAG-GEFI, which represents the key regulator of Rap GTPases activation downstream of PLC activation [25–28]. The expression of low levels of other Rap1GEFs, as PDZ-GEF and CalDAG-GEFIII, has also been reported, but in some cases their presence remains controversial [29, 30]. The only GAP specific for Rap GTPases identified in platelets is Rap1GAP2, whereas Rap1GAP1, SPA-1, and E6TP1 have not been found [29]. It has been shown that Rap1GAP2 is able to associate with 14-3-3 and to modulate cell adhesion when overexpressed in HeLa cells [31]; however the importance of this regulator in platelet function is still unknown.

3. Rap GTPases as Regulators of Platelet Integrins and Integrin-Mediated Functions: Insights from Rap1b and CalDAG-GEFI Knockout Mice

The role of Rap GTPases, and in particular of Rap1, in the regulation of cell adhesion is well documented by a number of observations in different cell types [32–35]. Rap GTPases participate to the conversion of integrins into a high-affinity state for their ligands, that in turn allows cells to interact, in a controlled fashion, with other cells and with extracellular matrix components. The ability of integrins to bind their ligands is regulated by inside-out signaling pathways that originate inside the cell and are then transmitted to the extracellular ligand-binding domain of the receptor [36].

Integrin-mediated platelet functions include adhesion, aggregation, and thrombus formation, and thus it appears reasonable for Rap1b to be involved in these responses. In this context, important information has been collected upon the generation of the genetically modified mice that do not express either Rap1b, or the main Rap1GEF present in platelets, CalDAG-GEFI. Rap1b knockout mice display a prolonged tail bleeding time and a marked protection from platelet-dependent arterial thrombosis, demonstrating an essential role of this GTPase in both haemostasis and thrombosis [37]. The importance of Rap1b activation in thrombus formation is also confirmed by analysis of CalDAG-GEFI knockout mice. Although the clotting parameters are normal, CalDAG-GEFI knockout mice display strong defects in haemostasis assessed by bleeding tail analysis [26]. In addition, platelets in whole blood collected from CalDAG-GEFI knockout mice fail to form thrombi when perfused over a fibrillar collagen surface both at low and high shear rates [26, 38]. Moreover, the lack of CalDAG-GEFI is also associated with defective *in vivo* thrombosis, that is virtually abolished in arteries and strongly reduced in venules [38]. These alterations of the haemostatic and thrombotic functions of platelets, which are driven by both cell-matrix and cell-cell adhesion, are indicative of a role for Rap1b in the regulation on platelet integrin function. Platelets express at least five different integrins ($\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_v\beta_3$) and in particular integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ play a predominant role in platelet adhesion and activation [2]. However, integrins $\alpha_5\beta_1$ and $\alpha_6\beta_1$ are required to mediate shear-resistant adhesion [39], whereas the function of integrin $\alpha_v\beta_3$ is less understood.

A number of "*ex vivo*" studies on human and murine platelets have demonstrated that Rap1b plays a critical role in the regulation of integrin $\alpha_{IIb}\beta_3$ affinity state and, therefore, controls platelet aggregation induced by soluble agonists

[26, 27, 37, 40]. Important information on the mechanism of inside-out integrin activation has been collected using transfected cell lines to reconstitute this signaling pathway. These studies indicate that integrin signaling involves the interaction of the head domain of the cytoskeletal protein talin with specific sites of integrin β-tail and with the plasma membrane [41, 42]. Talin can synergize with kindlin-1 and -2 to mediate integrin $\alpha_{IIb}\beta_3$ conversion into the active state; however, the overexpression of kindlin alone is not sufficient to promote integrin activation [43]. In talin- and integrin $\alpha_{IIb}\beta_3$-expressing CHO cells, the coexpression of PKC at levels comparable to those present in platelets induces a strong responsiveness to PMA exposure, and the overexpression of a constitutive active form of Rap1a bypasses the requirement of PKC, indicating that, in this model, Rap1 lies downstream of PKC [44]. Among different potential Rap1 effectors, RIAM (Rap1-interacting adaptor molecule) was shown to be involved in integrin regulation, and its overexpression in cell lines bypasses the requirement of Rap1. Moreover, RIAM knockdown suppresses Rap1-mediated integrin activation, as well as the association between talin and integrin β-tail [45, 46]. However, the importance of RIAM in platelet physiology and integrin $\alpha_{IIb}\beta_3$-mediated platelet aggregation still needs to be determined and will require the development of RIAM knockout mouse models.

