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Beyond the Borders: Signaling in Cell Adhesion and Migration

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Magdalena Joanna Lorenowicz

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Preface

Scope of the thesis

Background

Inflammation is the response of the body to infection and tissue damage. This process was recognized already in prehistoric times and was studied and treated for over 3000 years [1]. The term 'inflammation' was introduced by Celsus, as a metaphoric description of the dermal response to injury, reminiscent of a fire, characterized by redness, heat, swelling and pain [2]. Although the metaphor is still used, the term oversimplifies the biological phenomenon. Inflammation is a very complicated process, governed by many different cell types and variety of molecules. The main components of inflammatory response are the reaction to tissue injury or the presence of invaders, removal of damaged structures and cells or clearing of foreign organisms, and finally cell and matrix repair, which results in restoration of the original cell and tissue structure and function. There is a delicate balance between the beneficial effect of inflammation cascades and their potential to induce tissue destruction. If not well-controlled, an inflammatory response can lead to the development of diseases such as asthma, chronic obstructive pulmonary disorder (COPD), rheumatoid arthritis, inflammatory bowel disease, psoriasis, atherosclerosis or cancer. In fact, the recognition of the involvement of inflammation in the pathology of these increasingly common disorders of modern society has created a new interest in the process.

Inflammation is mainly associated with the innate immune responses of the body; however, it can be also seen as the key element for initiating adaptive immunity. Although most tissues can sense the foreign organisms or tissue injury and initiate inflammation, the cells of hematopoietic origin are the main line of defense of the body during an inflammatory response. The migration of white blood cells to the site of the infection or injury is therefore an essential component of the inflammatory process. It is well accepted that recruitment of leukocytes to the site of inflammation occurs in multiple steps and is orchestrated by many adhesion molecules (Figure 1) [3]. This process starts with reversible tethering to and subsequent rolling of inflammatory cells along the vessel wall, which is directed by interactions of selectin family of proteins on leukocytes and endothelial cells, with their glycosylated ligands on the opposite cell type [4]. Next, integrins on leukocytes are activated via chemokines presented on the apical side of the vessel wall, followed by firm adhesion through their binding to endothelial cell ligands such as ICAM-1 and VCAM-1 [5,6]. Once adherent, leukocytes migrate out of the vasculature. This is a dynamic process, coordinated by very specific signaling events involving, among others, the regulation of the endothelial

junctional proteins, e.g., Vascular-Endothelial (VE)-cadherin and the endothelial actin cytoskeleton. This signaling involves small GTPases such as Rac1, Rho or Rap1, as well as second messengers and tyrosine kinases [7-10]. In many inflammatory disorders, after crossing the vascular endothelium, leukocytes migrate across a polarized epithelium and accumulate in the lumen of the inflamed organ. In some aspects, the mechanism of leukocyte migration through epithelia seems to be similar to the one that governs transendothelial migration; however, much less is known about the way in which inflammatory cells pass the epithelial barrier. Similar to migration across the endothelium, transepithelial migration involves leukocyte attachment to epithelium via integrins [11-13]. Epithelial adherens junction proteins and tight junction components such as occludin, seem to play an important role in leukocyte transepithelial passage as well [14].

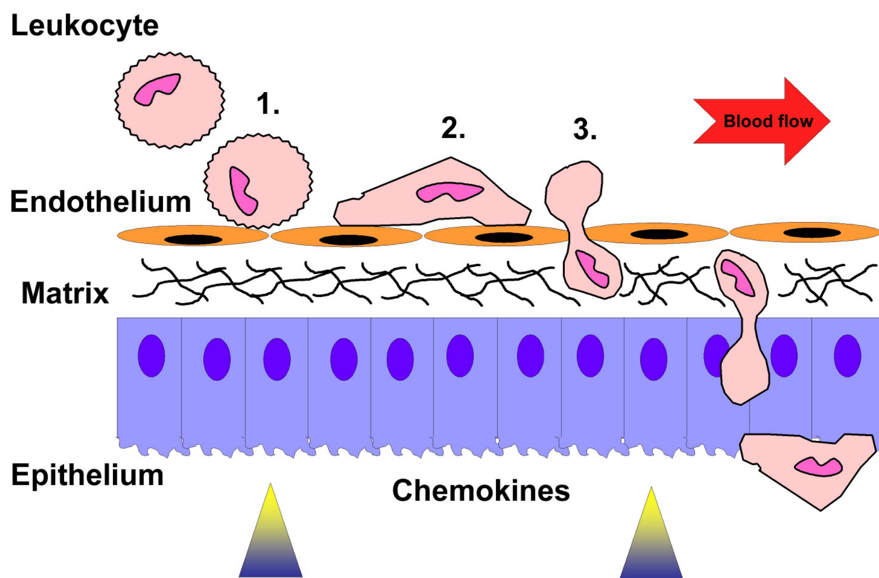

Figure 1. Multistep model of leukocyte extravasation.
Leukocyte diapedesis to sites of inflammation starts with reversible tethering, which slows down the leukocytes circulating in the blood stream and which is followed by selectin-mediated rolling of leukocyte along the vascular endothelium (1). Subsequently, the presence of chemoattractants on the endothelium triggers the activation of integrins on the leukocytes, which results in the arrest of leukocytes on the endothelium (2). Firm adhesion is followed by leukocyte transmigration across the vascular endothelium, the subendothelial matrix and epithelium (3).

Despite the significant progress that has been made during the past decades in understanding the process of leukocyte migration across vascular endothelium and polarized epithelium, the

molecular mechanisms that govern the travel of inflammatory cell to sites of injury are still not completely clear.

Scope of the thesis

The research described in this thesis was designed to better understand the molecular basis of the inflammatory response. We aimed to unravel signaling pathways that control leukocyte chemotaxis and endothelial as well as epithelial barrier function, with special emphasis on signaling events that occur downstream of cAMP, a well known second messenger, and its effectors Epac (Exchange protein directly activated by cAMP) and protein kinase A (PKA).

Chapter I discusses the recent advances in the field of cAMP-mediated signaling, with special focus on the role of signaling triggered downstream of two cAMP effectors PKA and Epac in the regulation of leukocyte transendothelial migration. In addition, this chapter provides an overview of increased insights to the growing complexity of cAMP signaling, which resulted from the discovery of new cAMP targets. It also points out the important questions that should be addressed in future studies to better understand the cAMP-mediated mechanisms that regulate leukocyte transendothelial migration.

Chapter II reports for the first time the expression of the Epac1 protein in different subsets of human leukocytes and hematopoietic progenitor cells. Moreover, it demonstrates the existence of the novel Epac1-Rap1 signaling pathway that governs monocyte adhesion and chemotaxis. In this chapter we also show that serotonin, a well established neurotransmitter known to induce cAMP production, stimulates monocyte adhesion and migration, most likely through the activation of Epac1-Rap1 signaling.

Chapter III provides insight into the regulation of endothelial barrier function and endothelial migration by signaling downstream of PKA and Epac1. In this chapter we used sensitive real-time measurement of transendothelial resistance and downregulation of Epac1 expression to demonstrate that PKA and Epac1 act independently in controlling both endothelial cell-cell adhesion and migration. Activation of either pathway by Epac-or PKA-specific cAMP analogues results in different cytoskeleton rearrangements. Moreover, in contrast to Epac1 signaling, regulation of endothelial integrity by PKA seems to involve integrins. Epac and PKA also seem to differentially control the focal adhesions during endothelial cell migration, which further indicates the independence of PKA and Epac signaling in endothelium.

Chapter IV continues on studies on the mechanism by which Epac1-Rap1-mediated signaling can control endothelial integrity. In this chapter, using siRNA technology, we provide evidence that AF-6, a potential effector of Epac1-Rap1 signaling, is required for proper Rap1 activation as well as for endothelial barrier function. However, our findings also indicate that AF-6 does not appear to act in the effector position of the Epac1-Rap1 signaling pathway, but rather may be situated upstream of or parallel to Rap1 in the signaling cascade that controls endothelial integrity.

Chapter V shifts the focus of the studies to the regulation of epithelial barrier function. Here we underscore the role of the microtubule cytoskeleton in the control of epithelial cell-cell adhesion. We also demonstrate the importance of RhoA/ROK-mediated signaling in the regulation of epithelial cell-cell contacts by microtubules. In addition, our data point to the small GTPase Rac1 as being an essential component of the signaling cascade that is required for the control of epithelial integrity.

Chapter VI summarizes the findings described in this thesis and defines the questions and future directions that are indicated by the research presented here and that may be interesting to continue in follow-up studies.

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CHAPTER I

Cyclic AMP SIGNALING IN LEUKOCYTE TRANSENDOTHELIAL MIGRATION

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Abstract

The migration of leukocytes across the vascular endothelium is crucial for immunosurveillance as well as for inflammatory responses. Uncontrolled leukocyte transendothelial migration results in pathologies such as asthma, rheumatoid arthritis and atherosclerosis. The molecular mechanisms that regulate leukocyte transendothelial migration involve signaling downstream of intracellular messengers such as cAMP, calcium, phospho-inositol lipids or reactive oxygen species. Among these, cAMP is particularly intriguing since it is generated in both leukocytes and endothelial cells and regulates leukocyte chemotaxis as well as endothelial barrier function. In addition, physiological stimuli that induce cAMP production generate both pro- and anti-inflammatory signals, underscoring the complexity of cAMP-driven signaling. This review discusses our current knowledge of the control of leukocyte transendothelial migration by two main cAMP effectors: protein kinase A and the Rap exchange factor Epac (Exchange protein directly activated by cAMP).

I. Introduction

A hallmark of chronic inflammation is the uncontrolled and excessive migration of leukocytes from the peripheral blood into the tissues. This leukocyte extravasation is a multistep process tightly regulated by bi-directional signaling in both leukocytes and vascular endothelium[1]. In the initial phase of the inflammatory response, locally produced pro-inflammatory cytokines trigger the activation of circulating white blood cells, followed by their reversible tethering and rolling over activated endothelium, a process controlled by selectins. Leukocytes subsequently firmly adhere to the endothelium through surface integrins and acquire a polarized shape. In the final phase of this process, leukocytes migrate across the vessel wall into the underlying tissues. This involves the regulated disassembly of endothelial junctional complexes in order to create gaps to allow leukocyte passage.

The molecular mechanisms that control leukocyte transendothelial migration (TEM) involve extensive signaling mediated by intracellular messengers such as cAMP and calcium, as well as by phospho-inositol lipids, small GTPases, reactive oxygen species and protein tyrosine kinases. All of this results in coordinated remodeling of the actin cytoskeleton, activation of integrins, and phosphorylation and transient inactivation of endothelial junctional proteins, allowing efficient TEM[2].

Cyclic AMP regulates a wide range of cellular processes, including differentiation, secretion, gene transcription, regulation of cell shape, cytoskeletal remodeling, proliferation,

apoptosis, adhesion and migration[3]. A large number of extracellular stimuli, including hormones, neurotransmitters and growth factors, induce intracellular cAMP production upon binding to their cognate G-protein-coupled receptors that trigger the activation of one of the several isoforms of adenylate cyclase[4]. In turn, phosphodiesterases (PDEs) degrade cAMP, preventing its diffusion in the cell, to ensure specific activation of nearby signaling complexes[5]. The different cellular responses to cAMP may be explained by 1) localized production and degradation of cAMP, regulated by adenylate cyclases and PDEs[4,5]; 2) the compartmentalization of the cAMP effector PKA through its interaction with specific A kinase anchoring proteins (AKAPs)[6]; and 3) the existence of a variety of additional cAMP effectors such as Epac (Exchange protein directly activated by cAMP), PDZ-GEFs, and cyclic nucleotide-gated channels (Figure 1)[7-9]. Of the latter two, information on their cAMP selectivity, mode of activation and role in cAMP signaling is currently limited.

Since their discovery in the 1950s it has become clear that cAMP and the agonists that trigger its production are important immunomodulators. Cyclic AMP-generating stimuli, such as prostaglandins (PGEs), serotonin (5-HT), β_2-adrenergic agonists and adenosine, are found at sites of inflammation, e.g. in atherosclerotic lesions[10,11]. This has led to the identification of some cAMP-inducing stimuli and phosphodiesterases as therapeutic targets to develop pharmacological treatments of asthma, chronic obstructive pulmonary disorder (COPD), rheumatoid arthritis, atherosclerosis and cancer[12]. Intriguingly, cAMP production mediates both pro- and anti-inflammatory effects. For example, 5-HT prevents vascular leakage as well as the stimulation of leukocyte chemotaxis[13,14]. Conversely, adenosine and β_2-adrenergic agonists act mainly as anti-inflammatory mediators by inhibiting adhesive and migratory properties of inflammatory cells, blocking degranulation and the respiratory burst in granulocytes and by reducing vascular leakage[14-17]. It is unclear which factors control the pro- or anti-inflammatory effects of cAMP, but these may include the duration and strength of the stimulus as well as the cell type involved, expressing its specific repertoire of cAMP-responsive effectors.

In this review we discuss the role of cAMP in the control of leukocyte transendothelial migration. Focusing on two major targets of cAMP, protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac), we provide an overview of the current knowledge on the regulation of leukocyte transendothelial migration in both leukocytes and endothelium by cAMP-driven signaling.

II. cAMP effectors

Protein kinase A (PKA)

The ubiquitously expressed and extensively studied serine/threonine kinase PKA is a heterotetramer, which combines two catalytic (C) with two regulatory (R) subunits[18]. Two PKA isozymes have been originally identified, PKA type I and type II, which differ in their regulatory subunits, RI and RII, respectively. Different isoforms of the regulatory subunit (RIα, RIβ, RIIα, RIIβ) and of the catalytic subunit (Cα, Cβ, Cγ, PrKX) have been identified by molecular cloning studies. The regulatory subunits are differentially expressed and can form homo- and heterodimers, further increasing complexity but likely also specificity of cAMP signaling[19]. Upon binding of two cAMP molecules to each regulatory subunit, the inactive tetramer dissociates into one dimer of R subunits and two active catalytic subunits that phosphorylate various target proteins.

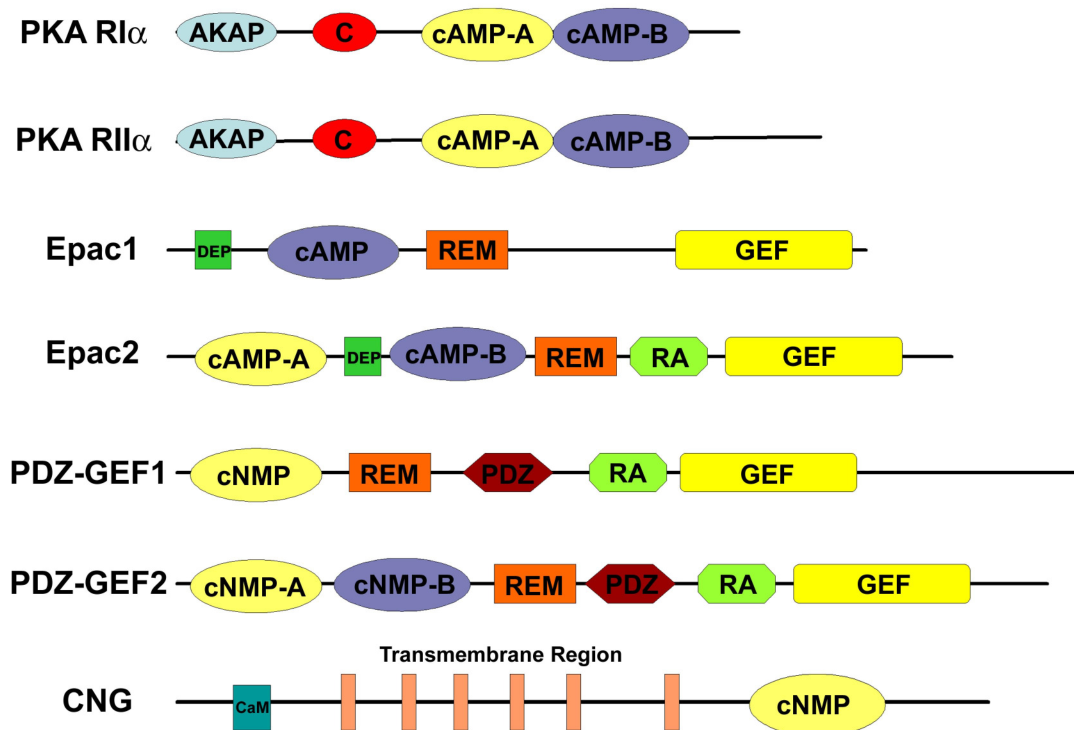

Figure 1. cAMP effector proteins.

Schematic representation of the domain structure of currently known cAMP effectors. The indicated domains are: cAMP, cyclic AMP-binding domain; cNMP, cyclic nucleotide-binding domain; DEP, Dishevelled, Egl-10, Plekstrin domain; REM, Ras-exchanger motif; RA, Ras-association domain; AKAP, A-Kinase anchoring protein-binding domain; C, region of interaction with catalytic subunit; GEF, guanine nucleotide exchange factor; PDZ, PDZ domain; CaM, CaM-binding domain.

Subcellular localization and compartmentalization of PKA is mainly determined by members of the functionally related, but structurally diverse, family of A kinase anchoring proteins (AKAPs)[6]. AKAPs target PKA to specific substrates and various subcellular regions and structures. In addition, AKAPs serve to co-localize PKA with other signaling enzymes, such as phosphatases and PDEs[6]. Such a spatial regulation of PKA by AKAPs is essential to generate specific cellular responses to cAMP, including cell adhesion and migration

Exchange protein directly activated by cAMP (Epac)

The small GTPase Rap1 is activated by growth factors, adhesion molecules and cytokines and has been implicated in the regulation of growth, secretion, integrin-mediated adhesion, neuronal differentiation and morphogenesis[20]. Like most other small GTPases, Rap1 acts as a molecular switch, cycling between a GDP-bound inactive, and a GTP-bound active state. This cycle is regulated by GTPase-activating proteins (GAPs), which enhance the hydrolysis of the bound GTP, and by guanine nucleotide-exchange factors (GEFs), which facilitate the release of the bound GDP and promote the binding of GTP. Epac is an exchange factor for Rap that is activated by cAMP[7,8]. By means of the Epac-selective cAMP analog 8-pCTP-2-O-Me-cAMP which activates Epac but not PKA[21], Epac was shown to regulate a variety of cellular processes previously attributed to PKA. These include E- and VE-cadherin-mediated cell-cell adhesion, integrin-mediated adhesion, monocyte chemotaxis, Ca^{++}-induced exocytosis, and Fcγ-receptor-mediated phagocytosis[2,13,20,22-25].

The two Epac variants, Epac1 and Epac2, show a different domain structure (Figure 1) and tissue expression pattern. Epac1 is expressed in kidney, ovary, thyroid and, at relative low levels, in leukocytes[7,13]. Epac2 is predominantly expressed in the brain and in the adrenal gland. Both Epac1 and Epac2 contain a C-terminal catalytic region, which comprises a CDC25 homology domain responsible for the nucleotide exchange of Rap, a Ras-association domain (RA) and a Ras exchange motif (REM) necessary for the stability of the GEF domain. The N-terminal part of Epac is the inhibitory regulatory region (RR) and contains a DEP (Dishevelled, Egl, Plekstrin) domain responsible for membrane localization, and a cAMP-binding domain that shares homology with the cAMP-binding domains of the regulatory subunit of PKA. Cyclic AMP binding results in a conformational change of Epac, which disrupts the intramolecular interaction between the N-terminal and the catalytic domain[20,21]. As a consequence, Epac becomes active and stimulates Rap activation. Epac2 contains a

second cAMP-binding domain, which binds cAMP with a relatively low affinity and is not required for cAMP-induced activation of Epac2. Its function is, so far, elusive[26]. Interestingly, a recent study shows that the Ras-association domain (RA) of Epac2 can bind to Ras. This study proposes that Epac2, but not Epac1, can be induced to translocate to the plasma membrane by co-stimulation with agonists that jointly activate Ras and increase cAMP levels[27].

III. Cyclic AMP-mediated signaling in leukocyte adhesion

PKA-The first steps in leukocyte transmigration are selectin-dependent rolling of leukocytes over the vascular wall and subsequent firm adhesion to the endothelial surface. Cyclic AMP-elevating agents inhibit rolling and adhesion of neutrophils through PKA-mediated signaling. Berends et al. demonstrated that the chemoattractant-stimulated shedding of L-selectin on human neutrophils and eosinophils can be attenuated by the PDE type IV inhibitor rolipram[28]. On the other hand, stimulation of neutrophils with cAMP analogues results in a blockade of integrin expression on the cell surface and a consequent loss of adhesion. Rolipram and cAMP-rising agents such as forskolin and isoproterenol also inhibit fMLP-induced adhesion of neutrophils to vascular endothelium by blocking the mobilization of the intracellular pool of αMβ2 integrin[15,29].

Pharmacological inhibition of PKA reverses the inhibition by cAMP of α4β1 and αMβ2 integrin surface expression on neutrophils and the adhesion of these cells to VCAM-1, thus implicating PKA in the control of integrin-mediated adhesion primarily through the modulation of surface integrin expression[30,31]. Interestingly, adhesion of TNF-treated neutrophils triggered a decrease in the intracellular level of cAMP, which could be reversed by the blocking of integrin-mediated adhesion with an anti-β2 antibody[32]. This indicates a feedback loop in which a reduction of cAMP activates integrins and where binding of activated integrins results in decreased levels of cAMP.

PKA may also negatively regulate integrin activation through the modulation of the actin cytoskeleton. Rovere et al. demonstrated that activation of PKA promotes T cell de-adhesion by disassembly of the actin cytoskeleton, thus dissociating integrins (αMβ2) from cytoskeletal anchoring proteins[33]. In line with this, PKA was also shown to inhibit the small GTPase RhoA, a critical regulator of the actin cytoskeleton[34]. Stimulation with a membrane-permeable cAMP analogue resulted in reduced RhoA activation and inhibition of α4β1-dependent adhesion of lymphocytes to VCAM-1 and of αMβ2-dependent adhesion of

neutrophils to fibrinogen. These effects were attenuated when cells were treated with selective PKA inhibitors. In conclusion, cAMP activation of PKA appears to negatively regulate leukocyte adhesion through the inhibition of surface integrin expression and integrin function (Figure 2).