Further support to the hypothesis that Rap1b regulates integrin affinity was provided by the analysis of Rap1b and CalDAG-GEFI knockout mice. Generally, the lack of Rap1b or CalDAG-GEFI is coupled to a substantial defect in integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation. Rap1b-deficient platelets display a reduced aggregation in response to a wide range of concentrations of agonists, as ADP and epinephrine, and to low doses of collagen, thrombin receptor-specific agonist AYPGKF, and Ca^{2+} ionophore [37]. The lack of CalDAG-GEFI is associated with a more severe reduction of platelet aggregation caused by the same set of agonists, whereas the DAG analogue PMA and high doses of thrombin induce a comparable extent of platelet aggregation in wild-type and CalDAG-GEFI-deficient platelets. The CalDAG-GEFI-independent pathway leading to platelet aggregation requires PKC activation, subsequent granule release, and P2Y12 stimulation by secreted ADP [26, 28, 47].

Similarly to integrin $\alpha_{IIb}\beta_3$, also integrin $\alpha_2\beta_1$ is present on the platelet surface in at least two different affinity states for its ligands, and conversion into the high-affinity state facilitates collagen-mediated platelet activation [48–50]. In this context, it has been recently shown that, under certain conditions, Rap1b expression is required to allow a proper integrin $\alpha_2\beta_1$ activation. Indeed, platelets from Rap1b knockout mice show defective integrin $\alpha_2\beta_1$ activation upon stimulation of the collagen receptor GPVI, but not upon stimulation of thrombin or ADP receptors. However, the extent of adhesion to collagen via integrin $\alpha_2\beta_1$ is normal in platelets from Rap1b knockout mice, suggesting that adhesion to collagen under static conditions does not involve Rap1b-dependent stimulation of integrin $\alpha_2\beta_1$ [51].

Interestingly, in contrast to what was observed for Rap1b-deficient platelets, platelets from CalDAG-GEFI knockout mice display a reduced ability to adhere to immobilized collagen under static conditions, when compared to wild-type cells [27]. Moreover, also the *ex vivo* analysis of thrombus formation on immobilized collagen under flow has revealed a reduced area coverage and thrombus growth in CalDAG-GEFI knockout platelets. Interestingly, the addition of ADP and TxA_2 increases the adhesion of CalDAG-GEFI knockout platelets to a collagen surface at low shear rates, without restoring thrombus growth [38]. In addition to the key role played in platelet adhesion to collagen, the expression of CalDAG-GEFI is also required for an efficient platelet interaction with other β_1 integrin ligands as laminin and fibronectin [52].

The observed differences between Rap1b and CalDAG-GEFI knockout mice, which are particularly evident in terms of phenotype severity, may be related to possible additional functions of CalDAG-GEFI, independent of Rap1b activation, that can be required for efficient integrin-mediated adhesion. Moreover, in Rap1b knockout platelets Rap1a or Rap2b could partially compensate the lack of Rap1b. Indeed, although expressed at lower level Rap1a can be activated by platelet stimulation in Rap1b knockout platelets [51]. By contrast, since CalDAG-GEFI also stimulates Rap2b activity in platelets [53], this isoform cannot compensate the lack of Rap1b, and this may explain the more severe phenotype of CalDAG-GEFI knockout mice. However, it should also be considered that some discrepancy in the results obtained from CalDAG-GEFI and Rap1b knockout mice simply reflects the different experimental conditions adopted.