Epac1-Rap1-The situation appears quite different for the Epac-Rap1 pathway. There is a large body of evidence corroborating the role of the small GTPase Rap1 in the stimulation of integrin-mediated adhesion. In T cells and B-cell leukemia cells, Rap1 stimulates αLβ2-dependent adhesion[35]. Moreover, Rap1 was found to increase the affinity of the αIIbβ3 integrin for its ligand in megakaryocytes[36], and Rap1 is required for direct activation of αLβ2 and α4β1 integrins by integrin-activating antibodies and manganese ions in Jurkat cells[37]. Conversely, expression of the RapGAP SPA-1 or Rap1N17 in Jurkat cells inhibit the ability of αLβ2 and α4β1 integrins to bind their ligands[38,39].

A role for Epac1 in integrin-mediated adhesion was first reported in ovarian carcinoma cells, in which activation of the Epac1-Rap1 pathway is required for α5β1- and αvβ3-mediated adhesion to fibronectin[22]. Similarly, Epac1-Rap1 signaling mediates α3β1-mediated adhesion of different types of adherent cells (e.g. keratinocytes) to laminin[40]. Interestingly, recent studies point to a role of Epac1 in the stimulation of integrin-mediated adhesion of leukocytes. Basoni et al. reported that TGFβ induced a loss of the Epac1 transcript in U937 monocytic cells, which was paralleled by reduced activation of Rap1 and of αMβ2[41]. We found that Epac1 is able to activate β1 integrins and to promote α4β1 and α5β1 (but not αMβ2) integrin-mediated adhesion of U937 cells to fibronectin and adhesion of primary monocytes to vascular endothelium under physiological flow[13]. Finally, also in $CD34^+$ hematopoietic progenitor cells, cAMP-mediated Rap1 activation results in increased α4β1-mediated adhesion[42]. Together, these data indicate that Epac1-Rap1 signaling positively regulates leukocyte adhesion, primarily through the activation of β1 integrins (Figure 2).

IV. Cyclic AMP-mediated signalling in leukocyte directional migration

PKA- Cyclic AMP or cAMP-generating agonists can exert opposite effects on leukocyte transendothelial migration. For example, PGE2 suppresses transendothelial migration of T lymphocytes and monocytes[43,44]. Similarly, stimulation of adenylate cyclase by forskolin or the inhibition of PDE attenuates chemotaxis of neutrophils and eosinophils[45,46]. These inhibitory effects of cAMP on leukocyte migration have been attributed to PKA, since a

competitive inhibitor of PKA, Rp-cAMP, reversed the inhibition of splenocyte migration induced by elevation of cAMP with forskolin or a PDE8 inhibitor[47]. Similarly, PKA has been suggested to inhibit migration of cytotoxic lymphocytes through inactivation of RhoA, thus modulating the actin cytoskeleton[48]. In contrast, forskolin or PGE2 stimulate cell polarization in adherent T lymphocytes, while inhibition of PKA abrogates chemokine-induced polarization, which suggests a positive role for PKA in leukocyte chemotaxis[49]. Accordingly, in neutrophils, activation of PKA mediates neutrophil migration induced by endothelins[50] or fMLP[51]. The latter effect correlated with increased adhesion, increased αMβ2 cell-surface expression and decreased fMLP-induced actin polymerization. Intriguingly, exposure to a gradient of PKA inhibitors also stimulated neutrophil migration and actin polymerization[51]. This suggests that neutrophil migration requires polarized PKA activity, possibly controlled by an intracellular gradient of cAMP. Several studies have shown that stimulation of neutrophils with chemotactic agents causes a small but consistent rise in cAMP, whereas the concentration of cAMP that inhibits chemotaxis is much higher[52,53]. In addition, inhibition of cell migration by cAMP appears to depend on the nature of chemotactic agent[54]. Thus, it is likely that PKA exerts both inhibitory and stimulatory effects on cell migration, depending on the type of cAMP-inducing stimulus and its capacity to induce low or high local concentrations of cAMP.

Epac1-Rap1 - Constitutively active Rap1 induces lymphocyte polarization through its effector RAPL, independently of spatial cues such as adhesion or chemokine gradients[55]. Constitutively active Rap1 localizes to the leading edge while wild type ‘cycling’ Rap1 is also present at a perinuclear area[56,57]. Similarly, chemokine stimulation of Rap1 is able to induce the translocation of RAPL from the perinuclear region to the leading edge, where it co-localizes with αLβ2 integrin. In turn, targeting of αLβ2 to the leading edge of the cell requires activation of Rap1 and RAPL[58]. This finding suggest that upon activation, the Rap1-RAPL complex moves to the cell’s leading edge and locally activates integrins, which initiates further cell polarization and migration.

In line with the effects of Rap1, activation of Epac1 also induces monocyte polarization. Epac1 localizes to the perinuclear region of polarized migrating cells, which suggests that Epac1 activates Rap1 in a perinuclear area where activated Rap1 may associate with RAPL followed by its binding to integrins, e.g. αLβ2, and translocation to the leading edge[13,59].

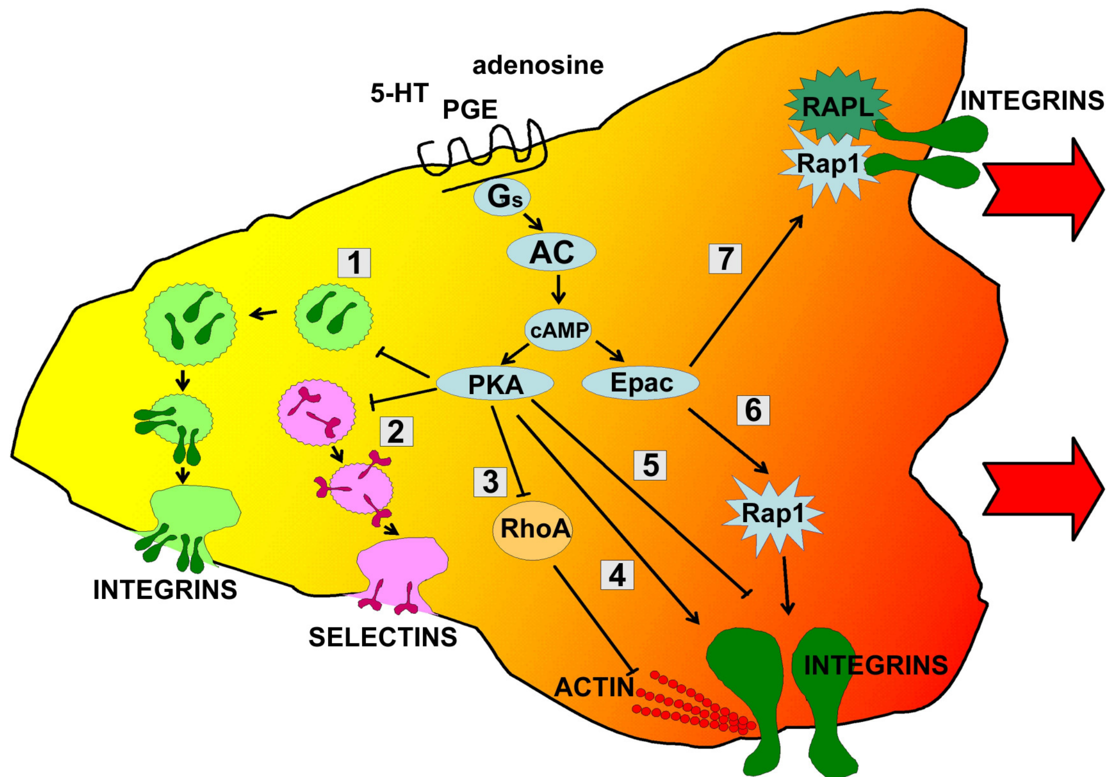

Figure 2. Schematic overview of cAMP signaling in a polarized leukocytes.

CyclicAMP-elevating agonists such as serotonin (5-HT), adenosine or prostaglandins (PGE) bind to their cognate Gs-coupled receptors, which trigger the activation of adenylate cyclase (AC) and production of cAMP. Cyclic AMP activates PKA and Epac signaling pathways. Activation of PKA results in inhibition of cell surface expression of integrins, by blocking the mobilization of intracellular pools of integrins, resulting in reduced adhesion (1). Similarly, PKA may regulate expression of selectins (as shown for platelets) to inhibit rolling of leukocytes over the endothelium (2). PKA also modulates leukocyte adhesion through the regulation of integrin anchorage to the actin cytoskeleton, by inhibiting RhoA (3). PKA has a dual role in the regulation of integrin activation. PKA signaling inhibits integrin activation during initiation of adhesion; however, PKA is necessary for stable, sustained adhesion mediated by clustered integrins (4, 5). Epac1 activated in leukocyte uropod activates Rap1, which together with its effector RAPL translocates to the leukocyte leading edge, locally activating integrins. Integrins activated by Epac1-Rap1 signaling mediate leukocyte adhesion (6, 7).

Expression of constitutively active Rap1 in T-lymphocytes stimulates cell migration on immobilized ICAM-1 and VCAM-1, comparable to the response induced by chemokines[55]. Conversely, inhibition of Rap1 by RapGAP significantly blocked the ability of B lymphocytes to migrate towards SDF-1[60]. The role of the Rap1-RAPL complex in leukocyte migration was recently underscored in an in vivo study demonstrating that lymphocytes from RAPL-deficient mice showed impaired chemokine-stimulated transendothelial migration under flow and a lack of proper homing to lymphoid tissues[58].

In line with these findings, we showed that Rap1 activation by Epac1, downstream of the serotonin receptor, promotes SDF-1-induced migration of monocytic U937 cells[13]. Interestingly, Goichberg et al. reported that also in $CD34^+$ hematopoietic progenitor cells, cAMP-induced activation of Rap1 downstream of the PGE2 receptor results in increased transendothelial migration. Moreover, cAMP-induced Rap1 signaling promotes homing of $CD34^+$-cells to the bone marrow[42]. Together, these data underscore the important role of (localized) cAMP signaling in leukocyte adhesion and directional migration.

V. cAMP signaling in vascular endothelium

PKA - It is generally accepted that the majority of leukocytes cross the endothelium by means of a paracellular pathway. This requires the formation of intercellular gaps between adjacent endothelial cells, which form upon the regulated disassembly of interendothelial tight and adherens junctions[2,61]. Particularly relevant in the context of leukocyte extravasation are the VE-cadherin-based adherens junctions (Figure 3). VE-cadherin is a homophilic transmembrane adhesion molecule that associates to cytosolic proteins such as β-, γ- and p120-catenin. This cadherin-catenin complex is dynamically connected to the actin cytoskeleton through another member of the catenin family, α-catenin[62,63].

It is well established that increases in intracellular cAMP promote endothelial barrier integrity. Observations that cAMP-raising agents decrease endothelial permeability date back to the 1980s, when Stelzner et al. showed that forskolin and cholera toxin, both being strong activators of adenylate cyclase, reduce the transfer of macromolecules, such as albumin, across endothelial monolayers. In addition, using the PKA antagonist Rp-cAMPS, these investigators demonstrated that PKA mediated the increase in endothelial barrier function induced by cAMP[64]. The work of Stelzner et al. initiated a series of studies in clustered cells, isolated microvessels, intact tissues and organs on the protective effect of β-adrenergic

agonists and PGEs. These agents were found to decrease endothelial permeability induced by stimuli such as thrombin, neutrophil-derived hydrogen peroxide and the inflammatory mediator bradykinin[65-67].

In agreement with the early observations by Stelzner et al., subsequent studies implicated PKA in the protective effect of cAMP on endothelial barrier function, mainly based on the use of selective PKA inhibitors such as H89 and upon expression of the PKA inhibitor (PKI) gene[68,69]. The mechanism by which PKA controls endothelial barrier function is not entirely clear, although a number of effectors have been identified that are all involved in the direct or indirect regulation of the actin cytoskeleton (Figure 3). These will be briefly discussed below.

MLCK - Myosin-based contractility, critical for the maintenance of endothelial integrity, is positively regulated by myosin phosphorylation. Myosin light chain (MLC) phosphorylation is controlled by MLC kinase (MLCK) and MLC phosphatase (MLCP)[70]. PKA phosphorylates MLCK, decreasing its affinity for calmodulin, which is essential for MLCK activation[71]. Moreover, PKA is also able to indirectly regulate MLCK through the phosphorylation and inhibition of phospholipase C. Consequently, Ca^{2+} release is reduced and the formation of calmodulin complexes is inhibited[72]. Accordingly, forskolin as well as cAMP analogues partially inhibit the basal and thrombin-induced phosphorylation of MLC[73]. In addition, inhibition of PKA results in increased co-localization of MLCK with actin and enhanced thrombin-induced endothelial permeability[74,75]. Thus, PKA activation may exert its protective effect on the endothelial barrier through the inhibition of actomyosin-based contractility.

RhoGTPases - Activation of the small GTPase RhoA induces endothelial permeability through the stimulation of actin polymerization and actomyosin-driven contraction. PKA is able to phosphorylate RhoA and inhibit RhoA activation in endothelial cells, thus counteracting the permeability-inducing contractile force[2,70,76]. Similar to RhoA, Rac1 is another GTPase which is important in the control of the actin cytoskeleton and endothelial barrier function. Recently, PKA was demonstrated to have both inhibitory and stimulatory effects on Rac1 activation in endothelial cells[77]. More specifically, PKA activation was reported to counteract the *Clostridium sordelli* lethal toxin (LT)-mediated Rac1 inhibition and to attenuate LT-induced endothelial permeability[78]. These findings implicate Rac1, as well as RhoA in the PKA-mediated control of endothelial permeability

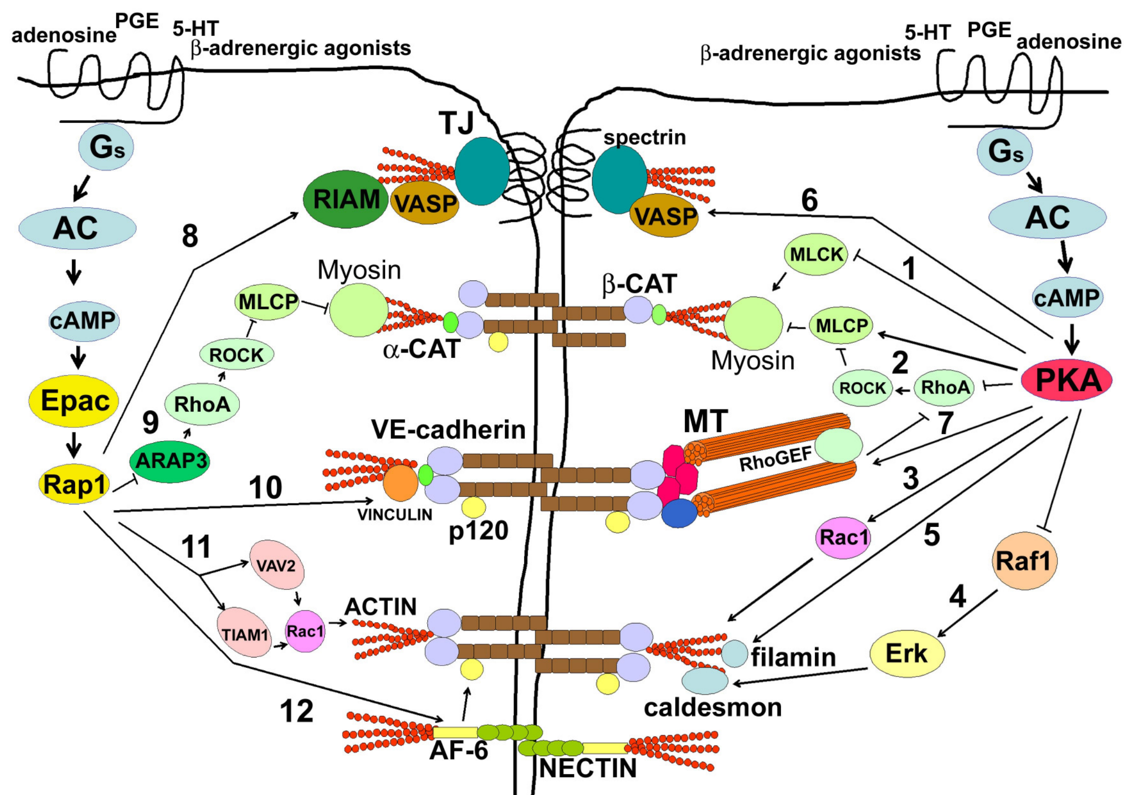

Figure 3. Control of endothelial cell-cell contact by PKA and Epac

Cyclic AMP-elevating agonists such as serotonin (5-HT), adenosine, β-adrenergic agonists or prostaglandins (PGE) bind to their Gs-coupled receptors, triggering the activation of adenylate cyclase (AC), production of cAMP and the activation of PKA and Epac signaling. PKA phosphorylates myosin light chain kinase (MLCK), thus decreasing its activity and inhibiting myosin-based contractility (1). On the other hand, PKA enhances activity of myosin light chain phosphatase (MLCP) directly, or indirectly by inhibition of RhoA and its downstream effector Rho kinase (ROCK), which also reduces myosin-based contractility (2). PKA may control endothelial integrity through activation of Rac1-mediated cytoskeletal rearrangements (3) or inhibition of Raf-1-ERK1/2-caldesmon induced formation of stress fibres (4). Regulation of endothelial cell-cell contact by PKA may also occur through direct phosphorylation of filamin (5). PKA stabilizes endothelial tight junctions (TJ) through phosphorylation of vasodilatator-stimulated phosphoprotein (VASP) (6) and stabilization of microtubule cytoskeleton (MT) and MT-associated RhoA-specific GEFs (7). Possible effectors of Epac1 in the regulation of endothelial integrity are: RIAM (8) which binds to VASP; ARAP3 (9), a GEF for RhoA; vinculin (10), supporting endothelial cell-cell contacts through association with α-catenin; (11) TIAM-1 and Vav2, GEFs for Rac1 and (12) AF-6 which associates with nectin and p120 catenin and reduces VE-cadherin endocytosis, thus promoting cell-cell adhesion.

ERK - Liu et al. showed that inhibition of PKA transiently enhances the activities of both ERK1/2 and of its upstream activator Raf-1 in pulmonary vascular endothelial cells[79]. This activation of the Raf-1-ERK1/2 pathway results in the phosphorylation of the cytoskeletal protein caldesmon and in stress fiber formation. The phosphorylation of caldesmon plays an important role in the regulation of smooth muscle contraction by modulating the dynamics of actin filament organization[80]. Thus, phosphorylation of caldesmon downstream of Raf-1/ERK1/2 signaling may contribute to the cytoskeletal reorganization that is controlled through PKA.

Actin-binding proteins –PKA directly phosphorylates actin-binding proteins such as dematin, adducin, filamin and VASP[81,82]. Filamin is particularly interesting in the context of the regulation of endothelial permeability, since it regulates the distribution of F-actin between cortical actin and actin stress fibers[83]. PKA constitutively phosphorylates filamin in unstimulated endothelial cells, which protects the protein from proteolysis by calpain and increases the capacity of filamin to crosslink actin filaments[82,84]. Another actin binding protein, phosphorylated by PKA, is the vasodilator-stimulated phosphoprotein (VASP). VASP stabilizes newly formed actin filaments and was originally discovered as a focal adhesion protein[85]. Phosphorylation of VASP by PKA at Ser 157 induces the localization of VASP to cell-cell junctions in endothelial and epithelial cells, where it associates with junctional proteins ZO-1, JAM-1 and occludin[86,87]. Transient expression of VASP mutants lacking the preferred PKA binding site increased endothelial permeability. Thus, PKA may promote endothelial cell-cell contact through the phosphorylation of VASP, stimulation of VASP interactions with junctional proteins and stabilization of junctional complexes.

Microtubules - Microtubules are important regulators of endothelial barrier function. In particular, the crosstalk between microtubules and the actin cytoskeleton is critical for the control of endothelial permeability. Depolymerization of the microtubule network with nocodazole results in the disruption of cortical actin, increased MLC phosphorylation, induction of RhoA activation and dissociation of endothelial cell-cell contacts[88,89]. Recently, Birukova et al. reported that elevation of cAMP by forskolin or cholera toxin attenuates the increase in permeability of human pulmonary endothelium induced by microtubule disassembly. In addition, activation of PKA inhibits nocodazole-induced stress fiber formation, RhoA activation and decreased MLC phosphorylation[88]. There is evidence that

PKA phosphorylates microtubule stabilizing proteins such as stathmin and MAP2[90,91]. These data indicate another mechanism for PKA-mediated endothelial barrier protection that involves stabilization of the microtubule cytoskeleton, resulting in inhibition of RhoA activity. This prevents MLC phosphorylation and actomyosin contractility (Figure 3).

Epac1-Rap1 - Recently, the role of Rap1 signalling in the regulation of the cadherin-catenin complex has received much interest. This was sparked by the finding that Rap1, independent of its effect on integrin-mediated adhesion, promotes cadherin-mediated adhesion and antagonizes hepatocyte growth factor-induced disruption of adherens junctions in MDCK cells. Conversely, inhibition of Rap1 activity resulted in loss of epithelial cell-cell contacts[92]. These initial findings have been corroborated by others, reporting that E-cadherin interacts with the RapGEF C3G and that Rap1 is necessary for proper targeting of E-cadherin and stabilization of E-cadherin complexes at the plasma membrane[93,94].

Rap1 plays a similar role in the stabilization of endothelial cell-cell adhesion (Figure 3). Cullere et al. found that cAMP-activated Epac1 markedly enhances endothelial barrier function, which is paralleled by an increase in cortical actin and a redistribution of adherens and tight junction proteins to cell-cell contacts[23]. In addition, activation of Epac1-Rap1 signaling blocked thrombin-induced endothelial permeability through inhibition of RhoA[23,24].

Similar findings were reported by Fukuhara et al. who found that activation of Epac1-Rap1 signaling by PGI2 results in rearranged cortical actin, accumulation of VE-cadherin at cell-cell contacts, increased amounts of actin-bound VE-cadherin and increased endothelial barrier function[24]. Moreover, inhibition of Rap1 by overexpression of Rap1GAP blocked PGI2-induced endothelial barrier function and decreased VE-cadherin-mediated adhesion.