4. Rap1b Activation Downstream of Platelet Adhesion Receptors

An increasing number of observations indicate that Rap GTPases and adhesive receptors are connected in a bidirectional fashion. As described above, a major role for Rap GTPases is the regulation of integrin affinity for the specific ligands. In this context Rap1b activation is triggered by stimulation of many GPCRs in platelets and participates to the organization of the signaling pathway for integrin inside-out activation. However, in the last years, an increasing number of studies have proved the involvement of Rap1b also in integrin outside-in signaling and have documented its activation downstream of many platelet adhesive receptors.

4.1. Integrin $\alpha_2\beta_1$-Mediated Rap1b Activation. Integrin $\alpha_2\beta_1$ is a collagen receptor important for a proper hemostasis in humans, as documented by the observation that mutations of this receptor are associated with bleeding disorders and reduced platelet responses to collagen [54, 55]. Integrin $\alpha_2\beta_1$ is a critical platelet adhesion receptor, that interacts with collagen, by recognizing hexapeptidic sequences, such as GFOGER, GLOGER, GASGER, GROGER, and GLOGEN [56–58], but it also binds tenascin [59], and mediates platelet adhesion to the small proteoglycan decorin [60].

Platelet adhesion via integrin $\alpha_2\beta_1$ directly stimulates Rap1b activation, without the need for further autocrine stimulation by secreted ADP or released thromboxane

A_2 [27]. Experiments performed with platelets from genetically modified mice have shown that CalDAG-GEFI is the only GEF required for Rap1b stimulation downstream of integrin $\alpha_2\beta_1$ [27]. The dissection of the signaling pathway involved indicates that PLCγ2 expression and activation are mandatory for integrin $\alpha_2\beta_1$-triggered Rap1b activation. Interestingly, recruitment of platelet integrin $\alpha_2\beta_1$ mediates the activation of PLCγ2 by two redundant mechanisms: a classical Src-mediated phosphorylation of PLCγ2 itself and a phosphorylation-independent mechanism involving the Rac GTPase [61]. These observations indicate the existence of important crosstalk between Rac1 and Rap1b GTPases in the control of platelet adhesion and activation, with Rac1 being potentially upstream of Rap1b. As it will be discussed later, the existence of a crosstalk between Rap and Rac has been recently confirmed also downstream of GPVI [53].

Further analysis of CalDAG-GEFI and PLCγ2 knockout mice demonstrated that the lack of Rap1b activation is accompanied by a significant inhibition of integrin $\alpha_{IIb}\beta_3$ conversion into the high-affinity binding state for fibrinogen, demonstrating that Rap1b activation plays a key role in the crosstalk between integrins and regulates integrin $\alpha_2\beta_1$-mediated platelet aggregation [27, 61]. More recently, it has been demonstrated that maximal Rap1b activation induced by integrin $\alpha_2\beta_1$ downstream of PLCγ2 requires the contribution of an additional Ca^{2+}-dependent signaling pathway involving the focal adhesion kinase Pyk2 and the subsequent stimulation of PI3Kβ. In fact, the lack or the impaired activation of Pyk2 and PI3Kβ causes a defective Rap1b activation triggered by integrin $\alpha_2\beta_1$ engagement [62]. These findings outline that, as previously observed in platelets stimulated with ADP or other soluble agonists [63–65], also in integrin $\alpha_2\beta_1$ outside-in signaling Rap1b activity is regulated by PI3K. Since virtually no residual Rap1b activity is detected in CalDAG-GEFI-deficient platelets, this observation points to a contribution of PI3K activity in the regulation of CalDAG-GEFI, but the molecular mechanism for this process is still to be defined.

4.2. GPVI-Mediated Rap Activation. GPVI is membrane glycoprotein specifically expressed in platelets, functionally associated with the ITAM-containing transmembrane adapter protein FcR γ-chain [66, 67]. It is well documented that platelet GPVI is responsible for the first set of signals induced by platelet interaction with collagen and that it strongly cooperates with integrin $\alpha_2\beta_1$ to mediate full collagen-induced response [50, 68]. The GPVI-FcR γ-chain complex initiates a tyrosine-kinase-based signaling cascade which involves Src and Syk kinases, the adaptor proteins LAT and SLP76, and leads to phosphorylation and stimulation of PLCγ2 [69]. Mouse or human platelets lacking GPVI–FcR γ-chain display severe defects in collagen-induced activation, integrin $\alpha_{IIb}\beta_3$ regulation and platelet aggregation [70–76].