These data were further supported by Kooistra et al. who, using Epac1-specific siRNA, showed that Rap1 activation by Epac1, but not by Epac2, was responsible for the increase in VE-cadherin-mediated cell-cell adhesion[95]. They also demonstrated that cytoskeletal rearrangements induced by the Epac1-Rap1 pathway were independent from the formation of VE-cadherin-mediated cell-cell contacts, since actin remodelling was still present in sparse cultures as well as in endothelial monolayers with reduced cell-cell contact. Finally, Wittchen at al. demonstrated that activation of the Epac1-Rap1 pathway not only promotes endothelial barrier function, but also inhibits transendothelial migration of differentiated HL60 cells[96]. In contrast, Cullere et al. did not observe any blocking effect on

neutrophil migration upon endothelial Epac1 stimulation. This discrepancy is currently unexplained.

The downstream effectors of Rap1 that regulate its effects on cadherin-mediated cell-cell adhesion remain to be identified. Potential candidates include the Rac1 GEFs Vav2 and Tiam1, that localize to sites of cell-matrix contact following Rap1 activation in fibroblasts[97]. Similarly, the CDC42 GEF FRG acts downstream of Rap1 in the control of E-cadherin-mediated cell-cell adhesion[94,96] and the RhoA GEF ARAP3 is also regulated by Rap1. Other potentially relevant effectors are cytoskeleton-associated regulatory proteins such as RIAM, which binds profilin as well as Ena/VASP[98], and vinculin, which relocalizes to VE-cadherin-mediated cell-cell contacts following Rap1 activation[99]. Finally, Cullere et al. suggested that Epac1-Rap1 regulates endothelial cell-cell adhesion through the actin-binding protein AF-6/afadin. Rap1 is known to associate with AF-6, and the complex interacts with p120 catenin. This promotes the binding of p120 catenin to E-cadherin, reduces E-cadherin endocytosis and induces formation of adherens junctions[100](Figure 3).

IV. Concluding remarks

Leukocyte transendothelial migration involves a large number of molecules and signalling events in both the migrating leukocyte and the endothelium. cAMP is an important and potent regulator of both leukocyte chemotaxis and endothelial barrier function. The identification of Epacs has complicated existing models on the role of cAMP in leukocyte transendothelial migration significantly. Earlier findings based on the use of cell-permeable, non-discriminating cAMP analogues need to be re-evaluated, since these compounds will activate both Epac and PKA. Currently available data clearly show that PKA and Epac both are relevant in leukocytes as well as in endothelial cells and act, most likely, in a parallel fashion. Important determinants for the outcome of cAMP signalling appear to be the subcellular localization of cAMP production, the local concentration of cAMP generated and the relative expression level of different cAMP effectors such as PKA or Epacs. These findings result in a series of new questions relevant for the molecular mechanism of leukocyte extravasation, related to the balance between Epac- or PKA- mediated events in both leukocytes and endothelial cells; potential redundancy between signaling through distinct cAMP targets; and crosstalk with other signaling pathways. Finally, we cannot exclude that, as with Epac, additional cAMP targets will be identified that are relevant for leukocyte chemotaxis. In conclusion, cAMP signaling in leukocyte transmigration represents a dynamic

and highly interesting area of investigation, with relevance for basic research as well as future therapies aimed at the control of inflammatory responses.

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CHAPTER II

EPAC1-RAP1 SIGNALING REGULATES MONOCYTE ADHESION AND CHEMOTAXIS

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Abstract

Extravasation of leukocytes is a crucial process in the immunological defence. In response to a local concentration of chemokines, circulating leukocytes adhere to and migrate across the vascular endothelium towards the inflamed tissue. The small GTPase Rap1 plays an important role in the regulation of leukocyte adhesion, polarization and chemotaxis. We investigated the role of Epac1 (a guanine nucleotide exchange factor for Rap1, directly activated by cAMP) in adhesion and chemotaxis in a pro-monocytic cell line and in primary monocytes. We found that Epac1 is expressed in primary leukocytes, platelets, CD34-positive hematopoietic cells and in the leukemic cell lines U937 and HL60. Epac activation with an Epac-specific cAMP analogue induced Rap1 activation, β1-integrin-dependent cell adhesion and cell polarization. In addition, activated Epac1 enhanced chemotaxis of U937 cells and primary monocytes. Similar to activation of Epac1, stimulation of cells with serotonin to induce cAMP production resulted in Rap1 activation, increased cell adhesion and polarization and enhanced chemotaxis. The effects of serotonin on U937 cell adhesion were dependent on cAMP production but could not be blocked by a PKA inhibitor, implicating Epac in the regulation of serotonin-induced adhesion. In summary, our work reveals the existence of previously unrecognized cAMP-dependent signaling in leukocytes regulating cell adhesion and chemotaxis through the activation of Epac1.

Introduction

Leukocyte extravasation from the blood stream is of key importance in physiological processes, such as immunosurveillance and acute inflammation. This process is tightly regulated by cytokines that activate the adhesive and migratory capacities of leukocytes. In pathological conditions, an excess of pro-inflammatory cytokines leads to excessive leukocyte extravasation. This is the main determinant in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis, asthma and atherosclerosis. Chemokine-induced leukocyte migration involves integrin-mediated adhesion of circulating leukocytes to the endothelium, polarization of leukocytes toward the source of chemokine and leukocyte migration across the endothelium (diapedesis) [1].

In lymphocytes, the small GTPase Rap1 plays a crucial role in the stimulation of integrin-mediated adhesion, cell polarization and cell motility [2]. Rap1 is activated upon GTP binding, which is induced by specific guanine nucleotide exchange factors (GEFs). Several GEFs for Rap1 have been identified, including the recently found Epac (exchange

protein directly activated by cAMP) [3]. Two Epac isoforms, Epac1 and 2, have been described. Epac1 was found to be expressed in most tissues, while Epac2 is expressed in the adrenal gland and in the brain [4]. Attempts to detect Epac expression in leukocytes showed the presence of Epac1 mRNA only in B cells and U937 cells, while Epac1 protein was detected in macrophages. Epac2 was undetectable in all hematopoietic cell types [5].

The second messenger cAMP is crucially involved in multiple cellular processes. Until Epac was identified, cAMP-dependent signaling was thought to be carried out by protein kinase A (PKA). Currently, a growing number of studies implicate Epac1 in the regulation of several cAMP-dependent effects, including substrate adhesion and cell-cell adhesion in adherent cells [6-10], Ca^{++}-induced exocytosis [11, 12], neurite extension [13] and Fcγ–receptor-mediated phagocytosis in macrophages [14]. However, the role of Epac in leukocyte adhesion and chemotaxis has not been established.

Epac becomes activated by stimuli that bind to receptors signaling via the heterotrimeric Gs proteins, which induce a rise in cAMP levels through the activation of adenylate cyclase. These stimuli include serotonin, prostaglandins and β_2–adrenergic agonists [6, 15-17]. In neurons, the Epac-Rap pathway regulates serotonin-induced secretion of amyloid precursor protein as well as ERK activation [15, 16]. Interestingly, in addition to being a neurotransmitter, serotonin plays an important role in inflammation. It is secreted by mast cells and platelets and induces chemotaxis of eosinophils, lymphocytes and macrophages [18-20]

In this study, we demonstrate that Epac1 is expressed in leukocytes, platelets and hematopoietic cells and we investigate its functionality in monocytic U937 cells and in primary monocytes. Our results show that activation of Epac1 promotes cell adhesion and polarization and enhances chemokine-induced migration.

Materials and Methods

Reagents

8CPT-2Me-cAMP and Rp-8-CPT-cAMPS were purchased from Biolog LSI (Germany). SQ22536 and 2',5'-Dideoxyadenosine were from Calbiochem (Darmstad, Germany). Serotonin (5- hydroxytryptamine, 5-HT), PMA and H89 were from Sigma. Stromal cell-derived factor 1 (SDF-1, CXCL12) and Monocyte chemoattractant protein-1 (MCP-1, CCL2)

were from Strathman Biotech (Hannover, Germany). Recombinant tumor necrosis factor (TNF)-α was purchased from Boehringer Mannheim (Germany).

Cell culture

All cell lines were purchased from ATCC (Manassas, VA, USA) and were cultured at 37ºC and 5% CO_2. U937 cells (monocytic cell line) were maintained in RPMI 1640 medium (GIBCO) containing 10% (v/v) heat-inactivated FCS (GIBCO), 2 mM L-glutamine, 100 U/ml penicilin and 100 μg/ml streptomycin. HL-60 cells (leukemic cell line) and CHO (Chinese hamster ovary) cells were cultured in IMDM medium (BioWhittaker, Brussels, Belgium) containing 10% (v/v) heat-inactivated FCS (GIBCO), 2 mM L-glutamine, 100 U/ml penicilin and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord veins as previously described [21]. Cells were cultured to confluency in M199 medium (GIBCO) containing 20% (v/v) heat-inactivated FCS (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml heparin and 50 μg/ml endothelial mitogen (Sanbio BV). Endothelial cells of second or third passage were used. HUVEC monolayers were stimulated for 16 h with 100 U/ml TNF-α prior to the perfusion experiments.

Cell isolation

Blood was obtained from healthy volunteers. Granulocytes and peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of 500 ml of blood by density gradient centrifugation over isotonic Percoll (Pharmacia, Uppsala, Sweden) with a specific gravity of 1.076 g/ml [22]. For further purification of monocytes and lymphocytes, the PBMC fraction was incubated with magnetic beads coated with anti-CD14 antibodies and monocytes were purified with a MACS separation system according to the manufacturer's instructions (Miltenyi Biotec GmgH, Bergisch Gladbach, Germany). Previous experiments performed in our lab have shown that this isolation protocol does not induce monocyte activation [24]. The remaining monocyte-depleted fraction of PBMCs was labelled with anti-CD3 and anti-CD19 antibodies and B and T cells were subsequently sorted with a Mo Flo sorter (Dako Cytomation; Denver, CO, USA).

After lysis of the erythrocytes with ice-cold lysis buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA (pH 7.4), the granulocyte fraction was incubated with an anti – CD16 antibody. Subsequently, neutrophils were sorted with a Mo Flo sorter. Eosinophils were isolated by means of the fMLP method [23]. In brief, granulocytes were incubated for 30 minutes at 37ºC to restore initial cell density. Cells were then washed and resuspended in PBS containing 0.5% (w/v) HSA (human serum albumin, Sanquin, Amsterdam, The Netherlands) and 13 mM trisodium citrate and incubated for 10 minutes at 37ºC after the addition of 10 nM fMLP to the cell suspension. Eosinophil and neutrophil fractions were separated by centrifugation (20 min., 1000x*g*) over isotonic Percoll (1.082 g/ml, pH 7.4). Platelets were isolated as previously described [24]. $CD34^+$ hematopoietic progenitor cells were isolated from cord blood by density gradient centrifugation over Ficoll-paque (1.077 g/ml) (Pharmacia Biotech, Upsala, Sweden) as previously described [25].

The purity of all the isolated cell populations was greater than 95%.

RT-PCR

RNA was isolated from purified leukocyte fractions, platelets and $CD34^+$ cells and the cell lines U937 and HL-60 by lysis of the cells in a solution containing guanidine isothiocyanate, followed by centrifugation over a layer of CsCl (5.7 M) in a Beckman OptimaTM L-100 XP Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA, USA) with an SW41 rotor. cDNA was synthesized using 2.5 µM oligo-dT primers and 10 U/ml Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) (5 minutes, 65°C). Quantitative PCR was performed with the FastStart DNA Master Plus SYBR Green I kit in the LightCycler Instrument (Hoffman-La Roche, Basel, Switzerland). Epac1 RNA was amplified by PCR with the following protocol: 10 minutes at 95°C, followed by 50 cycles of 5 seconds at 95°C, 30 seconds at 65°C and 15 seconds at 72°C. The following Epac1-specific primers were used: forward primer-5'TTGGAGAATGGCTGTGGGAATGCATC3' (exon 14); reverse primer 5'CCGAGTTGCTGAGGCCAAACATGAC3' (exon 19). The mRNA of the house-keeping gene β-glucuronidase was amplified as internal control using specific primers [26]. Amplified Epac1-specific cDNA was compared to the standard β-glucuronidase. The specificity of the PCR products was checked by DNA sequencing with the Big-dye Terminator Sequencing kit v1.1 and analyzed on a Genetic Analyzer 3100 Platform (both from Applied Biosystems, Foster City, CA, USA).

Western blot analysis - Isolated primary leukocytes, platelets and cell lines were lysed in Laemmli sample buffer containing a protease inhibitor cocktail (Roche) and incubated for 15 minutes at 95°C. Cell lysates were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes (BioRad Laboratories, Hercules, CA, USA). Epac1 was detected with a rabbit polyclonal anti-Epac1 antibody (Upstate). Actin was detected with a mouse monoclonal antibody against actin (Ab1, Oncogene, Darmstadt, Germany).

Integrin activation

U937 cells were resuspended in IMDM medium containing 0.25% (w/v) BSA and stimulated with 8CPT-2Me-cAMP for various time periods at 37ºC. Stimulation was stopped by the addition of ice-cold PBS containing 0.5% (w/v) BSA, and cells were pelleted by centrifugation (490 *g*, 5 minutes, 4ºC). Cells were then resuspended in ice-cold PBS containing 0.5% BSA and incubated with a mouse monoclonal antibody against the activated conformation of β1 integrins (12G10, IMGEN, The Netherlands; [27]) or β2 integrins (CBRM1/5, a kind gift from Dr. Kevin L. Moore; University of Oklahoma, USA, [28]) for 30 minutes at 4ºC. Total surface expression of β1 and β2 integrins was detected with mouse monoclonal antibodies specific for β1 or β2 integrins (Sanquin, Amsterdam, The Netherlands). After washing with ice-cold PBS-0.5% (w/v) BSA, bound monoclonal antibodies were detected with PE-labeled goat-anti-mouse-Ig (DakoCytomation, Denmark) for 30 minutes at 4ºC. The fluorescence intensity of labelled cells was measured with a FACScan flow cytometer (Becton and Dickinson, San Jose, CA, USA). Integrin activation was calculated by correcting for the amount of total integrins in every sample and was expressed as fold increase over control-unstimulated cells.

Adhesion assay

Flat-bottom 96-well plates (Maxi Sorp Nunc; Denmark) were coated with 20 μg/ml of human fibronectin (Sigma) for 16 hours at 4ºC and blocked with 0.5% BSA (Sigma) for at least 1 hour at 37ºC. U937 cells were washed in IMDM medium containing 0.25% BSA, labeled with Calcein-AM, according to the manufacturer's instructions (Molecular Probes, Leiden, The Netherlands) for 30 minutes at 37ºC, and washed twice with IMDM-BSA. Labeled U937

cells were pre-incubated or not with 8CPT-2Me-cAMP for 15 minutes and subsequently added to the fibronectin-coated 96-well plates (2×10^5 cells per well). Plates were centrifuged at 40 *g* for 1 minute and stimuli were immediately added. After a further incubation for 30 minutes at 37°C, non-adhered cells were removed by washing three times with warm PBS. Adherent cells were lysed with 0.5% (w/v) Triton X-100 and fluorescence was measured on a GENios Plus plate reader (TECAN; Salzburg, Austria). The percentage of adhesion was calculated by dividing the measured fluorescence intensity by the fluorescence intensity of the input cells (set to 100%).

VLA-5 and CD11b blocking experiments were performed by pre-incubating cells for 30 minutes with the monoclonal antibodies SAM-1 (Sanquin, Amsterdam, The Netherlands) and 44a (ATCC, Rockville, MD, USA), respectively. In order to block undesirable Fc receptor activation by the blocking VLA-4 antibody HP 2/1, cells were incubated for 10 minutes with the anti-Fc receptor antibodies anti-CD32 (Medarex, Annandale, NJ, USA) and anti-CD16b (Sanquin, Amsterdam, The Netherlands), prior to the incubation with a mixture of HP 2/1 and anti-Fc receptor antibodies for additional 30 minutes.

Perfusion assay

Perfusion experiments were performed as previously described [24]. In brief, monocyte suspension (2×10^6 cell/ml in incubation buffer) was aspirated from a reservoir through plastic tubing and perfused through a chamber (containing the HUVEC monolayer) with a Harvard syringe pomp (Harvard Apparatus, USA) at flow rate of at 0.8 dyn/cm^2. During perfusions the flow chamber was mounted on a microscope stage (Axiovert 25, Zeiss, Germany), equipped with a B/W CCD video camera (Sanyo, Osaka, Japan) and coupled to a VHS video recorder [29]. Video images were evaluated for the number of adherent monocytes and the rolling velocity per cell, with dedicated routines made in the image analysis software Optimas 6.1 (Media Cybernetics Systems, USA). The number of surface-adherent monocytes was measured after 5 minutes of perfusion at a minimum of 25 randomized high-power fields. To automatically determine the velocity of rolling cells, custom-made software was developed in Optimas 6.1. A sequence of 50 frames representing an adjustable time interval (δt, with a minimal interval of 80 milliseconds) was digitally captured. The position of every cell was detected in each frame, and for all subsequent frames the distance traveled by each cell and the number of images in which a cell appears in focus were measured. The cut-off value to

distinguish between rolling and static adherent cells was set at 1μm/s. With this method, static adherent cells, rolling and free-flowing cells could be clearly distinguished and quantified.

Transmigration assay

Migration assays were performed as previously described [25]. In brief, Transwells of 6.5 mm diameter, with 5-μm pore size filters (Costar, Cambridge, MA, USA) were coated with 20 μg/ml fibronectin (Sigma). Before use, cells were washed once with migration medium (IMDM containing 0.25% BSA). At the start of the assay, 10^5 cells were placed in the upper compartment of the Transwells and allowed to migrate for 1 hour at 37 ºC to chemokine-containing medium added to the lower compartment. Migrated cells were collected from the lower compartment and quantified by flow cytometric analysis in the presence of a fixed amount of control cells labeled with Calcein-AM (Molecular Probes, Leiden, The Netherlands). The percentage of migrated cells was calculated as a fraction of the total cell input as follows: % of migrated cells = [(number of transmigrated cells/number of input labeled cells)]/[number of not labeled input cells/ number of labeled input cells)] x 100%.

For primary monocyte migration, transmigrated cells were collected from the lower compartment of the Transwell as well as from the bottom of the Transwell filter (since a fraction of transmigrated monocytes remains adhered to the bottom of the filter). Monocytes in the lower compartment were quantified by flow cytometric analysis as indicated above. Cells adhering to the bottom side of the filters were counted under a fluorescent microscope (three random fields) after removing cells on the top side of the filters with a cotton swab, followed by fixation and staining with Hoechst (Molecular Probes). The percentage of transmigrated monocytes that adhered to the filter was calculated as follows: % of migrated cells adhered to the filter = [(number of transmigrated cells counted per field/surface of the field)]/[number of input cells/ surface of the filter)] x 100%. The calculated percentages of the two fractions were added to give the total percentage of transmigrated monocytes.

Rap1 activation assays

Rap1 pull-down experiments were performed as previously described [30]. In brief, following stimulation U937 cells were lysed in ice-cold lysis buffer containing 10 mM Tris-HCl, 150 mM NaCl, 1% (w/v) NP-40, 0.5% (w/v) deoxycholic acid, 0.1% (w/v) SDS, 1 mM NaF, 2

mM $NaVO_3$ and protease inhibitor cocktail (Roche) for 10 minutes on ice. Lysates were clarified by centrifugation at 10,000 *g* for 10 minutes at 4ºC. GST-RalGDS-RBD coupled to glutathione-Sepharose beads (Amersham Biosciences) was added to the supernatants and incubated for 1 hour at 4ºC. Beads were washed three times in lysis buffer and bound proteins were eluted with Laemmli sample buffer. Rap1 in total cell lysates and precipitates was detected by Western blotting with a mouse monoclonal anti-Rap1 antibody (Santa Cruz Biotechnology). Densitometric analysis was performed with a CanoScan LiDE20 scanner (Canon) and Gene Tools Analysis Software version 3.03.03 (SynGene, Cambridge, UK).

Electroporation and Immunofluorescence

A plasmid containing HA-Epac1 (pMT2SM-HA-Epac1, 30 µg) [3] was added to U937 cells (12 x 10^6 cells) resuspended in RPMI medium. Cells were subsequently electroporated with a BioRad Gene Pulser II electroporator (950 µF, 250 V) and cultured in RPMI medium containing 20% FCS for forty-eight hours. Subsequently, cells were collected by centrifugation, washed and resuspended in IMDM medium containing 0.25% (w/v) BSA. Transfected cells were allowed to adhere to fibronectin-coated coverslips for 10 minutes at 37ºC followed by a 20 minute incubation in the presence of stimuli. Cells were then washed with PBS containing 0.5% (w/v) BSA, fixed with 3.7% (w/v) formaldehyde for 10 minutes at RT and permeabilized with 0.1% (w/v) Triton X-100 for 5 minutes. For immunofluorescence staining of HA-Epac-1, cells were incubated with a mouse monoclonal antibody to HA (12CA5; Boehringer Mannheim Corp., Indianapolis, IN) followed by a goat-anti-mouse-Ig antibody labeled with Alexa 488 (Molecular Probes, Leiden, The Netherlands). F-actin was visualized with TexasRed-labelled phalloidin (Molecular Probes, Leiden, The Netherlands). Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope. Fluorescence distribution profiles were created with Zeiss LSM 510 confocal laser scanning microscope software.

Statistical analysis

All results were expressed as a mean ± SEM of at least three independent experiments. Where applicable, values were compared with paired two-tailed Student t-test. Multiple comparisons were analyzed with a two-way ANOVA test. A p value lower than 0.05 was considered significant. All statistical analyses were performed with GraphPad Prism version 3.0 software.

Results

Expression of Epac1 in human primary leukocytes and leukocytic cell lines

We examined Epac1 mRNA and protein expression in peripheral blood leukocytes, platelets, cord blood-derived CD34-positive hematopoietic cells and two human myelocytic cell lines (HL60 and U937) by RT-PCR and Western blot analysis. RT-PCR was performed under optimized conditions for maximal sensitivity to enable the analysis of low copy number transcripts. In contrast to previous reports [5], we detected Epac1 mRNA in all cell populations examined, with C_T values in the range between 30-34 (Figure 1A and Table1). The analysis of the expression of Epac2 transcript resulted in very high C_T values (C_T higher than 38; [31]), indicating that Epac2 is not expressed in leukocytes (data not shown). Western blotting experiments showed expression of Epac1 protein in all leukocyte populations examined with the exception of neutrophils, despite the presence of Epac1 mRNA in these cells (Figure 1B). Similarly, eosinophils and platelets showed low Epac1 protein expression levels, while C_T values for Epac1 transcript in these cells were comparable to C_T values of the other cell populations analyzed. These data suggest that Epac1 expression is differentially regulated among leukocytes at the posttranslational level.

Epac1 enhances monocyte adhesion through integrin activation

To study Epac signaling in leukocytes, we made use of the Epac-specific cAMP analogue 8CPT-2Me-cAMP, which does not activate PKA [32, 33]. This analogue has been extensively used as a tool to study the role of Epac as a regulator of a variety of cellular processes [5-14]. We verified the functionality of Epac1 in the monocytic cell line U937 by assaying Rap1 activation upon cell incubation with 8CPT-2Me-cAMP. U937 cells stimulated with 8CPT-2Me-cAMP for 0.5, 1 and 5 minutes were lysed, and GTP-bound Rap1 was precipitated in a GST-RalGDS-RBD pull-down assay. Thirty seconds stimulation of U937 cells with 8CPT-2Me-cAMP increased Rap1 activation. This induction was transient and declined to basal levels after 5 minutes of stimulation (Figure 2A). Notably, the activation kinetics of Rap1 by Epac in U937 cells is similar to the previously described kinetics in adherent cells [6]. These data show that U937 cells express functional Epac1.

Table 1. C_T values of Epac1 and β–glucuronidase

Cell type	Epac 1* C_T	β– glucuronidase† C_T
neutrophils	33.02	19.78
monocytes	31.51	18.53
B cells	31.83	21.40
T cells	33.74	17.81
eosinophils	33.63	27.04
platelets	34.62	29.48
CD34+	31.50	25.25
U937	27.50	21.41
HL60	33.80	25.12

* slope -4.556; intercept 27.37

†slope -3.457; intercept 18.08

Epac was recently shown to regulate integrin-mediated adhesion in ovarian carcinoma cells [6, 7]. To investigate whether Epac1 has a similar function in U937 cells, we analyzed cell adhesion to fibronectin after thirty minutes incubation with 8CPT-2Me-cAMP. In addition, stimulation with PMA was included as a positive control. Epac1 activation induced a 60% increase in adhesion when compared to control untreated cells (Figure 2B). This indicates that Epac1 activation promotes adhesion of U937 cells to fibronectin. To further substantiate the role of Epac1-Rap1 signaling in monocyte adhesion we studied the effect of 8CPT-2Me-cAMP in the adhesion of freshly isolated monocytes to cultured human umbilical vein endothelial cells under flow. Epac1 activation resulted in a clear induction of monocyte firm adhesion to endothelial cells, without affecting rolling, which is mainly mediated by selectins (Figure 2C) [34-36]. This provides evidence for a role of Epac1-Rap1 signaling in the regulation of primary monocyte adhesion to endothelial cells under flow, and thus suggests that this pathway may also function in vivo.

To gain insight into the mechanism by which Epac1 enhances U937 cell adhesion to fibronectin we analyzed the surface expression of total and activated β1 and β2 integrins upon treatment with 8CPT-2Me-cAMP. Epac1 activation resulted in a rapid and significant increase of activated β1 integrins on the cell surface followed by their down-modulation (Figure 3A). In contrast, no changes were observed for β2 integrin activation (data not shown). Our results show a rapid but transient activation of integrins by Epac, while the effects on cell adhesion are prolonged for at least 30 minutes. This may be explained by the fact that integrin activation was measured in cells in suspension, where no integrin

engagement takes place. Upon seeding, stable engagement of the activated integrins by fibronectin may induce a prolonged effect on adhesion.

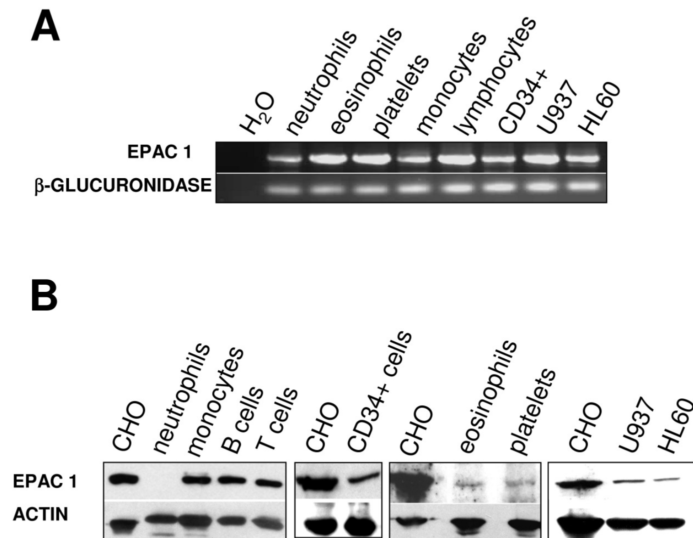

Fig. 1. Expression of Epac1 in primary leukocytes and leukocytic cell lines. (A) RT-PCR for Epac1 of cDNA derived from purified human primary neutrophils, monocytes, lymphocytes, CD34+ cells, eosinophils, platelets and the myelocytic cell lines U937 and HL60. The size of the amplified PCR fragment was 735 bp. As a control, cDNA for β-glucuronidase was amplified. Representative results of 3 independent experiments are shown. (B) Western blot detection of Epac1 in cell lysates from purified human primary neutrophils, monocytes, B cells, T cells, CD34+ cells, eosinophils, platelets and the myelocytic cell lines U937 and HL60. CHO cells were used as a positive control for Epac1 protein expression. Bands corresponding to Epac1 are ~ 110 kDa. β-actin was used as a control for equal protein loading. Representative results of four independent experiments are shown.

To confirm the role of β1 integrins in Epac1-mediated U937 cell adhesion to fibronectin, cells were incubated with blocking anti-integrin antibodies before their addition to fibronectin-coated wells. A blocking anti-VLA-5 (α5β1) antibody completely abrogated adhesion of both control and 8CPT-2Me-cAMP-treated cells (Figure 3B), indicating that VLA-5 is the main integrin involved in U937 cell attachment to fibronectin. However, an anti-VLA-4 (α4β1) antibody specifically reduced 8CPT-2Me-cAMP-induced increase of adhesion, which returned to control levels (Figure 3C), whereas an anti-Mac1 (αMβ2) antibody showed no inhibitory effect (Figure 3D). From these experiments we conclude that Epac1 activation triggers inside-out signaling resulting in the activation of β1 integrins, which leads to VLA-4-, and likely VLA-5-, mediated adhesion of U937 cells to fibronectin.

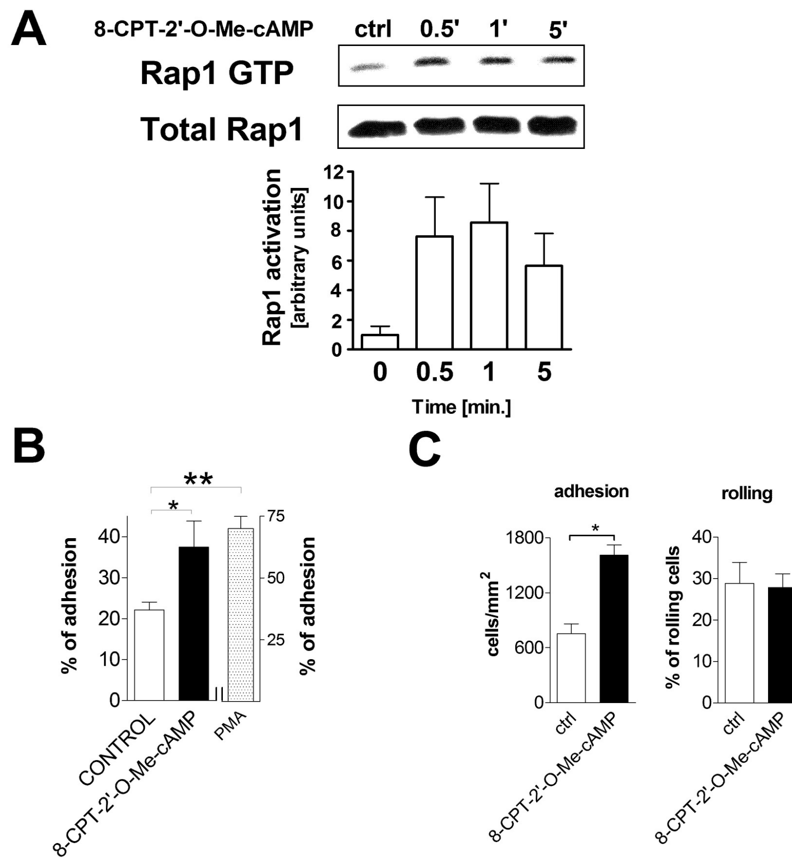

Fig. 2. Epac1 activates Rap1 in U937 cells and promotes cell adhesion to fibronectin and endothelial cells. (A) U937 cells were treated with 100 µM 8CPT-2Me-cAMP for the indicated time periods, and GTP-bound Rap1 was precipitated with GST-Ral-GDS-RBD, followed by western blotting and Rap1 detection by immunoblotting (upper panel). Total levels of Rap1 in whole cell lysates are shown (lower panel). Representative results of three independent experiments are shown. Bar graph represents densitometric analysis of Rap1 activation. Data are mean of three independent experiments (+/- SEM). (B) U937 cells were pre-treated or not with 100 µM 8CPT-2Me-cAMP for 15 minutes and placed in a fibronectin-coated plate for 30 minutes. As a positive control, PMA (100 ng/ml) was added at the time of plating. The percentage of adhesion was determined as described in Materials and Methods. Data are mean (+/- SEM) of five independent experiments performed in triplicate. * $p < 0.05$; ** $p < 0.005$ (C) Primary monocytes were pre-treated or not with 100 µM 8CPT-2Me-cAMP for 30 minutes and perfused over TNF-α-stimulated monolayers of human umbilical vein endothelial cells for 5 minutes at 0.8 dyn/cm^2. Video images were evaluated for the number of adherent monocytes and cell rolling as described in Materials and Methods. Data are mean (+/- SEM) of five independent experiments performed in duplicate. * $p < 0.05$.

Epac1 induces cell polarization and localizes to the uropod

Cell polarization plays a crucial role in directional cell movement. Polarized cells develop a leading edge where membrane extension and lamellipodia formation occur, and a retracting rear (uropod). Since Rap1 is proposed to play a central role in lymphocyte polarization [37], we analyzed whether Epac1 activation triggers polarization of U937 cells. Fibronectin-adherent U937 cells were treated with 8CPT-2Me-cAMP, fixed and stained for F-actin. The percentage of polarized cells was quantified by microscopy according to morphological criteria: non-polarized cells are round in shape whereas polarized cells have a morphologically-defined leading edge and a uropod (cell images in Figure 4A). Quantitative analysis indicated that 8CPT-2Me-cAMP induced a two-fold increase in the number of polarized cells (Figure 4A). We next investigated the intracellular distribution of Epac1 in polarized versus non-polarized cells. U937 cells were transfected with HA-tagged Epac1, seeded on fibronectin and stained with HA antibodies for microscopy analysis. Epac1 localized at the cell periphery in non-polarized cells, whereas it concentrated at the uropod of polarized cells (Figure 4B). These observations suggest that Epac1 activates Rap1 at a perinuclear location.

Epac1 promotes U937 cell and primary monocyte chemotaxis

Rap1 plays a role in the regulation of chemokine-induced lymphocyte migration [38, 39]. To assess the role of Epac1 in monocyte chemotaxis, we analyzed the effect of 8CPT-2Me-cAMP on chemokine-induced migration of monocytic U937 cells and of freshly isolated monocytes. 8CPT-2Me-cAMP was not chemotactic by itself (data not shown). However, treatment with 8CPT-2Me-cAMP resulted in a 80% increase in migration of U937 cells to CXCL12 (stromal cell-derived factor 1, SDF-1) (Figure 5). To exclude the possibility that the Epac1-induced migration was due to the upregulation of chemokine receptors, we analyzed whether 8CPT-2Me-cAMP modulated the cell surface levels of CXCR4 in U937 cells. No differences in CXCR4 surface levels were detected between untreated or 8CPT-2Me-cAMP-treated cells (not shown). We next examined the effect of 8CPT-2Me-cAMP on the chemotaxis of freshly isolated human monocytes toward CCL2 (monocyte chemoattractant protein 1, MCP-1). Similar to the findings in U937 cells, Epac activation resulted in an 80% increase in CCL2-induced chemotaxis. These data show that, although Epac1 activation does induce cell migration by itself, it significantly promotes chemokine-induced monocyte migration.

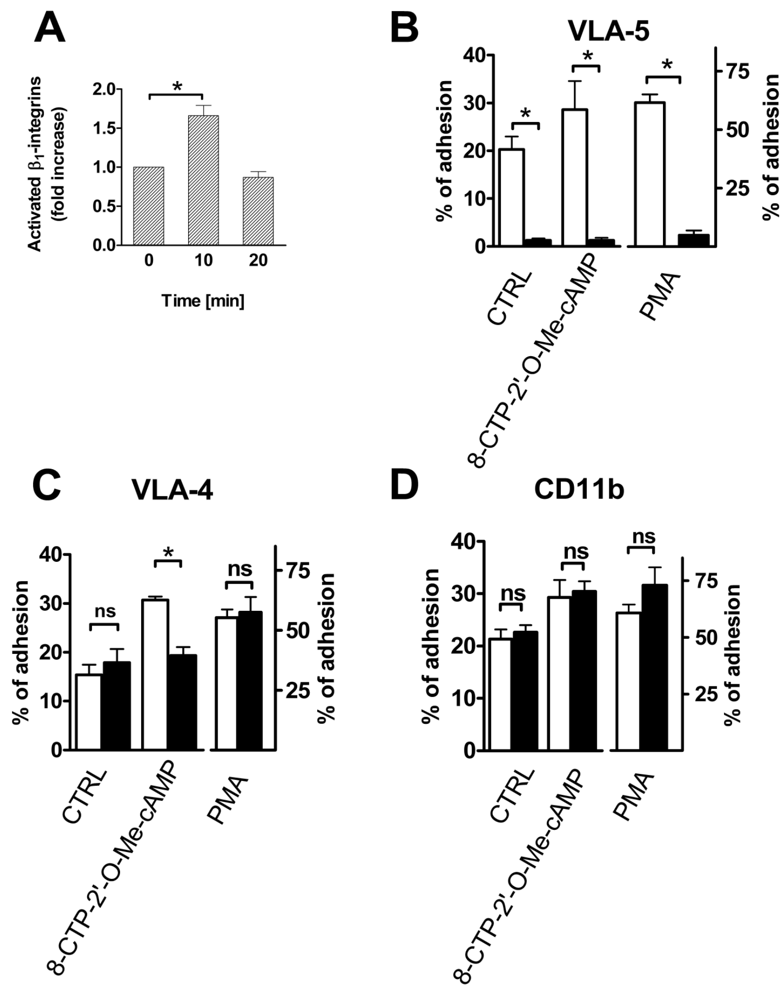

Fig. 3. Epac1 induces β1-integrin activation and β1 integrin-mediated adhesion. (A) U937 cells were treated with 100 µM 8CPT-2Me-cAMP for the indicated time periods. Surface expression of activated β1 integrins was determined by flow cytometry using a mouse monoclonal antibody that recognizes the activated state of β1 integrins (12G10). The activation of β1 integrins is expressed as fold increase over control untreated cells and is corrected for total β1 integrin surface expression. Data are mean (+/- SEM) of three independent experiments. * $p < 0.05$. (B-D) U937 cells were pre-incubated with integrin-blocking monoclonal antibodies to VLA-5 (B), VLA-4 (C) or Mac-1 (D) for 30 minutes before addition of the cells to fibronectin-coated plates. Data are mean (+/- SEM) of three independent experiments performed in triplicate. * $p < 0.05$.

Serotonin activates Rap1 and promotes CXCL12-induced adhesion and chemotaxis of U937 cells

We next investigated whether stimulation of leukocytes with cAMP-raising receptor agonists would induce similar effects as direct Epac1 activation. To test this, we used serotonin (5-

hydroxytryptamine, 5-HT), a neurotransmitter that has been shown to be an inflammatory mediator [18, 40, 41]. Serotonin is released by activated platelets and can be found at micromolar concentrations within the inflammatory site. Notably, Epac1 has been implicated in signaling from serotonin receptors in neurons [15, 16]. We hypothesized that serotonin could enhance leukocyte chemotaxis through the Epac1-Rap1 pathway. To test this hypothesis we analyzed the effect of serotonin on U937 cell chemotaxis towards CXCL12. Serotonin was not chemotactic by itself, but significantly enhanced CXCL12-induced migration of U937 cells (Figure 6A). Furthermore, serotonin induced a small but significant increase in cell adhesion to fibronectin and cooperated with CXCL12 to enhance cell adhesion (Figure 6B).

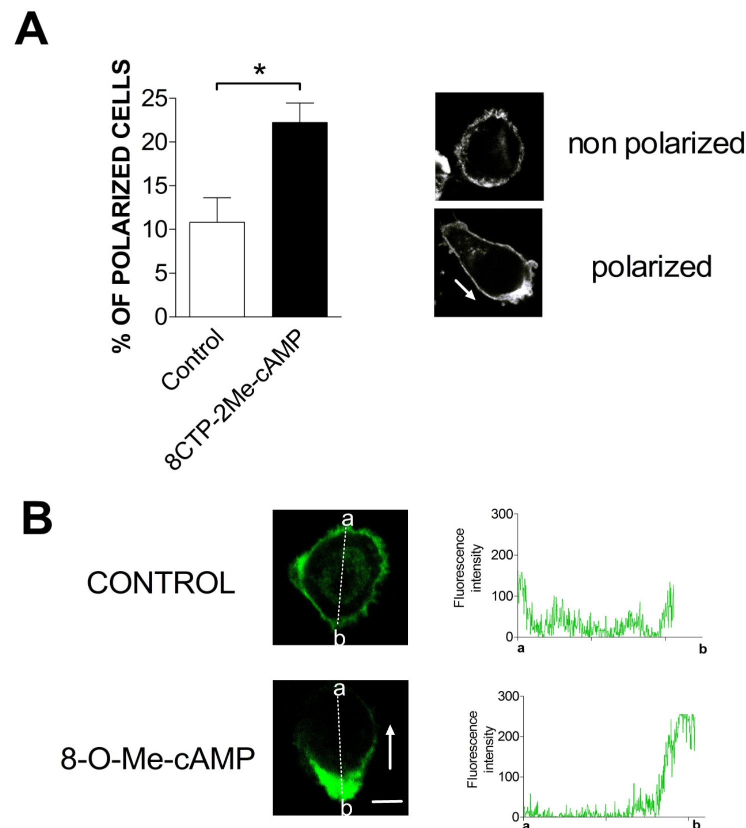

Fig. 4. Epac1 induces cell polarization and redistributes to the uropod of polarized cells. (A) U937 cells were allowed to adhere to fibronectin-coated coverslips, and were stimulated or not with 100 µM 8CPT-2Me-cAMP for 20 minutes, fixed, stained for F-actin and analyzed by confocal microscopy. Data represent the percentage of polarized cells, scored on the basis of morphology, of a total of 100 to 120 cells per condition (left panel). Representative images of a polarized and a non-polarized cell stained for F-actin are shown (right panel, arrow

indicates direction of migration). Data are mean (+/- SEM) of three independent experiments. (B) U937 cells were transiently transfected with HA-tagged Epac1 and allowed to adhere to fibronectin-coated coverslips. Thereafter, U937 cells were stimulated or not with 100 μM 8CPT-2Me-cAMP for 20 min, fixed and stained for Epac1. Fluorescence intensity profiles along the indicated dashed line between the points marked as **a** and **b** are shown. White arrows on images indicate direction of cell polarization. Images are representative of three independent experiments. Bars: 5 μm. * $p < 0.05$

We next investigated whether serotonin was able to trigger cell polarization, similar to Epac activation. U937 cells seeded on fibronectin-coated coverslips were stimulated with serotonin for 30 minutes. Cells were fixed and stained for F-actin for confocal microscopy analysis. The number of polarized versus non-polarized cells was determined as described above. The results showed that serotonin induced a significant increase in the number of polarized cells (Figure 6C). Thus, serotonin is able to induce a similar phenotype as activation of Epac. This suggested that serotonin signals through Epac to promote cell adhesion, polarization and chemotaxis. To test this hypothesis we first investigated whether serotonin is able to induce Rap1 activation. Treatment of U937 cells with serotonin resulted in the rapid activation of Rap1, observed already after 30 seconds of stimulation. This activation was sustained for at least 5 minutes, decreasing to basal levels after 10 minutes stimulation (Figure 6D). Interestingly, the kinetics of Rap1 activation by serotonin is similar to the kinetics observed upon Epac activation with 8CPT-2Me-cAMP (Figure 2A). In contrast, stimulation of cells with CXCL12 resulted in a strong but short activation of Rap1. The kinetics of Rap1 activation upon stimulation with serotonin and CXCL12 together resembled that induced by serotonin alone (Figure 6D). Thus, the enhancing effects of serotonin on CXCL12-induced adhesion and migration might be due to the ability of serotonin to induce a more sustained Rap1 activation than CXCL12 alone, and therefore improved integrin-mediated adhesion.