As part of the signaling pathways for collagen-induced platelet activation, GPVI stimulation triggers Rap1b activation. This process, however, is at least partially dependent on ADP secretion and the subsequent stimulation of P2Y12 receptor, both in human and murine platelets [24, 65, 77]. Nevertheless, the existence of a direct, P2Y12-independent pathway of GPVI-mediated Rap1b activation has been confirmed by the analysis of aggregation of platelets collected from wild-type and Rap1b knockout mice, performed in the presence of ADP receptors antagonists. These experiments show that the lack of Rap1b is associated with a reduced ADP-independent, GPVI-mediated platelet aggregation, demonstrating that this GTPase is required for an efficient GPVI signaling [78]. Interestingly, direct Rap1b-activation-mediated downstream of GPVI depends on the activity of PI3K, and it has been demonstrated that the contribution of both the α and β isoforms of PI3K is required [65, 77, 78].

As for the other platelet collagen receptor, integrin $\alpha_2\beta_1$, CalDAG-GEFI is a specific regulator of Rap1b activation also downstream of GPVI [28, 53]. In addition, a number of GPVI-dependent responses have been found to be impaired in CalDAG-GEFI-deficient platelets. Some of these, such as integrin $\alpha_{IIb}\beta_3$ activation, and platelet aggregation are consistent with the well-documented role for Rap1b, and other defects, such as the reduction of ERK signaling leading to a decreased TxA_2 synthesis, or the impaired granule secretion, point to possible novel implication for this GTPase in platelet function [28, 53, 79].

The GPVI-mediated signaling pathway leading to Rap1b activation involves the small GTPase Rac1 [53]. The observation that Rac1 is involved in PLCγ2-dependent stimulation of Rap1b downstream of integrin $\alpha_2\beta_1$[61] has been extended in a recent work by Stefanini and coauthors showing that the two GTPases exert a mutual influence also downstream of GPVI. Indeed, Rap1b signaling sustains Rac1 activation, and, in turn, Rac1 provides a feedback regulation of Rap1 through CalDAG-GEFI and P2Y12.

Interestingly, the ability of GPVI-FcR γ-chain complex to stimulate Rap1b has been directly compared to the stimulation of the closely related Rap2b [24]. It has been reported that GPVI ligation results in a time-dependent Rap2b activation, that is not influenced by platelet aggregation (i.e., integrin $\alpha_{IIb}\beta_3$-mediated fibrinogen binding) and actin cytoskeleton remodelling. Differently to what was observed for Rap1b, secreted ADP plays only a negligible role in Rap2b activation triggered by GPVI, whereas Ca^{2+} mobilization and PKC activation are both required. Another remarkable difference between activation of Rap1b and Rap2b downstream of GPVI is that Rap2b stimulation is largely independent from PI3K activity. However, it is important to note that PI3K inhibitors suppress thrombin-induced Rap2b activation [24]. Rap1 and Rap2 are therefore differently regulated by PI3K, depending on the nature of the stimulus. Recently, it has been shown that Rap2b activation depends on CalDAG-GEFI and P2Y12 signaling, similarly to what was already demonstrated for Rap1b [53]. According to the lower sensitivity to GAPs, Rap2b displays a higher baseline activation that is also a more sustained in time, compared to that of Rap1b [53, 80]. Unfortunately, Rap2b knockout mice have not been generated yet. Therefore, our current information on the contribution of Rap2b to platelet adhesion is still really limited.

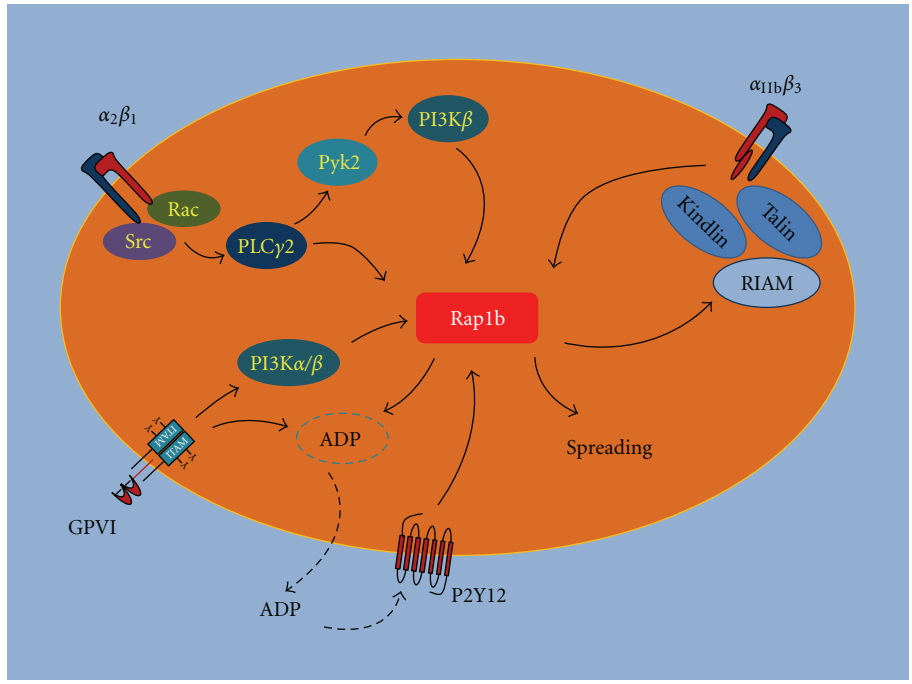

Figure 1: Schematic diagram of the main pathways regulating Rap1b activation during platelet adhesion. The figure summarizes the major players involved in Rap1b activation mediated by platelet adhesion receptors. Downstream of integrin $\alpha_2\beta_1$, PLCγ2 plays a critical role for Rap1b activation, which, however, requires also the Pyk2-mediated stimulation of PI3Kβ. Rap1 stimulates inside-out activation on integrin $\alpha_{IIb}\beta_3$ and thus it is a central player in the crosstalk between these two integrin receptors. The collagen receptor GPVI stimulates Rap1b both directly, through PI3Kα and β, and indirectly through the autocrine stimulation of the P2Y12 receptor by secreted ADP. Moreover, activated Rap1b facilitates platelet granule secretion and ADP release. Rap1b-mediated inside-out activation of integrin $\alpha_{IIb}\beta_3$ involves the Rap1 effector RIAM and the cytoskeletal proteins talin and kindlin. In turn, integrin $\alpha_{IIb}\beta_3$ binding to fibrinogen stimulates an outside-in signaling able to promote Rap1b activation, which is an essential step for platelet spreading on fibrinogen.

4.3. Rap1b Activation Mediated by Integrin $\alpha_{IIb}\beta_3$ Outside-In Signaling. Integrin $\alpha_{IIb}\beta_3$ (also known as GPIIb/IIIa) is the most abundant platelet membrane receptor and is responsible of the binding of platelets to soluble fibrinogen, a process that mediates platelet aggregation [2]. As introduced before, integrin $\alpha_{IIb}\beta_3$ undergoes a conformational change upon platelet activation, that increases the receptor affinity for fibrinogen. This inside-out activation of the integrin function involves Rap1b stimulation and its association with RIAM, talin and other signaling and cytoskeletal proteins, such as vinculin and kindlin [2, 44, 46]. However, in addition to the key role in platelet aggregation, integrin $\alpha_{IIb}\beta_3$ is also able to mediate platelet adhesion to immobilized fibrinogen and to other RGD-containing ligands, including VWF [81], vitronectin [82], fibronectin [83], and thrombospondin [84]. Integrin $\alpha_{IIb}\beta_3$ interaction with its ligands initiates an outside-in signaling pathway, that contributes to the regulation of the later phases of platelet activation and is required for firm platelet adhesion and spreading on extracellular matrices [85–87], fibrin clot retraction [88], platelet procoagulant activity, and microparticle release [89, 90].