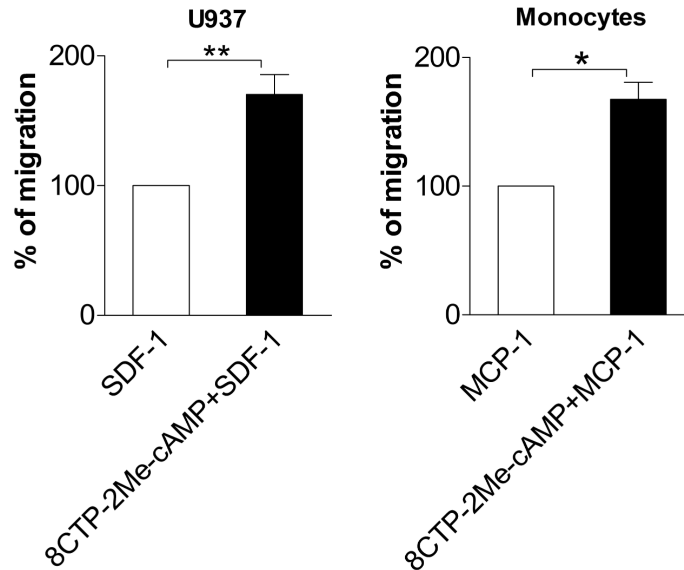

Fig. 5. Epac1 promotes chemotaxis of U937 cells and primary monocytes. U937 cells (left panel) or primary monocytes (right panel) were allowed to migrate for 1 hour to 100 ng/ml CXCL12 (SDF-1) or 10 ng/ml CCL2 (MCP-1), respectively, in the presence or absence of 100 μM 8CPT-2Me-cAMP added to the cell suspension in the upper compartment of Transwell system. When no chemoattractant was present 1-2% U937 cells or primary monocytes migrated regardless of the presence of 8CPT-2Me-cAMP. In the presence of chemoattractant, 30-40 percent of untreated U937 cells and 6-7% of untreated monocytes migrated. Data represent the percentage of cell migration compared to the untreated cells (set at 100%). Data are mean (+/- SEM) of three to five independent experiments. * $p < 0.05$; ** $p < 0.005$

To unequivocally implicate Epac in the mediation of serotonin effects on adhesion and chemotaxis, we transfected U937 cells with a dominant negative Epac mutant. However, no quantitative data could be obtained due to very low transfection efficiencies. We then followed an indirect approach by demonstrating that serotonin-induced adhesion requires cAMP production but is PKA-independent. U937 cells were treated with two different adenylate cyclase inhibitors, SQ22536 and 2'-5'-deoxyadenosine, to block cAMP generation upon stimulation with serotonin. Treatment with either inhibitor abrogated the differences in adhesion between serotonin-stimulated and unstimulated cells and between CXCL12-stimulated and CXCL12/serotonin-stimulated cells (Figure 7A). These data suggest that cAMP production is required for the enhancing effects that serotonin has on adhesion. Since cAMP can activate both Epac1 and PKA, we excluded the possibility that serotonin-induced Rap1 activation and cell adhesion were mediated by PKA by using the specific PKA inhibitor H89. Pre-treatment of cells with H89 did not inhibit serotonin-induced Rap1 activation

(Figure 7B) or prevent the effects of serotonin on cell adhesion (Figure 7C). Similar results were obtained with the competitive PKA inhibitor Rp-cAMPS (data not shown). These data indicate that PKA is not involved in serotonin-induced Rap1 activation and adhesion of U937 cells to fibronectin.

Together, these results suggest that serotonin enhances U937 cell adhesion and chemotaxis through the activation of the Epac1-Rap1 pathway.

Discussion

Our report provides the first comprehensive analysis of Epac expression in leukocytes and hematopoietic cells. We show that Epac1 is functional in primary monocytes and in monocytic U937 cells where it regulates β1 integrin dependent cell adhesion, cell polarization and chemotaxis.

The small GTPase Rap1 is activated by almost all receptor types and regulates adhesion-related functions such as cell-cell contact and integrin-mediated adhesion [42, 43]. In lymphocytes, Rap1 plays a crucial role in integrin-mediated adhesion, polarization and transendothelial migration downstream of chemokine receptors [38, 39]. Recently, Epac1 was identified as a Rap1 exchange factor directly activated by cAMP and shown to be involved in integrin-mediated adhesion in ovarian carcinoma cells through Rap1 activation [6]. Although Epac1 is expressed in most tissues, previous studies failed to show Epac1 expression in primary leukocytes with the exception of B cells and macrophages [5]. Here, we have used optimized RT-PCR conditions to detect low copy number transcripts and found Epac1 mRNA in circulating leukocytes (monocytes, eosinophils, neutrophils and B and T cells), platelets, CD34-positive hematopoietic cells and the myelocytic cell lines U937 and HL60. Importantly, we found Epac1 protein expression in all leukocytes with the exception of neutrophils. Epac1 protein levels did not always correlate directly with Epac1 mRNA levels, e.g. in eosinophils and platelets. This might be due to differential posttranslational regulation of Epac1 expression in different types of leukocytes and may have functional consequences during the response of different leukocyte types to cAMP-elevating agents.

In this study, we have investigated the function of the Epac1-Rap1 pathway in the pro-monocytic cell line U937 and in primary monocytes. We found that a cAMP analogue (8CPT-2Me-cAMP) that specifically activates Epac1, and not PKA, was able to induce cell adhesion, polarization and chemotaxis. In U937 cells, Epac1 activation induced β1 integrin activation

and VLA-4-mediated adhesion to fibronectin. Thus, Epac1 appears to have a similar role in leukocytes as in adherent cells, namely the activation of Rap1 and the consequent 'inside-out' signaling toward β1-integrins [6, 7].

In line with previous studies showing that Rap1 activation induces T cell polarization [37], we show that activation of the Epac1-Rap1 pathway induces polarization of U937 cells. Additionally, ectopically expressed Epac1 redistributes from the cell periphery to the perinuclear area upon cell polarization. This suggests that Epac1 activates Rap1 at a perinuclear location and that activated Rap1 subsequently translocates to the plasma membrane. Accordingly, wild type (active and inactive) Rap1 was shown to localize to a perinuclear vesicular compartment and to the plasma membrane in T cells, wheras activated GTP-bound Rap1 was found exclusively at the plasma membrane [44].

We have used monocytic U937 cells for most of our studies; however, we have demonstrated that the Epac pathway is functional in primary human monocytes. We show that Epac1 activation upregulates adhesion of freshly isolated monocytes to endothelial cells under flow as well as monocyte migration towards CCL2 (MCP-1). This chemokine is a potent monocyte chemoattractant that has a key role in the recruitment of monocytes to atherosclerotic lesions. Based on these data we postulate that Epac1 activation by cAMP-raising agonists plays a role in the pathophysiology of atherosclerosis. Supporting this notion, we have shown that Epac1 activation induces β1 integrin-mediated adhesion to fibronectin. β1 integrins mediate the arrest and initial adhesion of monocytes to the endothelium [35]. In addition, β1 integrins can bind to the alternatively spliced connecting segment-1 (CS-1) domain of fibronectin, which contributes to monocyte-endothelium interactions [45, 46]. Both endothelial β1 integrins and CS-1-containing fibronectin have been suggested to play a crucial role in atherogenesis through the recruitment of circulating monocytes [46-48]. Additionally, minimally modified LDL (MM-LDL) induces the deposition of CS-1 on the endothelial surface and the induction of β1 integrin-mediated monocyte binding to this integrin fragment [46]. Interestingly, treatment of endothelial cells with MM-LDL has been demonstrated to cause a rapid increase in cAMP that is necessary for the induction of monocyte binding by MM-LDL [49]. The same study showed that other cAMP-elevating agents were also inducing monocyte, but not neutrophil, binding to endothelium. This is interesting, since we show here that neutrophils do not contain Epac1 protein. Together, these data suggest a model in which local concentrations of cAMP-elevating agonists in atherosclerotic lesions induce endothelial activation and monocyte recruitment, which is

mediated by Epac1-induced activation of monocyte β1 integrin-mediated adhesion to fibronectin.

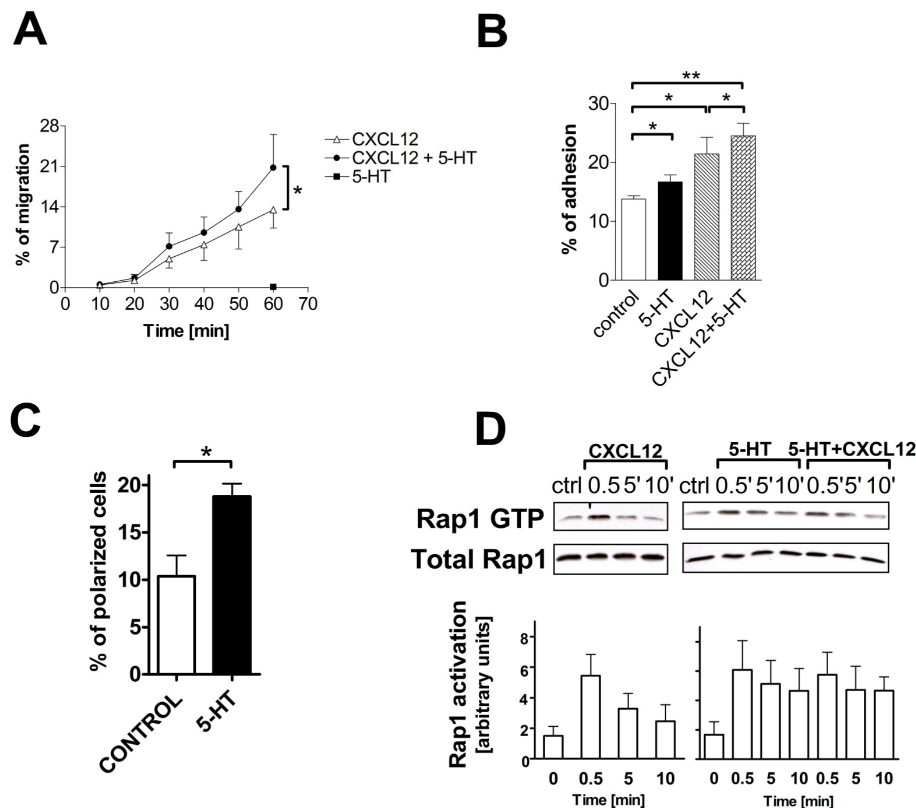

Fig. 6. Serotonin activates Rap1, induces U937 cell adhesion and increases CXCL12-induced chemotaxis. (A) U937 cells were allowed to migrate across fibronectin-coated filters towards CXCL12 (10 ng/ml), serotonin (5-HT; 1 μM) or a combination of both for the indicated time periods. The percentage of migration was determined as described in Material and Methods. Data are mean (+/- SEM) of three independent experiments. The percentage of migration toward CXCL12 was compared with the percentage of migration toward the combination of 5-HT and CXCL12 by a two-way ANOVA test ($p < 0.05$). (B) U937 cells were assayed for adhesion to fibronectin in the presence of CXCL12 (100 ng/ml), 5-HT (10 μM) or a combination of both. The percentage of adhesion was determined as described in Materials and Methods. Data are mean (+/- SEM) of five independent experiments performed in triplicate. * $p < 0.05$; ** $p < 0.005$. (C) U937 cells were allowed to adhere to fibronectin-coated coverslips, and were stimulated or not with 10 μM serotonin for 30 minutes, fixed, stained for F-actin and analyzed by confocal microscopy. Data represent the percentage of polarized cells, scored on the basis of morphology, of a total of 100 to 120 cells per condition. (D) U937 cells were stimulated with 5-HT (10 μM), CXCL12 (100 ng/ml) or combination of both stimuli for the indicated time periods and Rap1 GTP-loading was assayed (upper panel). Total levels of Rap1 in whole cell lysates are shown (lower panel). Representative results of three independent experiments are shown. Bar graph represents densitometric analysis of Rap1 activation. Data are mean of three independent experiments (+/- SEM).

Serotonin is a cAMP-elevating agonist secreted by activated platelets and mast cells, and increased plasma levels of serotonin are associated with the pathophysiology of atherosclerosis and asthma [19, 50]. Interestingly, serotonin was recently shown to be a chemotactic factor for eosinophils [18] and to modulate cytokine and chemokine release by monocytes [51]. We show here that serotonin is able to induce adhesion, polarization and chemokinesis of U937 cells similarly to Epac activation. Although we could not directly implicate Epac1 in these effects, we show that serotonin-induced adhesion requires cAMP but is PKA independent, which suggests that Epac1 activation by cAMP mediates serotonin-induced adhesion of monocytes to fibronectin. Accordingly, Epac1 has previously been shown to be activated by serotonin receptors in neuronal cells [15, 16, 52]. In the concentrations used in our study, serotonin did not show chemotactic properties for U937 cells, similar to the Epac activator 8CPT-2Me-cAMP. However, both agents increased CXCL12-induced chemotaxis, indicating that other signaling pathways engaged by chemokines are likely to be required for cell movement [53-55]. The enhancing effects of serotonin on migration could be due to its ability to induce a more sustained Rap1 activation than does CXCL12. This may result in the improvement of Rap1-mediated functions, such as polarization and adhesion, which are pre-requisites for directional migration. In conclusion, our data support a pro-inflammatory role for serotonin as an enhancer of monocyte adhesion and chemotaxis.

Previous reports have shown that agents that increase cAMP such as forskolin, IBMX or prostaglandin E2 inhibit chemokine-induced monocyte adhesion and migration [56, 57]. However, other studies demonstrated that u-PA and relaxin stimulate monocyte adhesion and migration through cAMP-dependent pathways [58, 59]. An explanation for these contradictory observations may be the compartmentalization of cAMP signaling in cells [60]. Different signaling receptors activate differentially located members of the adenylate cyclase family and specific phosphodiesterases degrade cAMP to prevent its diffusion. This results in the formation of cAMP 'clouds' at discrete sites within the cell, which activate only nearby located effectors. Thus, it may be that cAMP more potently activates either PKA or Epac1, depending on the stimulus, resulting in different outcomes for adhesion and migration. Interestingly, cAMP is known to consistently inhibit neutrophil migration, which may be explained by the fact that they do not express Epac1 protein, as shown here.

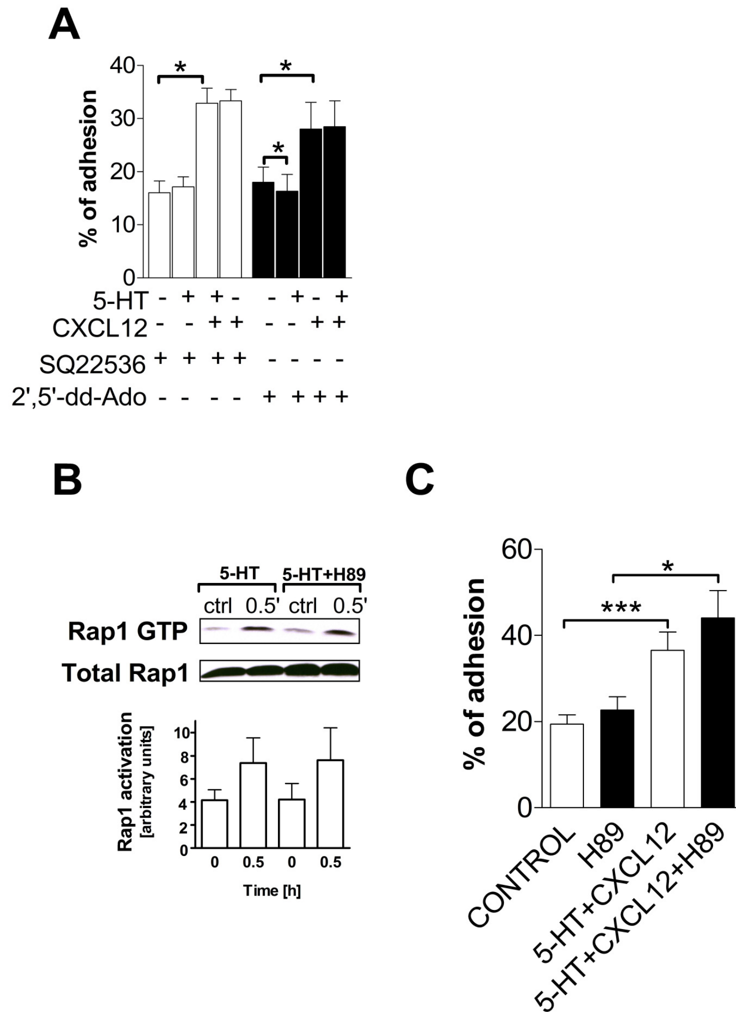

Fig. 7. Serotonin increases adhesion in a cAMP-dependent, PKA-independent manner. (A) U937 cells were pre-incubated with the adenylate cyclase inhibitors SQ22536 or 2'5'-deoxyadenosime (2'-5'-dd-Ado) for 1 hour before addition of the cells to fibronectin-coated plates. The percentage of adhesion was determined as described in Materials and Methods. Data are mean (+/- SEM) of three independent experiments performed in triplicate. (B) U937 cells were pre-treated or not with 10 μM H89 for 30 minutes, stimulated with 5-HT (10 μM) and Rap1 GTP-loading was assayed (upper panel). Total levels of Rap1 in whole cell lysates are shown (lower panel). Representative results of three independent experiments are shown. Bar graph represents densitometric analysis of Rap1 activation. Data are mean of three independent experiments (+/- SEM). (C) U937 cells pretreated or not with H89 (10 μM) for 30 minutes were assayed for adhesion to fibronectin in the presence of CXCL12 (100 ng/ml) and 5-HT (10 μM). The percentage of adhesion was determined as described in Materials and Methods. Data are mean (+/- SEM) of five independent experiments performed in triplicate. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$

In conclusion, our work reveals a previously unrecognized cAMP-dependent signaling pathway in monocytes, regulating cell adhesion, polarization and chemotaxis through the

activation of Epac1. Finally, our data suggest that cAMP-elevating receptor agonists may regulate inflammatory processes through the activation of Epac1-Rap1 signaling in monocytes.

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CHAPTER III

PKA AND EPAC1 REGULATE ENDOTHELIAL INTEGRITY AND CELL MIGRATION THROUGH INDEPENDENT PATHWAYS

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Abstract

The vascular endothelium provides a semipermeable barrier, which restricts the passage of fluid, macromolecules and cells to the surrounding tissues. Chronic inflammatory disorders such as arteriosclerosis, diabetes or asthma are accompanied by increased vascular permeability and vascular injury. Cyclic AMP, a well established secondary messenger, promotes endothelial barrier function. We used real time measurement of transendothelial electrical resistance to investigate the relative contribution of two major cAMP targets Epac1 and PKA in the regulation of endothelial barrier function and endothelial migration in a wound closure model. We found that activation of either PKA or Epac1 with specific cAMP analogues increased endothelial barrier function and promoted endothelial cell migration. In addition, reduction of Epac1 expression showed that Epac1 and PKA control endothelial integrity by two independent signaling pathways. We demonstrate that PKA but not Epac1-Rap1 signaling exerts its effects on endothelial barrier function through engagement of integrins. However, both PKA- and Epac-stimulated endothelial cell migration is dependent on integrin-mediated adhesion. Based on these data, we propose a model where Epac1 and PKA activation by cAMP results in the stimulation of two independent signaling pathways that regulate endothelial integrity and cell migration.

Introduction

Vascular integrity is a critical parameter for chronic inflammation, edema and tumor angiogenesis and is regulated by the level of endothelial cell-cell contact. A decrease in endothelial integrity leads to matrix exposure, leakage of plasma proteins from the circulation, and extravasation of activated immune cells. For these reasons, insight into the mechanisms that control endothelial barrier function is important to define potential therapeutic targets for treatment.

Many receptor agonists (including thrombin, histamine and VEGF) affect endothelial integrity mostly by promoting permeability through reduction of endothelial cell-cell adhesion [1-4]. The molecular mechanisms by which these different stimuli reduce endothelial cell-cell contact include activation of Rho-like GTPases and Src-like tyrosine kinases, as well as the production of Reactive Oxygen Species. This causes increased contractility of the actin cytoskeleton and a reduction of Vascular-Cadherin (VE)-cadherin-mediated cell-cell contact

[5, 6]. Stimuli that promote endothelial barrier function include beta-adrenergic receptor agonists, as well as the lipid sphingosine-1-phosphate (S1P) [7, 8]. Downstream of beta-adrenergic receptors, the generation of cyclic AMP is the key event known to reduce vascular permeability both in vitro and in vivo, providing an anti-inflammatory signal [9-12].

The mechanism by which cAMP stabilizes endothelial cell-cell junctions is not entirely clear. Downstream of the prime effector for cAMP, protein kinase A (PKA), inhibition of RhoGTPase-mediated cytoskeletal contractility, vasodilator-stimulated phosphoprotein-mediated cytoskeletal changes, reduction of myosin light chain phosphorylation and regulation of Rac1 GTPase activation, have been implicated [13-16].

Cyclic AMP also activates the guanine nucleotide exchange factor Epac1 (exchange protein directly activated by cAMP) [17]. Epac1 activates the small GTPase Rap1 that has previously been shown to stimulate integrin function through inside-out signaling [18, 19], to induce chemotaxis [20, 21] and to promote epithelial and endothelial cell-cell adhesion [22-28]. Thus, there are at least two cAMP-activated pathways that lead to increased endothelial barrier function: Epac1-Rap1 and PKA signaling.

cAMP-mediated activation of PKA has been implicated in both negative and positive regulation of endothelial cell migration [29-32], which is essential for tumor angiogenesis as well as for collateral formation and is governed by a balance between cell-matrix and VE-cadherin-mediated cell-cell adhesion. Importantly, Rap1 was shown to regulate endothelial wound closure and to localize to the leading edge of migrating endothelial cells [33]. However, the relative contribution of the PKA and Epac1-Rap1 pathways to endothelial cell motility is unclear.

Here, we used sensitive real-time measurement of transendothelial resistance to study the effects and potential cross-talk of the Epac1-Rap1 and PKA pathways in the control of endothelial integrity and migration. Using an Epac1 knockdown approach and selective inhibition of PKA, we find that these two proteins act independently to promote endothelial barrier function and to stimulate endothelial cell migration. We provide evidence that the PKA- but not Epac1-Rap1 signaling-mediated increase in endothelial integrity is dependent on integrin-mediated cell adhesion. In contrast, both pathways require integrin engagement to stimulate endothelial cell migration Together, these findings suggest that cAMP promotes endothelial cell integrity and migration through the activation of two independent pathways regulated by PKA and Epac1.

Materials and Methods

Reagents

8CPT-2Me-cAMP, N6-Benzoyl-cAMP and Rp-8-CPT-cAMPS were purchased from Biolog LSI (Germany). Poly-L-lysine and H89 were from Sigma. NS23766 was purchased from Calbiochem (Germany).

Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord veins as previously described [34]. Alternatively, HUVECs pooled from several donors were used (purchased from Cambrex, Walkersville, MD, U.S.A.). Cells were cultured in EBM-2 Bulletkit culture medium (EBM-2 supplemented with EGM-2 SingleQuots containing fetal bovine serum, hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, hEGF, GA-1000, heparin) (Clonetics). Endothelial cells of second to sixth passage were used.