The first evidence for the involvement of Rap1b in integrin $\alpha_{IIb}\beta_3$ outside-in signaling was obtained from studies with thrombin-stimulated platelets in the presence of integrin antagonists, including the peptide GRGDS, that prevent fibrinogen binding and platelet aggregation. In this context it was initially shown that sustained Rap1b activation mediated by thrombin requires the interaction between integrin $\alpha_{IIb}\beta_3$ and fibrinogen, as it was inhibited by the RGDS peptide [23].

The activation of Rap1b by integrin $\alpha_{IIb}\beta_3$ has been confirmed by the direct observation that platelet adhesion to immobilized fibrinogen stimulates the accumulation of GTP-bound Rap1b [61]. Integrin $\alpha_{IIb}\beta_3$-mediated Rap1b activation is regulated by multiple intracellular effectors, including Src kinases, PKC, and cytosolic Ca^{2+}. Moreover, Rap1b-deficient platelets display a reduced spreading on fibrinogen compared with wild-type controls, whereas clot retraction is abolished, indicating that stimulation of Rap1b is important for integrin $\alpha_{IIb}\beta_3$-mediated platelet responses [37, 79].

4.4. GPIb-IX-V and VWF in Rap1b Activation. The GPIb-IX-V receptor complex, which contains four transmembrane proteins, GPIbα, GPIbβ, GPIX, and GPV, mediates platelet binding to VWF in a shear-dependent fashion. The VWF-GPIb-IX-V interaction is required to slow down circulating platelets on the site of injury and is strongly involved in the regulation of integrin $\alpha_{IIb}\beta_3$ and in the formation of arterial thrombi [91]. The adhesive function of GPIb-IX-V is coupled to the generation of intracellular signals that support platelet activation mainly through the phosphorylation of the ITAM-bearing FcγIIA receptor, which is

physically associated with it [92]. Moreover, FcγIIA receptor-independent signal transduction pathways occurring downstream of GPIb-IX-V and leading to protein phosphorylation, calcium oscillation, and integrin $\alpha_{IIb}\beta_3$ activation have been identified [93]. GPIb-IX-V ligation triggers the subsequent activation of integrin $\alpha_{IIb}\beta_3$ that mediates firm platelet adhesion and initiates thrombus formation.

Despite the relevance of the initial platelet adhesion through GPIb-IX-V at the site of arterial injury for the whole process of thrombus formation, the information about the ability of this adhesion receptor to trigger Rap GTPases activation is extremely limited. It has been shown that platelet stimulation with VWF triggers the activation of both Rap1b and Rap2b and promotes their association with the cell cytoskeleton through a process involving the FcγIIA receptor [23]. VWF-induced Rap2b translocation to the cytoskeleton, in particular, is dependent on integrin $\alpha_{IIb}\beta_3$ as it is prevented in the presence of anti-integrin $\alpha_{IIb}\beta_3$-specific antibodies, as well as in patients affected by Glanzmann's thrombasthenia, a genetic disorder associated with the lack of expression of integrin $\alpha_{IIb}\beta_3$ [22].

Antibody-mediated clustering of FcγIIA receptor, that induces its tyrosine phosphorylation and mimic the signaling pathway triggered by VWF-mediated GPIb-IX-V stimulation, is coupled to Rap1b activation in a fashion completely dependent on secreted ADP [94]. Unfortunately, studies that used transgenic mouse models to elucidate the role of Rap1b in GPIb-IX-V-mediated platelet responses have not been reported.

5. Conclusions

The great effort devoted to understand the roles of Rap GTPases in the regulation of platelet function produced a huge amount of evidence demonstrating their critical role in haemostasis and thrombosis. As schematically summarized in Figure 1, the involvement of Rap GTPases in adhesion dynamics is complex and bidirectional, as they are both activated by adhesive receptors and essential for the regulation of the adhesive properties of integrins. The crucial implication of Rap GTPases in the control of cell adhesion suggests that the identification of their effectors may help to define novel possible targets for effective antiplatelet therapies for the treatment of cardiovascular diseases.

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