Cell transfection

Sixteen hours after plating, cells were transfected with control siRNA (siCONTROL™ Non-Targeting siRNA#1, Dharmacon, Chicago, IL, U.S.A.) or siRNA against Epac1 (CCATCATCCTGCGAGAAGA, Dharmacon, Chicago, IL, U.S.A) using Oligofectamine (Invitrogen, Carlsbad, CA) according to manufacturer instructions. Transfection was repeated after 24 h [25]. For the measurement of transendothelial resistance and quantitative wounding assays transfected cells were seeded on fibronectin-coated gold ECIS electrodes. For immunofluorescence analysis cells were grown and directly transfected on fibronectin-coated coverslips.

Transendothelial electrical resistance measurement

Endothelial cells were seeded at $1.5x10^5$ cells per well (0.8 cm^2) on fibronectin or poly-L-lysine-coated electrode arrays and grown to confluency. Measurements of transendothelial electrical resistance were performed in real time at 37ºC, 5% CO_2, using an electrical cell-substrate impedance sensing system (ECIS; Applied BioPhisics Inc., Troy, NY, U.S.A.) as previously described [35]. Briefly, the small measuring electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier, and 1-V, 4000 Hz signal was supplied by a 1-MΩ resistor to approximate a constant current source of 1μA. The in-phase and out-phase voltages between the electrodes were monitored in real time followed by

conversion to scalar measurements of transendothelial impedance. The presented electrical resistance values were normalized to the initial baseline resistance.

Electrical cell-substrate impedance sensing based wounding

HUVEC monolayers grown on fibronectin or poly-L-lysine coated gold electrodes were subjected to a high field current of 3V at a frequency of 40 kHz for 15 seconds. Such a electrocution results in the death of cells covering the electrode, leaving the rest of the cell monolayer in the array well intact. Subsequently, the increase in transendothelial resistance resulting form cells migrating into the wound was recorded to measure endothelial cell migration [36] . Maximal increase in the resistance (maximal resistance change) was calculated to determine the maximal speed of cell migration. Monolayer recovery curves were analyzed by nonlinear regression sigmoidal fit. This analysis was followed by calculation of the derivative from the sigmoidal fit function. The maximum of this function represents maximal increase in the resistance, which is the parameter that describes the maximum speed of cell migration.

Immunofluorescence

Cells were cultured on fibronectin or on poly-L-lysine-coated glass coverslips. After stimulation, cells were fixed with 3.7% formaldehyde for 10 minutes at RT and permeabilized with 0.1% Triton X-100 for 5 minutes. Thereafter, cells were incubated with a mouse monoclonal antibody to VE-cadherin (Transduction Laboratories), followed by the incubation with a chicken-anti-mouse-Ig antibody labeled with Alexa 488 (Molecular Probes, Leiden, The Netherlands). F-actin was visualized with TexasRed-labeled phalloidin (Molecular Probes, Leiden, The Netherlands). Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope. Fluorescence distribution profiles were created with Image-Pro Plus 6.0 software, Media Cybernetics.

'Scratch' assay

Cells were cultured to confluence on fibronectin- or on poly-L-lysine-coated glass coverslips. Thereafter, endothelial monolayers were wounded with a yellow pipette tip, and after 2 hours cells were stimulated, fixed with 3.7% formaldehyde for 10 minutes at RT and permeabilized with 0.1% Triton X-100 for 5 minutes. Cells were immunostained with a mouse monoclonal antibody to paxillin (Transduction Laboratories), followed by the incubation with a chicken-

anti-mouse-Ig antibody labeled with Alexa 488 (Molecular Probes, Leiden, The Netherlands). F-actin was visualized with TexasRed-labelled phalloidin (Molecular Probes, Leiden, The Netherlands). Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope.

Western blot analysis

HUVEC were lysed in Laemmli sample buffer and incubated for 10 minutes at 95°C. Cell lysates were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes (BioRad Laboratories, Hercules, CA, USA). Epac1 was detected with a previously described [27] mouse monoclonal antibody (5D3). Actin was detected with a mouse monoclonal antibody against actin (Ab1, Oncogene, Darmstadt, Germany).

Statistical analysis

All results were expressed as a mean ± SEM of at least three independent experiments. Where applicable, values were compared with paired two-tailed Student t-test. A *p* value lower than 0.05 was considered significant.

Results

Activation of either Epac or PKA promotes endothelial barrier function

To establish the relative contribution of PKA and Epac1 in the cAMP-induced stimulation of endothelial barrier function we made use of cyclic AMP analogues that specifically activate PKA (N6-Benzoyl-cAMP) or Epac (8-CTP-2Me-cAMP) [37, 38] . To quantify changes in transendothelial resistance (TER) in real time, we seeded primary human umbilical vein endothelial cells (HUVEC) on fibronectin-coated gold electrodes and measured electrical resistance by ECIS (Electrical Cell-substrate Impedance Sensing [35]). Monolayers with stable TER were treated either with N6-Benzoyl-cAMP or with 8-CTP-2Me-cAMP and changes in TER were followed over a five hour period. Activation of either Epac or PKA in primary endothelial cells resulted in a rapid increase in transendothelial resistance (Figure 1A) that levelled off after 20 minutes, and remained very stable during the five-hour period of measurement. No additional increase in electrical resistance was recorded

when HUVEC monolayers were treated with both cAMP analogues simultaneously (data not shown), suggesting no additive effect of Epac1 and PKA stimulation on endothelial integrity.

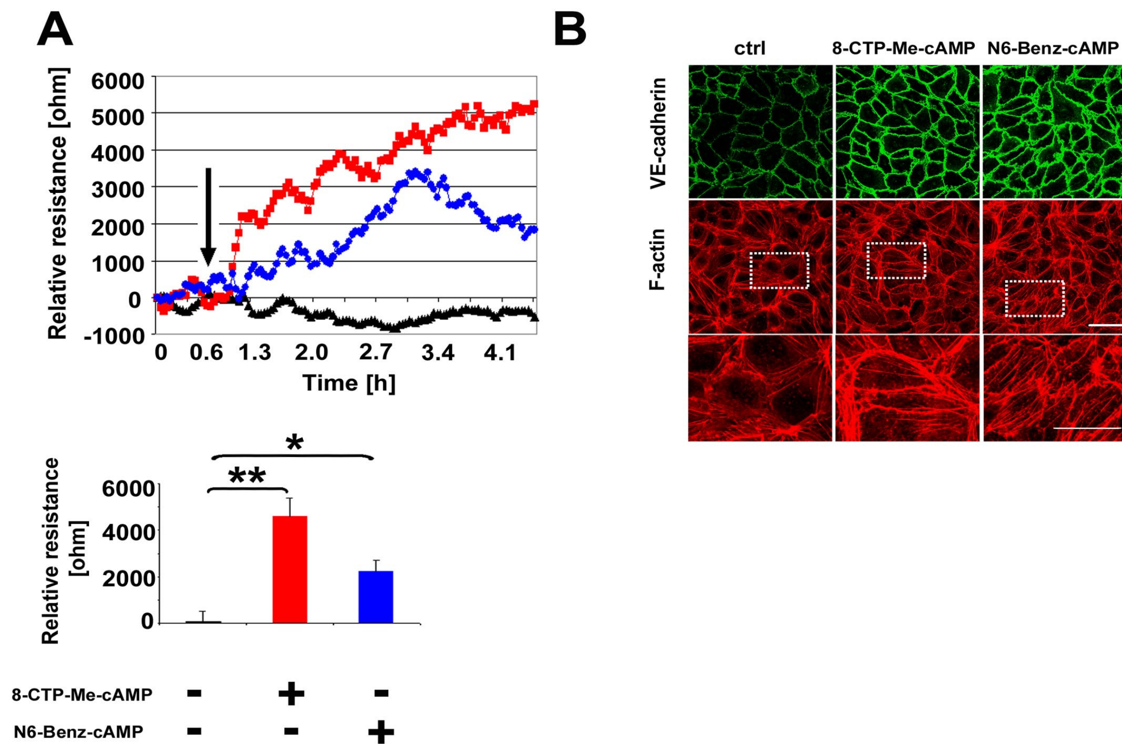


Figure 1. Epac1 and PKA promote endothelial barrier function. **(A)** HUVEC were plated on fibronectin-coated golden electrodes and grown to confluence. Transendothelial resistance was measured in real time as described in Materials and Methods. Cells were stimulated or not with 200 µM 8CTP-2'-O-Me-cAMP or 200 µM N6-Benzoyl-cAMP at the time indicated by the arrow (upper panel). *Black triangle* – control; *red square-* 8CTP-2'-O-Me-cAMP; *blue circle-* N6-Benzoyl-cAMP. Representative results from at least 5 independent experiments are shown. Bar graph (lower panel) represents the changes in resistance at the 3-hour time point, when the resistance in most of the experiments reached a plateau. Data are means (+/- SEM) of 5 independent experiments performed in duplicate. *p<0.05; **p<0.005 **(B)** HUVEC were plated on fibronectin-coated glass coverslips and grown to confluence. Cell monolayers were treated or not with 200 µM 8CTP-2'-O-Me-cAMP or 200 µM N6-Benzoyl-cAMP for 30 minutes fixed and stained for VE-cadherin and F-actin. Fluorescence intensity profiles along the indicated dashed line between points indicated as **a** and **b** are shown. Images are representative of 3 independent experiments. Bars: 50 µm

In endothelial cells, monolayer integrity and TER are critically dependent on Vascular-Endothelial (VE-) cadherin, which is located at the adherens junctions [39] and is indirectly linked to the endothelial actin cytoskeleton. As a result, VE-cadherin-mediated cell-

cell adhesion is regulated by cytoskeletal dynamics. To investigate whether the increase in transendothelial resistance induced by activation of PKA or Epac1 was accompanied by changes in adherens junction organisation, we examined the distribution of VE-cadherin in endothelial monolayers treated with 8-CTP-2Me-cAMP or N6-Benzoyl-cAMP. Immunofluorescence analysis of HUVEC monolayers, stimulated with either cAMP analogue, showed increased levels of VE-cadherin at cell-cell contacts (Figure 1B), in good agreement with the increased TER (Figure 1A). In addition to VE-cadherin recruitment to cell-cell contacts, both Epac1 and PKA activation induced cytoskeletal changes. Activation of Epac1 promoted the formation of cortical actin, whereas activation of PKA resulted in an increase in the formation of actin stress fibres. These results suggest that both Epac and PKA promote TER by stimulating VE-cadherin accumulation at cell-cell junctions. However, Epac1 and PKA clearly have differential effects on the actin cytoskeleton, suggesting that they operate through distinct and potentially independent pathways.

Epac1 and PKA promote endothelial barrier function through independent pathways.

To establish whether PKA and Epac1 promote endothelial TER through independent pathways, we tested the effects of 8-CTP-2Me-cAMP and N6-Benzoyl-cAMP in cells in which Epac1 expression was reduced by transfection of siRNA specific for Epac1(Figure 2A, upper panel). In contrast to cells transfected with control siRNA, no increase in transendothelial resistance was observed upon 8-CTP-2Me-cAMP addition in Epac1-negative cells (Figure 2A, lower panel). Moreover, reduction of Epac1 expression caused a small but significant decrease in the basal resistance of endothelial monolayers (Figure 2A). This suggests that, even in unstimulated cells, a low level of Epac1 activation stabilises endothelial cell-cell contacts. Importantly, activation of PKA in Epac1-negative cells still resulted in a similar increase in TER as in control cells (Figure 2B).

Complementary analysis of VE-cadherin distribution in Epac1-negative cells showed that 8-CTP-2Me-cAMP was no longer able to induce an increase in VE-cadherin levels at cell-cell junctions or to promote the polymerization of cortical actin (Figure 2C). The effects of Epac1 stimulation were unaffected in control siRNA-transfected cells (Figure 2C). In addition, reduction of Epac1 expression did not inhibit the PKA-mediated increase in VE-cadherin at cell-cell junctions or block the PKA-induced formation of actin stress fibres (Figure 2B).These results show that the reduction of Epac1 expression does not affect the PKA-mediated increase in endothelial resistance, VE-cadherin accumulation and stress fibres

formation. Thus, Epac1 and PKA regulate the endothelial barrier function through independent signalling pathways.

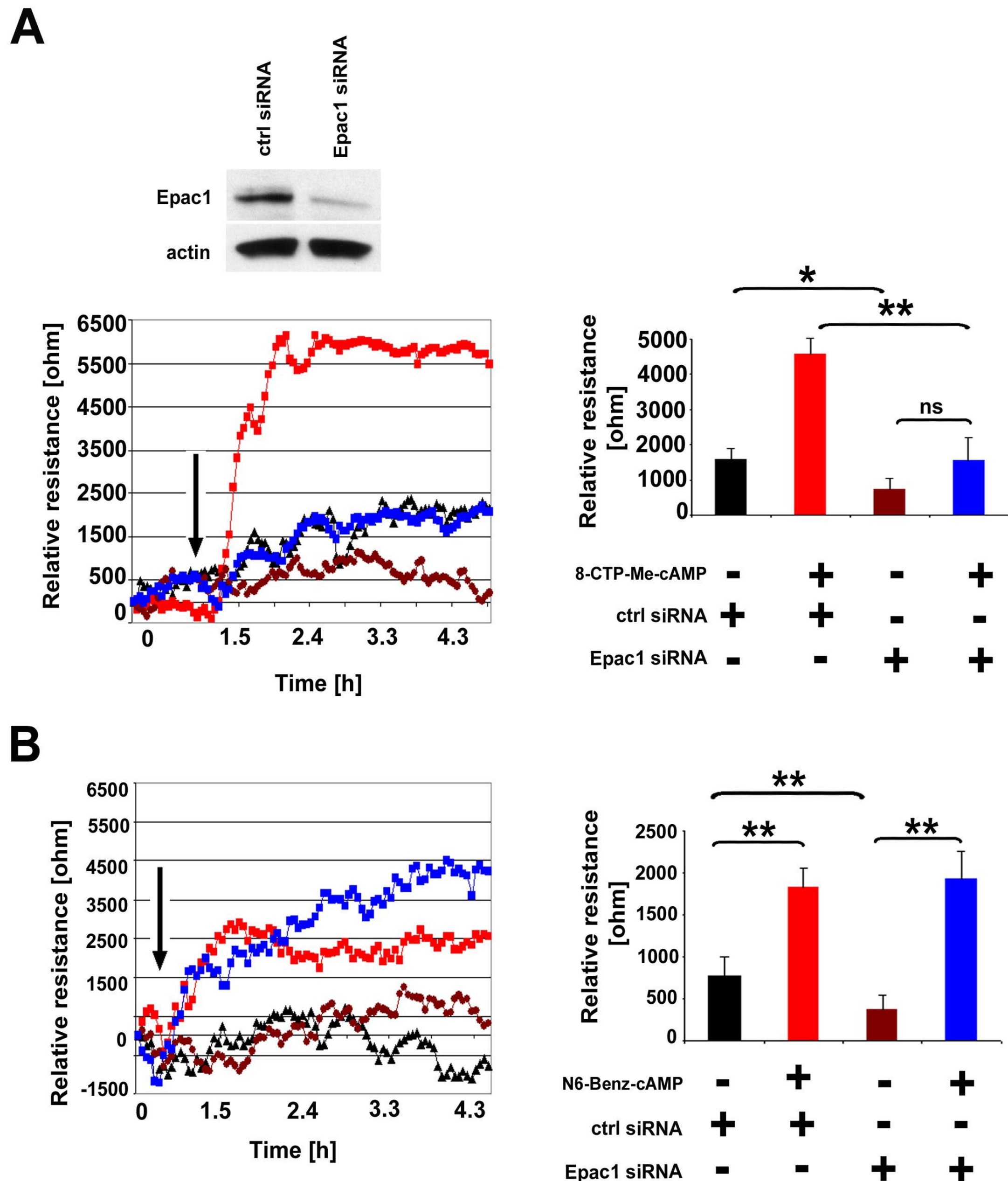
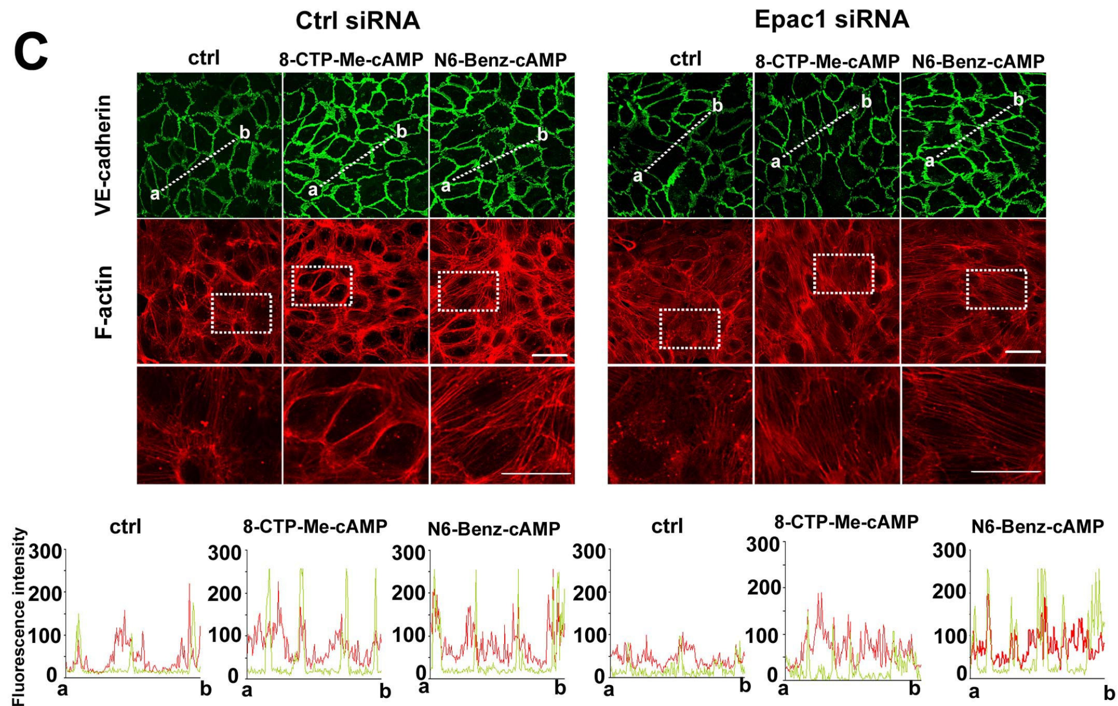


Figure 2. Stimulation of PKA but not Epac1 induces an increase in endothelial barrier function in Epac1-negative cells. HUVEC were transfected with control siRNA or siRNA against Epac1, plated on fibronectin-coated golden electrodes and grown to confluence. Transendothelial resistance was measured in real time as described in Materials and Methods. **(A)** Western blot detection of Epac1 in lysates of control and Epac1-negative cells are shown (Upper panel). Bands corresponding to Epac1 are ~ 100 kDa. β-actin was used as a control for equal loading. Representative result of 10 independent experiments is shown. Cells were stimulated or not with 200 µM 8CTP-2'-O-Me-cAMP at the time indicated by the arrow. *Black triangle* – control siRNA; *red square-* control siRNA and 8CTP-2'-O-Me-cAMP; *brown circle-* siRNA against Epac1; *blue square-* siRNA



against Epac1 and 8CTP-2'-O-Me-cAMP (lower left panel). Representative results from 5 independent experiments are shown. Bar graph (lower right panel) represents the changes in resistance at the 3-hour time point, when the resistance in most of the experiments reached a plateau. Data are means (+/- SEM) of 5 independent experiments performed in duplicate. *p<0.05; **p<0.005. **(B)** Cells were stimulated or not with 200 µM N6-Benzoyl-cAMP at the time indicated by the arrow (right panel). *Black triangle* – control siRNA; *red square-* control siRNA and N6-Benzoyl-cAMP; *brown circle-* siRNA against Epac1; *blue square-* siRNA against Epac1 and N6-Benzoyl-cAMP. Representative results from 7 independent experiments are shown. Bar graph (left panel) represents the changes in resistance at the 3-hour time point, when the resistance in most of the experiments reached a plateau. Data are means (+/- SEM) of 7 independent experiments performed in duplicate. *p<0.05; **p<0.005..**(C)** HUVEC were transfected with control siRNA or siRNA against Epac1, plated on fibronectin-coated glass coverslips and grown to confluence. Cell monolayers were treated or not with 200 µM 8CTP-2'-O-Me-cAMP or 200 µM N6-Benzoyl-cAMP for 30 minutes, fixed and stained for VE-cadherin and F-actin. Fluorescence intensity profiles along the indicated dashed line between points indicated as **a** and **b** are shown. Images are representative of 3 independent experiments. Bars: 50 µm

Epac1 activation compensates loss of endothelial barrier function caused by inhibition of PKA.

To further investigate the relation between PKA and Epac1-mediated stimulation of endothelial integrity we tested whether activation of Epac1 could rescue a loss of endothelial

barrier function caused by inhibition of PKA activity. To test this, we measured transendothelial resistance in cells treated with H89, a specific PKA inhibitor, in the absence or presence of Epac1 activation. As shown in Figure 3A, inhibition of PKA resulted in a fast and pronounced decrease in transendothelial resistance. This response was completely prevented when cells were simultaneously stimulated with 8-CTP-2Me-cAMP to activate Epac1 (Figure 3A). In fact, the relative effect of Epac1 stimulation was comparable to that in untreated cells (Figure 1A), further indicating that PKA activity is not required for the response of endothelial cells to Epac1. Similar results were obtained with the competitive PKA inhibitor Rp-cAMPS (data not shown).

Immunofluorescence analysis showed that the VE-cadherin distribution at the cell-cell junctions was less regular in H89-treated cells versus control, untreated cells. However, no major differences in VE-cadherin levels at cell-cell junctions were observed (Figure 3B). Importantly, inhibition of PKA resulted in an almost complete loss of actin stress fibres, further underscoring the notion that PKA regulates endothelial integrity through reorganisation of the cytoskeleton. VE-cadherin distribution at cell-cell junctions changed from jagged to more continuous in H89-treated cells upon Epac1 activation. This was accompanied by a clear increase in cortical actin at the cell-cell contact area (Figure 3B). This further suggests that Epac1 promotes cell-cell adhesion through the enhanced formation of cortical actin. Together, these data provide additional evidence that Epac1 and PKA regulate cytoskeletal dynamics endothelial integrity through parallel rather than sequential signalling events.

Epac1 and PKA regulate endothelial cell migration

The migration of endothelial cells is crucial for the formation of new blood vessels and for the restoration of endothelial integrity following endothelial damage [40]. Several reports have implicated PKA in both negative and positive regulation of cell migration in different cell types, including endothelial cells [29-32, 41-43]. So far, there is no information on a role for Epac1 in endothelial cell migration. However, active Rap1 has been reported to localize to the leading edge of migrating endothelial cells and control chemotaxis and wound healing [33]. Therefore, we set out to investigate the relative contribution of Epac1 and PKA to endothelial cell migration in a model based on endothelial wound closure. Endothelial monolayers were locally wounded by ECIS-mediated high-current electrocution and wound closure was followed by real time measurement of transendothelial electrical resistance. This

recording reflects the ingrowth of cells that surround the electrode and the subsequent restoration of endothelial barrier function [36].

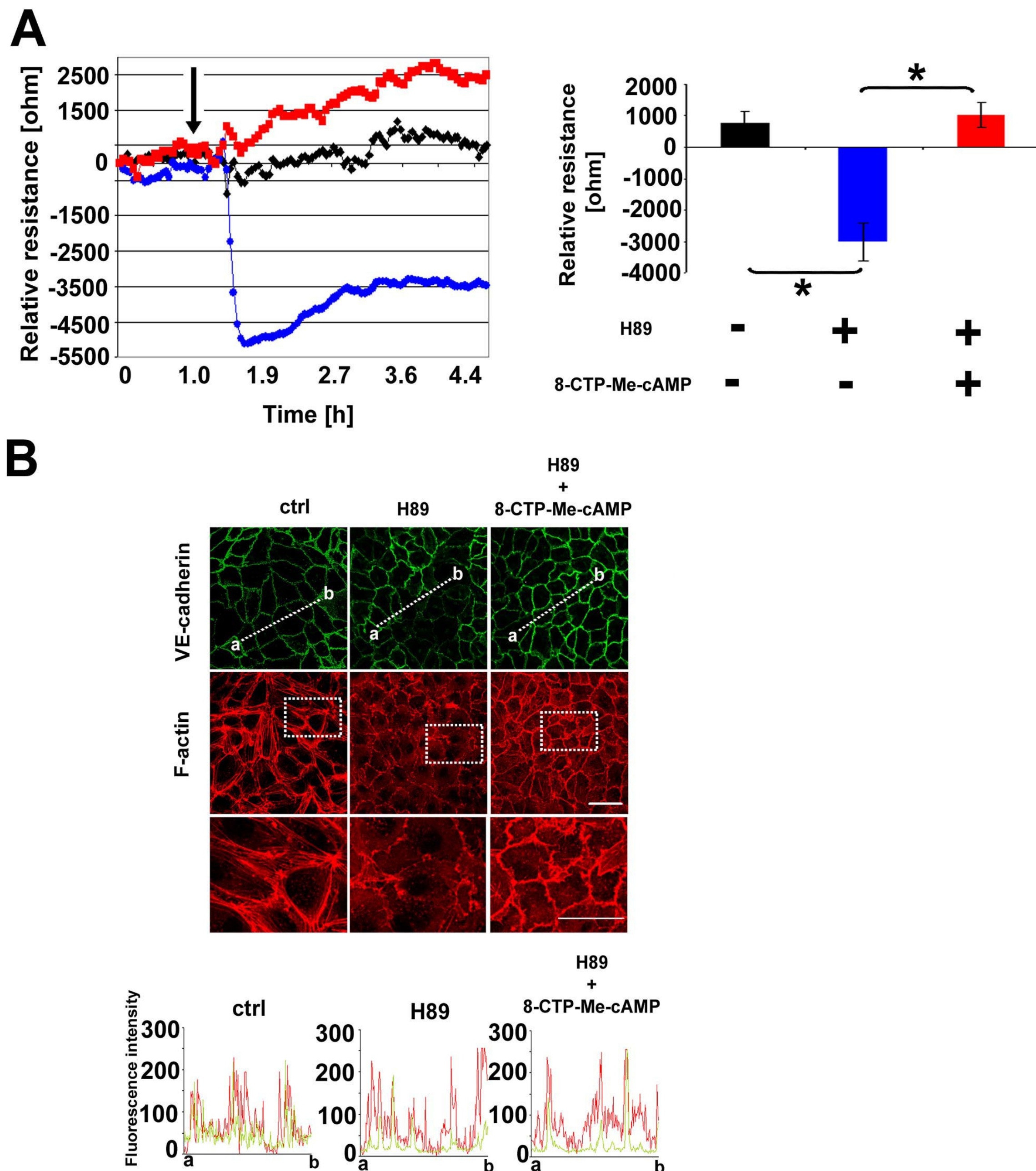


Figure 3. Epac1 activation prevents loss of endothelial barrier function caused by PKA inhibition. **(A).** HUVEC were plated on fibronectin-coated golden electrodes and grown to confluence. Transendothelial resistance was measured in real time as described in Materials and Methods. Cells were treated or not with 10 μM H89 or combination of 10μ M H89 and 200 μM 8CTP-2'-O-Me-cAMP at the time indicated by the arrow (left panel). *Black diamond* – control; *blue circle-* H89; *red square-* H89 and 8CTP-2'-O-Me-cAMP. Representative results from 5 independent experiments are shown. Bar graph (right panel) represents the changes in resistance at the 3-hour time point, when the resistance in most of the experiments reached a plateau. Data are means (+/- SEM) of 5 independent experiments performed in duplicate. *$p<0.05$ **(B)** HUVEC were plated on fibronectin-coated glass

coverslips and grown to confluence. Cell monolayers were treated or not with 10 μM H89 or combination of 10 μM H89 and 200 μM 8CTP-2'-O-Me-cAMP for 30 minutes, fixed and stained for VE-cadherin and F-actin. Fluorescence intensity profiles along the indicated dashed line between points indicated as **a** and **b** are shown. Images are representative of 3 independent experiments. Bars: 50 μm

Stimulation of PKA or Epac1 in wounded endothelial monolayers accelerated cell migration and the restoration of monolayer integrity (Figure 4A), indicating that both Epac1 and PKA are involved in this process. To further substantiate this, we transfected endothelial cells with siRNA against Epac1 and monitored the wound healing response. The recovery of Epac1-negative monolayers was delayed compared to monolayers transfected with control siRNA (Figure 4B). This delay in Epac1-negative cell migration was consistent, albeit statistically not significant. Importantly, reduced Epac1 expression ablated the stimulatory effect of 8-CTP-2Me-cAMP, but not of N6-Benzoyl-cAMP, on endothelial cell migration (Figure 4B, Figure 4C).

These data suggest that both PKA and Epac1 are important regulators of endothelial cell migration that act through independent signalling pathways, similar to the control of endothelial barrier function.

Epac1 and PKA reorganise focal adhesions

Interactions of cells with the extracellular matrix are essential for the control of cell migration. These interactions are orchestrated by integrins that are indirectly linked to the actin cytoskeleton and can translate mechanical force, generated in the cell, into increased adhesion [44]. Moreover, focal adhesions serve to cluster and concentrate signalling molecules including adaptor proteins, protein kinases and small GTPases. As a result, integrin activation by extracellular matrix molecules initiates a range of intracellular signalling events. Conversely, specific intracellular signalling, e.g. through the Rap1 GTPase, regulates integrin function [18].

In order to gain insight into the mechanism by which PKA and Epac1 regulate migration of endothelial cells, we performed a so called 'scratch' assay. Two hours after wounding we analysed by immunofluorescence the distribution of paxillin, one of the major proteins in focal adhesions. In cells treated with 8-CTP-2Me-cAMP, paxillin-positive focal adhesions were clearly redistributed towards the leading edge, compared to control, untreated cells (Figure 4D). Moreover, Epac1 stimulation resulted in an increase in size of focal adhesions in wounded monolayers, suggestive for integrin clustering. Interestingly, activation

of PKA did not affect the overall distribution of focal adhesions, but induced an increase both in number and size of focal adhesions (Figure 4D).

Next, we examined the paxillin distribution in Epac1-negative cells. Treatment of cells transfected with control siRNA with 8-CTP-2Me-cAMP induced paxillin redistribution to the leading edge (Figure 4E, left panel). However, in Epac1-negative cells treated with 8-CTP-2Me-cAMP, paxillin distribution did not change (Figure 4E, right panel). In contrast, the overall increase in number and size of focal adhesions induced by PKA activation was not affected by the downregulation of Epac1 (Figure 4E).

To further investigate the relation between PKA and Epac1-mediated signalling in the regulation of endothelial cell migration we treated electrically wounded endothelial monolayers with the PKA inhibitor H89 or the combination of H89 and 8-CTP-2Me-cAMP and monitored wound closure by ECIS. H89 significantly inhibited endothelial monolayer recovery (Figure 5A). However, activation of Epac1 in H89-treated cells abrogated the blocking effect of H89 on endothelial cell migration (Figure 5A).

To assess the extent to which the inhibition of PKA affects the organisation of focal adhesions, we made a scratch in monolayer of HUVEC and subsequently added H89. Immunofluorescence analysis of paxillin distribution showed a dramatic decrease in both the number of focal adhesions and actin stress fibres in cells treated with H89 (Figure 5B). Interestingly, the stimulation of H89-treated cells with 8-CTP-2Me-cAMP still induced the recruitment of paxillin to the leading edge of wounded monolayers. However, the distribution of paxillin was continuous and not punctuate as in control cells, suggesting that PKA is required for proper organisation of focal adhesions**.** In addition, wounded monolayers treated with the combination of H89 and 8-CTP-2Me-cAMP showed an increase in actin cables next to the edge of the wound (Figure 5B**).**

These results suggest that regulation of endothelial cell migration by Epac1 and PKA involves reorganization of focal adhesions through cytoskeletal rearrangement.

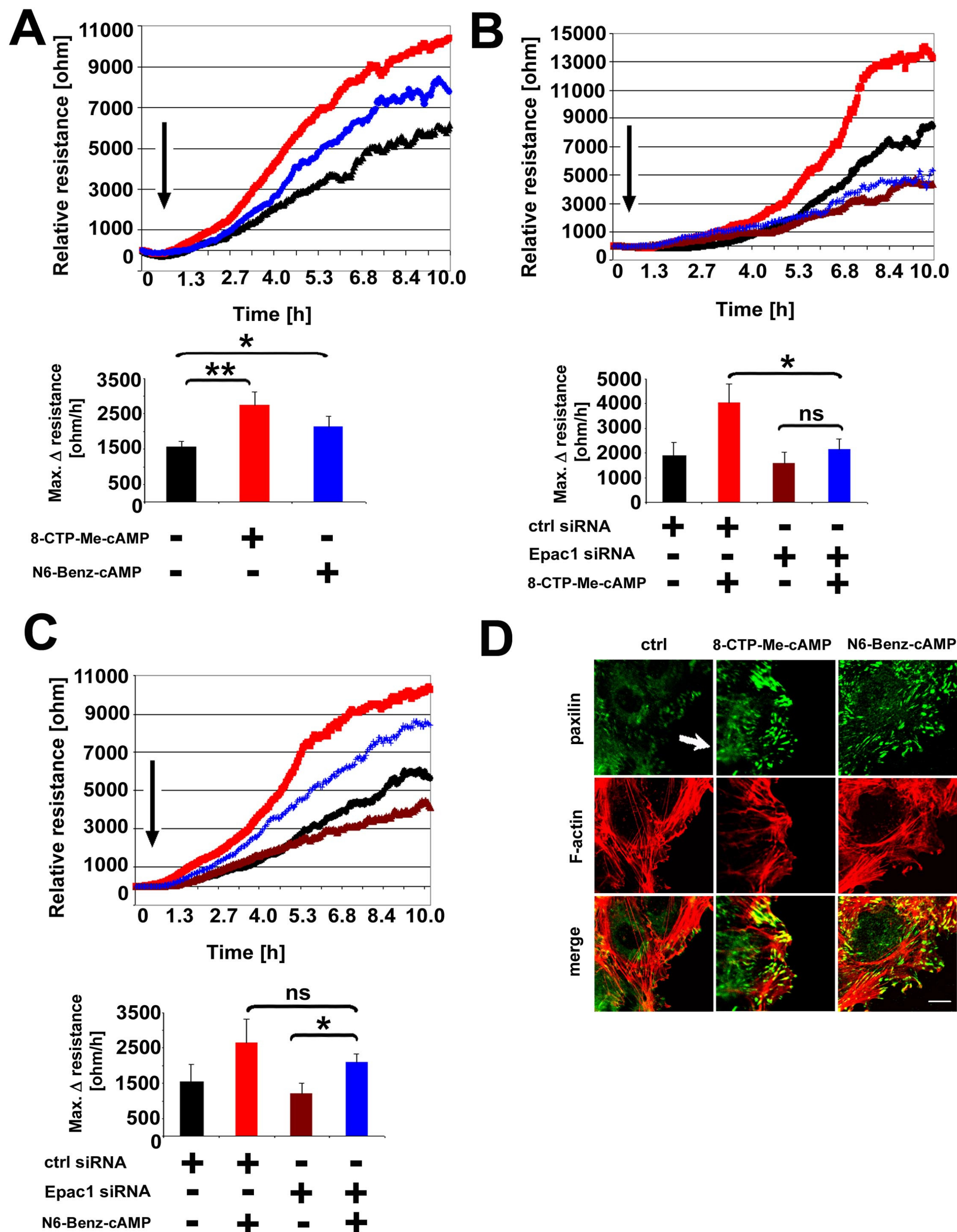


Figure 4. Epac1 and PKA regulate endothelial cell migration. HUVEC were plated on fibronectin-coated golden electrodes and grown to confluence. Thereafter, the cells were electrically wounded and the transendothelial resistance of recovering cell monolayer was measured in real time as described in Materials and Methods. **(A)** Cells were stimulated or not with 200 µM 8CTP-2'-O-Me-cAMP or 200 µM N6-Benzoyl-cAMP directly after the wounding (indicated by the arrow, upper panel). *Black triangle* – control; *red square-* 8CTP-2'-O-Me-cAMP; *blue circle-* N6-Benzoyl-cAMP. Representative results from at least 6 independent experiments are shown. Bar graph (lower panel) represents the maximal changes in the resistance. Data are means (+/- SEM) of 6

independent experiments performed in duplicate. *p<0.05; **p<0.005 **(B) and (C)** HUVEC were transfected with control siRNA or siRNA against Epac1, plated on fibronectin-coated golden electrodes and grown to confluence. Thereafter, the cells were electrically wounded and the transendothelial resistance of recovering cell monolayer was monitored. **(B)** Cells were stimulated or not with 200 µM 8CTP-2'-O-Me-cAMP at the time indicated by the arrow (upper panel). *Black circle* control siRNA; *red square-* control siRNA and 8CTP-2'-O-Me-cAMP; *brown triangle* - siRNA against Epac1; *blue cross* -siRNA against Epac1 and 8CTP-2'-O-Me-cAMP. Representative results from 6 independent experiments are shown. Bar graph (lower panel) represents the maximal changes in resistance. Data are means (+/- SEM) of 6 independent experiments performed in duplicate. *p<0.05. **(C)** Cells were stimulated or not with 200 µM N6-Benzoyl-cAMP at the time indicated by the arrow (upper panel). *Black circle* – control siRNA; *red square-* control siRNA and N6-Benzoyl-cAMP; *brown triangle* - siRNA against Epac1; *blue cross* -siRNA against Epac1 and N6-Benzoyl-cAMP. Representative results from 6 independent experiments are shown. Bar graph (lower panel) represents the maximal changes in resistance. Data are means (+/- SEM) of 6 independent experiments performed in duplicate. *p<0.05. **(D)** HUVEC were plated on fibronectin-coated glass coverslips and grown to confluence. Thereafter, the cells were wounded as described in Materials and Methods and stimulated with 200 µM 8CTP-2'-O-Me-cAMP or 200 µM N6-Benzoyl-cAMP for 30 minutes, fixed and stained for paxillin and F-actin. Images are representative of 3 independent experiments. White arrow indicates the direction of cell migration. Bars: 10 µm

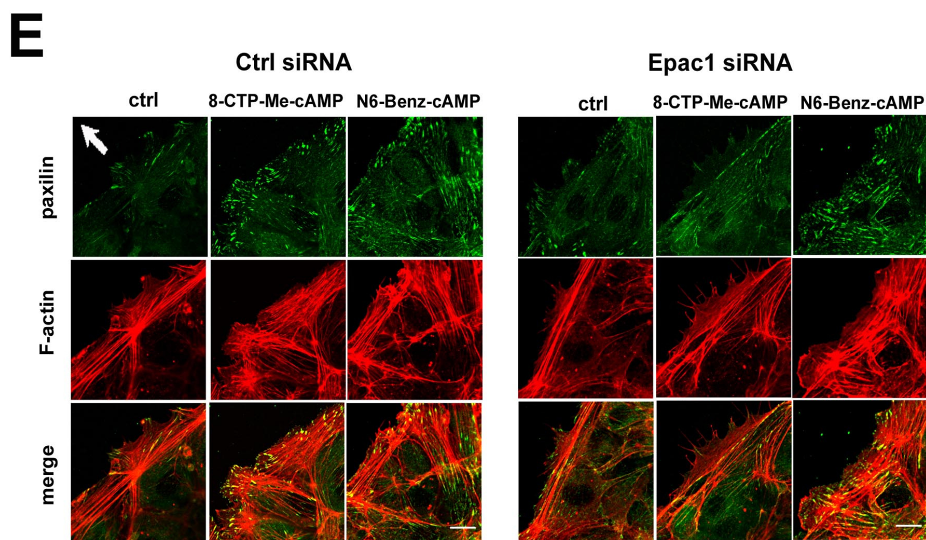


Figure 4. Epac1 and PKA regulate endothelial cell migration. HUVEC were plated on fibronectin-coated golden electrodes and grown to confluence. **(E)** HUVEC were transfected with control siRNA or siRNA against Epac1, plated on fibronectin-coated glass coverslips and grown to confluence. Thereafter, the cells were wounded as described in Materials and Methods and stimulated with 200 µM 8CTP-2'-O-Me-cAMP or 200 µM N6-Benzoyl-cAMP for 30 minutes, fixed and stained for paxillin and F-actin. Images are representative of 3 independent experiments. White arrow indicates the direction of cell migration. Bars: 10 µm

Epac1 and PKA regulate endothelial integrity through the engagement of integrins

Both PKA and Epac1 were previously shown to regulate integrin-mediated adhesion [32, 45-48]. Moreover, PKA was reported to be involved in integrin-dependent endothelial cell spreading and migration [29, 31]. Therefore, we addressed the question whether Epac1 and PKA control endothelial integrity in an integrin-dependent manner. To test this we plated cells on poly-L-lysine-coated electrodes to reduce integrin-mediated cell attachment. Next, we performed real time analysis of transendothelial resistance of monolayers stimulated with 8-CTP-2Me-cAMP or with N6-Benzoyl-cAMP. Surprisingly, only the activation of Epac1 resulted in increased transendothelial resistance (Figure 6A), similar to what we found in cells seeded on fibronectin (Figure 1A). N6-Benzoyl-cAMP failed to induce an increase in transendothelial resistance,, indicating that PKA regulation of endothelial integrity is dependent on integrin function.

Next, we studied the distribution of VE-cadherin in cells plated on poly-L-lysine and stimulated with 8-CTP-2Me-cAMP or with N6-Benzoyl-cAMP. Similar to endothelial monolayers plated on fibronectin, Epac1 activation resulted in increased levels of VE-cadherin at cell-cell junctions (Figure 6B). Moreover, plating the cells on poly-L-lysine did not prevent the increase in cortical actin induced by 8-CTP-2Me-cAMP (Figure 6B). In line with the results obtained from the real-time resistance measurements, N6-Benzoyl-cAMP was unable to induce an increase of VE-cadherin at cell-cell junctions in cells plated on poly-L-lysine (Figure 6B and Figure 1B), implicating again integrin engagement in PKA-mediated regulation of endothelial integrity.

We next explored the role of integrins in the Epac1- and PKA-mediated regulation of endothelial migration. We electrically wounded endothelial monolayers plated on poly-L-lysine-coated electrodes and followed wound closure in the presence/absence of 8-CTP-2Me-cAMP or N6-Benzoyl-cAMP. As shown in Figure 6C, the plating of cells on poly-L-lysine did not inhibit the capacity of the endothelial cells to close the wound, but blocked the stimulatory effect of both cAMP analogues on cell migration, previously observed in cells seeded on fibronectin (Figure 4A). Plating cells on poly-L-lysine inhibited the Epac1-mediated redistribution of paxillin to the leading edge as well as blocked a general increase in number and size of focal adhesion stimulated by PKA (Figure 6D and Figure 4D).

These results show that integrin-mediated adhesion is required for proper wound closure of endothelial cells, induced through Epac1 or PKA. Since integrin function is particularly relevant for PKA-, rather than for Epac1-, mediated increases in endothelial

monolayer integrity, these data dissociate the regulation of endothelial motility from that regulating the formation of strong cell-cell contacts.

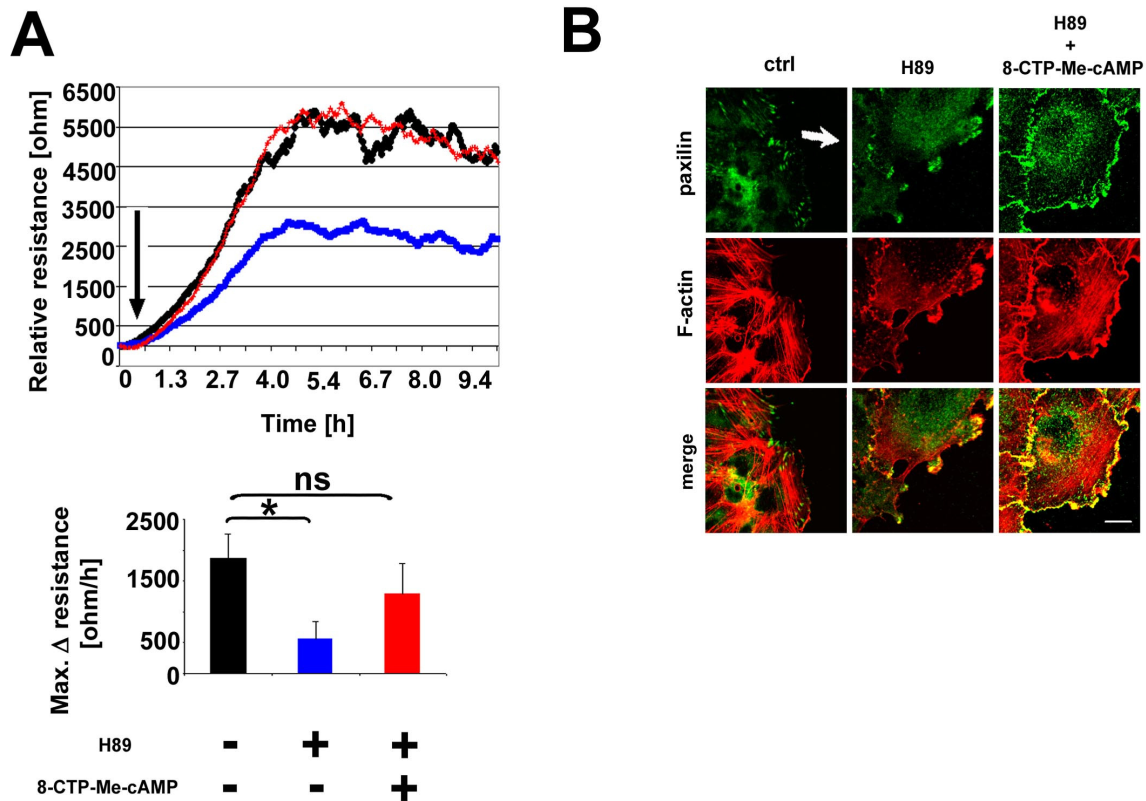


Figure 5. Epac1 and PKA regulate endothelial focal adhesions. **(A)** HUVEC were plated on fibronectin-coated golden electrodes and grown to confluence. Thereafter, the cells were electrically wounded and the transendothelial resistance of recovering cell monolayer was measured in real time as described in Materials and Methods. Wounded monolayers were treated or not with 10 µM H89 or a combination of 10 µM H89 and 200 µM 8CTP-2'-O-Me-cAMP (indicated by the arrow, upper panel). *Black circle* – control; *blue square*- H89; *red cross*- H89 and 8CTP-2'-O-Me-cAMP. Representative results from at least 5 independent experiments are shown. Bar graph (lower panel) represents the maximal changes in the resistance. Data are means (+/- SEM) of 5 independent experiments performed in duplicate. *p<0.05. **(B)** HUVEC were plated on fibronectin-coated glass coverslips and grown to confluence. Thereafter, the cells were wounded as described in Materials and Methods and treated with 10 µM H89 or a combination of 10 µM and 200 µM 8CTP-2'-O-Me-cAMP for 30 minutes, fixed and stained for paxillin and F-actin. Images are representative of 3 independent experiments. White arrow indicates the direction of cell migration. Bars: 10 µm

Discussion

This study was designed to define the effects and interdependence of the two major cAMP effectors in human endothelial cells, Epac1 and PKA, on endothelial integrity and endothelial cell migration. Our observations indicate that Epac1 and PKA act independently but not co-operatively, since the simultaneous stimulation of both Epac1 and PKA does not result in an additional increase of endothelial barrier function. The independence of these two pathways is underscored by our findings that in the absence of Epac1, the PKA pathway is still intact and that in the absence of PKA activity, the Epac1-Rap1 mediated increase in endothelial integrity was not reduced. Stimulation of either pathway results in cytoskeletal rearrangements. However, Epac1 induces cortical actin formation, while PKA stimulates stress fibre formation. Moreover, the PKA- but not the Epac1-mediated increase in barrier function was dependent on integrins, which further underscores the independence of Epac1 and PKA signalling.

It is not clear why a cell would require two parallel pathways to control the barrier function. PKA and Epac1 were reported to have the same affinity for cAMP [17, 49]. However, half-maximal activation of Epac1 occurs at about forty times higher concentrations of cAMP than required for PKA [50-52]. Thus, it may be that the different sensitivity of Epac1 and PKA for cAMP allows a cell to respond to a broad range of cAMP concentrations, generated by different stimuli. Another explanation could be that Epac1 and PKA pathways are differentially localized and are controlled by local generation of cAMP and the proximity of downstream targets [53-56].

The mechanism by which the Epac1-Rap1 and PKA pathways regulate endothelial integrity is not entirely clear. Cullere et al. concluded that Epac1-Rap1 as well as PKA acts by inhibiting thrombin-induced Rho activation [22]. At which point in the signaling cascade this inhibition occurs has not been determined. However, there is no direct evidence for inhibition of unstimulated Rho activity in endothelium by either PKA [15] or Epac1, suggesting that Rho might not necessarily be a direct target of either signaling pathway. We have seen clear cytoskeletal arrangements induced by PKA activation, i.e. formation of stress fibers, that do not suggest inhibition of Rho. This is in line with data from Whittard et al., who showed that integrin-induced PKA activation results in F-actin polymerization and integrin clustering, which in turn leads to increased cell-matrix and cell-cell adhesion [47]. Similarly, our analyses of transendothelial resistance support the role for integrins in the PKA- but not the

Epac1-mediated increase in endothelial barrier function. Conversely, in an earlier study, we found that raising cAMP by stimulating HUVEC with isoproterenol induced a loss of actin stress fibers, indicating that selective stimulation of PKA does not entirely mimic the effects of elevating intracellular cAMP [57].

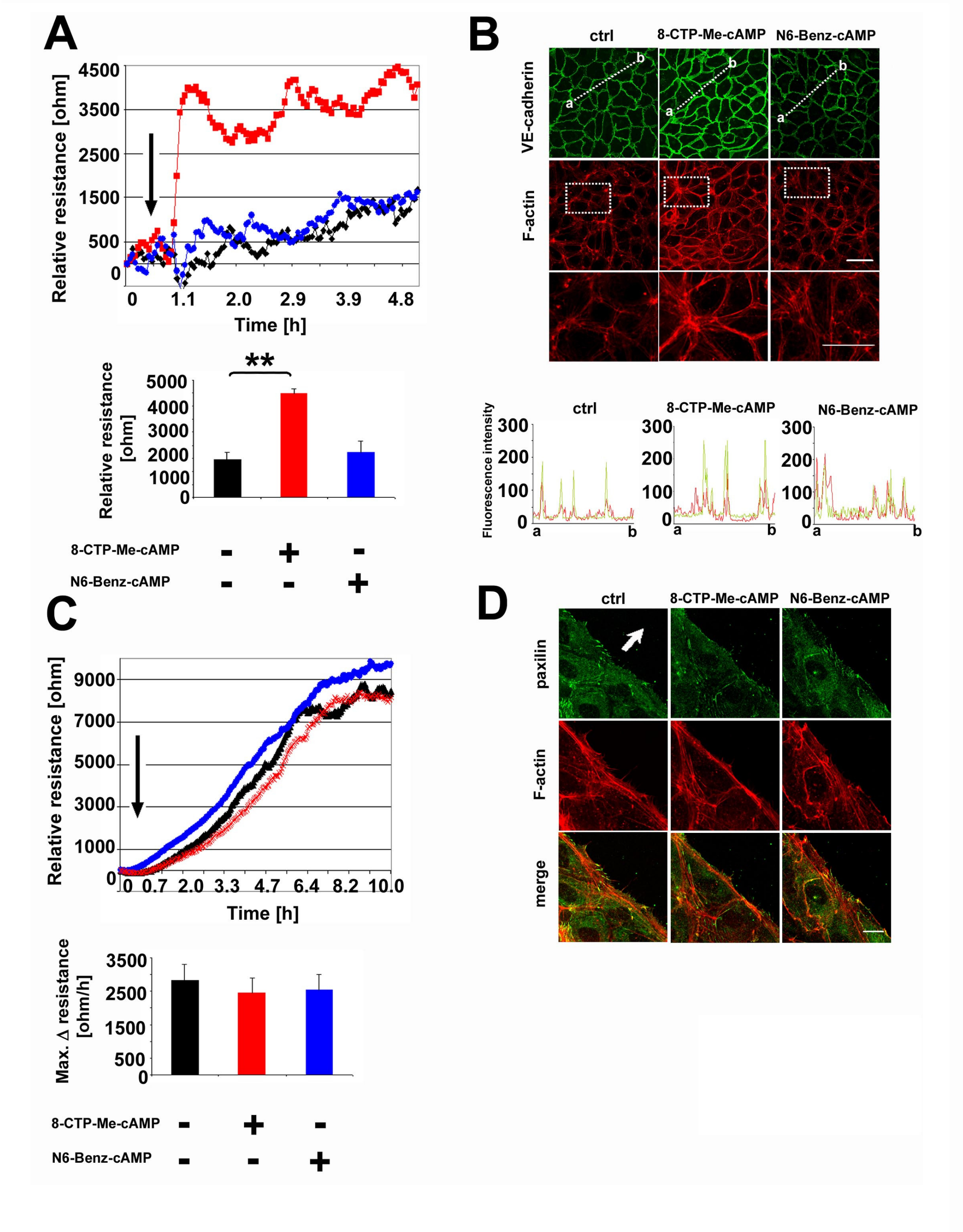


Figure 6. Epac1 and PKA regulate endothelial integrity through the engagement of integrins. **(A)** HUVEC were plated on poly-L-lysine-coated golden electrodes and grown to confluence. Transendothelial resistance was

measured in real time as described in Materials and Methods. Cells were stimulated or not with 200 µM 8CTP-2'-O-Me-cAMP or 200 µM N6-Benzoyl-cAMP at the time indicated by the arrow (upper panel). *Black diamond* –control; *red square-* 8CTP-2'-O-Me-cAMP; *blue circle-* N6-Benzoyl-cAMP. Representative results from at least 4 independent experiments are shown. Bar graph (lower panel) represents the changes in resistance at the 3-hours time point, when the resistance in most of the experiments reached a plateau. Data are means (+/- SEM) of 4 independent experiments performed in duplicate. **p<0.005 **(B)** HUVEC were plated on poly-L-lysine-coated glass coverslips and grown to confluence. Cell monolayers were treated or not with 200 µM 8CTP-2'-O-Me-cAMP or 200 µM N6-Benzoyl-cAMP for 30 minutes, fixed and stained for VE-cadherin and F-actin. Fluorescence intensity profiles along the indicated dashed line between points indicated as **a** and **b** are shown. Images are representative of 3 independent experiments. Bars: 50 µm **(C)** HUVEC were plated on fibronectin-coated golden electrodes and grown to confluence. Thereafter, the cells were electrically wounded and the transendothelial resistance of recovering cell monolayer was measured in real time as described in Materials and Methods. Wounded monolayers were stimulated or not with 200 µM 8CTP-2'-O-Me-cAMP or 200 µM N6-Benzoyl-cAMP directly after the wounding (indicated by the arrow, upper panel). *Black triangle* – control; *red cross-* 8CTP-2'-O-Me-cAMP; *blue circle-* N6-Benzoyl-cAMP. Representative results of 3 independent experiments are shown. Bar graph (lower panel) represents the maximal resistance changes. Data are means (+/- SEM) of 3 independent experiments. **(D)** HUVEC were plated on poly-L-lysine-coated glass coverslips and grown to confluence. Thereafter, the cells were wounded as described in Materials and Methods and stimulated with 200 µM 8CTP-2'-O-Me-cAMP or 200 µM N6-Benzoyl-cAMP for 30 minutes, fixed and stained for paxillin and F-actin. Images are representative of 3 independent experiments. White arrow indicates the direction of cell migration. Bars:10 µm

PKA promotes barrier function primarily in macrovascular, but not in microvascular cells [58]. This has been linked to a reduction of myosin light chain phosphorylation and a reduction of isometric tension in these cells. Our data suggest that, in addition, redistribution of junctional proteins also underlies the stimulatory effects of PKA. Others have also reported that cAMP promotes HUVEC spreading, which would be in line with the increased barrier function that we observed [29].

The downstream effector(s) of the Epac1-Rap1 pathway that mediate the increase in TER are presently undefined. The tight junction protein AF-6 has been implicated in the control of endothelial barrier function through Rap1 [22], but definitive proof for this notion is currently lacking. The Rap1 effector Riam has been shown to promote integrin-mediated adhesion and cell spreading in T cells as well as in HEK293 cells [59, 60]. However, the Epac1-Rap1-mediated increase in endothelial integrity appears not to be critically dependent on integrin-mediated adhesion. Another Rap1 effector, RapL, has recently been implicated in localized Rap1 activation and directional migration in endothelial cells [33]. Selective single

or double knockouts of these effectors could answer the missing link of the pathway. Although Epac1-induced formation of cortical actin and PKA-mediated stress fiber formation strongly indicate that cytoskeletal rearrangements are central to the effects on endothelial barrier function, it cannot be excluded that Epac1 and PKA promote integrity by stimulating recruitment of VE-cadherin to cell-cell junctions through vesicle trafficking.

Our data demonstrate that Epac1 and PKA also control restoration of endothelial integrity (wound closure) through separate signaling pathways. However, the mechanism by which PKA and Epac1 regulate endothelial wound closure appears different from that controlling tight cell-cell adhesion. Our data indicate that both PKA and Epac1-induced endothelial cell migration requires integrin-mediated adhesion. This is in line with previous reports pointing out the role for Epac1 in the control of ovarian carcinoma cell adhesion and spreading [45, 46] and in the agreement with reports showing the importance of PKA for integrin-mediated endothelial cell migration [29, 31, 32]. Our experiments suggest that PKA and Epac1 regulate endothelial cell motility through reorganization of focal adhesions. Epac1 appears important for targeting of focal adhesions to the leading edge of migrating cells, while PKA is necessary for organization of focal complexes, since simultaneous activation of Epac1 and inhibition of PKA results in a continuous rather than punctuate distribution of paxillin at the leading edge (Figure 5B). This is in line with a study by Goldfinger et al. showing that PKA regulates interaction between α4-integrin and paxillin at focal contacts, modulating α4-dependent cell migration [61]. Importantly, activated Rap1 was shown to localize to the leading edge of migrating endothelial cells and to regulate endothelial cell migration [33]. Moreover, Rap1 was reported to be an important regulator of focal adhesions in fibroblasts [62, 63], supporting a mechanism in which Epac1 regulates cell migration through focal adhesion reorganization.

Taken together, our data support a model for cAMP–induced endothelial integrity, in which PKA and Epac1 act through independent signalling pathways to control endothelial barrier function and endothelial cell migration. This study may contribute to further understanding the regulation of complex cAMP signaling in vascular endothelium and may open possibilities for new anti-inflammatory therapies.

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CHAPTER IV

ACTIVATION OF EPAC1 RESCUES AF6 KNOCK-DOWN-INDUCED ELECTRICAL RESISTANCE DEFECT IN ENDOTHELIAL CELL-CELL JUNCTIONS

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Abstract

Regulation of endothelial cell-cell contacts is essential for the integrity of the endothelium. We showed previously that Rap1 and its guanine-nucleotide exchange factor Epac1 regulate endothelial cell-cell junctions, resulting in increased endothelial integrity. Here, we studied the role of the Rap1-binding protein AF6 in this process. AF6 is a junction protein that can bind to active Rap1 and Ras and is required for proper cell-cell junctions in epithelial cells. We found that knock-down of AF6 in Human Umbilical Vein Endothelial Cells (HUVEC) had no effect on the recruitment of VE-cadherin, JAM-A and ZO-1 to the junctions, but resulted in a reorganization of the cytoskeleton. This reorganization was also observed in single cells, indicating that the effect of AF6 on actin is independent of the formation of cell-cell junctions. AF6 knock down also resulted in a decreased trans-endothelial resistance, showing that also in endothelial cells AF6 is involved in the formation of cell-cell junctions. However, AF6 knock-down did not affect Epac1-mediated increase in endothelial resistance, suggesting that activation of Rap1 can overcome a defect in AF6. We conclude that AF6 is involved in the regulation of endothelial cell-cell junction formation and that the AF6 knock-down-induced electrical resistance defect of endothelial cell-cell junctions can be rescued by activation of Epac1. This suggests that AF6 operates in a pathway parallel to Rap1 in the control of endothelial integrity.

Introduction

The small GTPase Rap1 is a member of the Ras family of GTPases, which plays a role in among others the control of integrin-mediated cell adhesion and cadherin-mediated cell junction formation. Rap1 cycles between an active GTP-bound and an inactive GDP-bound form. Activation of Rap1 is induced by guanine-nucleotide exchange factors (GEF), wheras GTPase-activating proteins (GAP) enhance the hydrolysis of GTP into GDP [1]. Recently one of the GEFs for Rap1, Epac1, was found to be an effector for cAMP–mediated control of the endothelial cell-cell junction formation. [2, 3]. Activation of Rap1 with the Epac1-specific cAMP-analog 8-pCPT-2O-Me-cAMP (007), resulted in an increase in resistance of the endothelial monolayer, indicating that the monolayer had reduced permeability for fluids. This coincided with increased adherens junction maturation as well as increased cortical actin formation [4-6]. Although the increased endothelial integrity depends on VE-cadherin, the reorganization of the actin cytoskeleton in HUVEC by 007 was independent of cell-cell

contacts [4, 7]. Also the transendothelial migration of HL-60 cells was inhibited by the increased junction formation upon activation of Rap1 [6]. However, a mechanism for the increase in endothelial resistance by Rap1 remained elusive.

An interesting candidate that could mediate Rap1-mediated junction formation is AF6/Afadin. AF6/Afadin is localized at cell-cell contacts of endothelial and epithelial cells and binds various junctional proteins, such as JAM-A, and ZO-1 and nectins [8-10] as well as the regulator of the actin cytoskeleton profilin [11]. Indeed, evidence is accumulating that AF6 plays an important role in the formation of cell-cell junctions. For instance, although E-cadherin and ZO-1 localize normally to the junctions in the intestine of AF6 knock-out mice, the integrity of the intestinal epithelium is affected [12] and knock-down of AF6 in epithelial cells results in loss of tight junctions [13]. The first Ras-Associating (RA)-domain of AF6 has been shown to bind to the effector region of both active Ras and Rap, marking AF6 as a putative target protein [11, 14]. However, most evidence indicates that AF6 is most likely an effector of Rap1. For instance, in *Drosophila melanogaster*, both Rap1 and the AF6 homolog canoe are required for dorsal closure of the embryo. Furthermore, active Rap1 could not rescue the phenotype of a canoe loss-of-function, suggesting that canoe is located downstream of Rap1 [15]. More recently, it was found that inhibition of endocytosis of E-cadherin *in vitro* by dominant-negative Rap1 can be rescued by a mutant of AF6 lacking the Rap1-binding domains but not by full-length AF6, suggesting that AF6 also functions as effector of Rap1 in mammalian epithelial cells [16]. AF6 also binds to actin and to the actin-binding protein profiling [11]. For instance, it was shown that the actin-binding domain of AF6, is required for its role in cell-cell junction formation and migration of epithelial cells [17]. Whether AF6 plays a role in endothelial cell-cell junction formation is currently unclear. Finally, AF6 also plays a role in the regulation of Rap1 activity. Indeed, AF6 binds Rap1GAP1 and the RapGAP Spa-1 to negatively control Rap1 activity, but binding of AF6 also protects Rap1 from GAP-induced GTP hydrolysis [18]

Here we investigated the role of AF6/Afadin in Epac1-induced junction enhancement in endothelial cells, using real-time analysis of endothelial resistance. We find that reduced levels of AF6 in endothelial cells result in decreased endothelial resistance. Although the constitution of the endothelial junctions is normal, we observed changes in the actin cytoskeleton. Finally we show that activation of Rap1 in endothelial cells rescued the reduction in endothelial resistance to basal values. Together, these data suggest that AF6 is required for the integrity of the endothelial monolayer and that activation of Epac1 can overcome this defect.

Materials and Methods

Cell culture

HUVEC were isolated and cultured in EBM-2 Bulletkit culture medium (EBM-2 supplemented with EGM-2 SingleQuots (hEGF, hydrocortisone, fetal bovine serum, VEGF, hFGF-B, R3-IGF-1, Ascorbic Acid, GA-1000, heparin) (Clonetics) as previously described [19, 20]. Second to fourth passage cells were used. Cells were cultured on 1% gelatin (Sigma) and experiments were performed on 7 µg/ml fibronectin (Sigma). 18 hours after plating HUVEC were transfected with siRNA oligos (Dharmacon) against AF6 (#1: GAAUAUAGUGAACCAAAGA; #2: UGAGAAACCUCUAGUUGUA) or Epac1 (GCACCUACGUCUGCAACAA) as indicated, with Oligofectamine (Invitrogen) according to the manufacturers' recommendation. Transfection was repeated after 24 hours. Cells were replated 24 hours later and analyzed 48 hours after the second round of transfection, unless stated differently.

Antibodies

A Mouse mAb against the extracellular domain of human VE-cadherin (clone TEA 1.31) was used for immunostainings [21, 22]. Rap1 polyclonal antibodies (Santa Cruz), JAM-A (Abcam), AF6 monoclonal antibody (Transduction labs), ZO-1 polyclonal antibodies (Transduction labs) were used for immunostainings and Western Blotting.

Immunofluorescence

Cells were replated 24 hrs after transfection on fibronectin-coated (7 µg/ml) glass coverslips (12 mm) for 24 hrs. After stimulation, cells were fixed with 3.8% formaldehyde, permeabilised with 0,2 % Triton X-100 and blocked with 2% BSA in PBS. Cells were incubated with indicated antibodies for 1 hour and subsequently incubated with Alexa-568-labeled phalloidin to stain F-actin and/or Alexa-488/568-labeled secondary antibodies (Molecular Probes). After mounting the coverslips onto slides, cells were examined using a Axiokop2 microscope (Zeiss).

Transendothelial electrical resistance measurement

Unless otherwise stated, HUVEC were seeded at $1.5x10^5$ cells per well ($0.8\ cm^2$) on fibronectin-coated electrode arrays and grown to confluency. Measurements of

transendothelial electrical resistance were performed in real time at 4000Hz, 37ºC, 5% CO_2, using an electrical cell-substrate impedance sensing system (ECIS; Applied Biophysics Inc.Troy. NY, USA) [23].

Results

Knock-down of AF6 affects the actin cytoskeleton independently of cell-cell junction formation

To investigate the role of AF6 in Epac1-mediated junction formation in endothelial cells, we used siRNA to knock down AF6 in human umbilical vein endothelial cells (HUVEC).

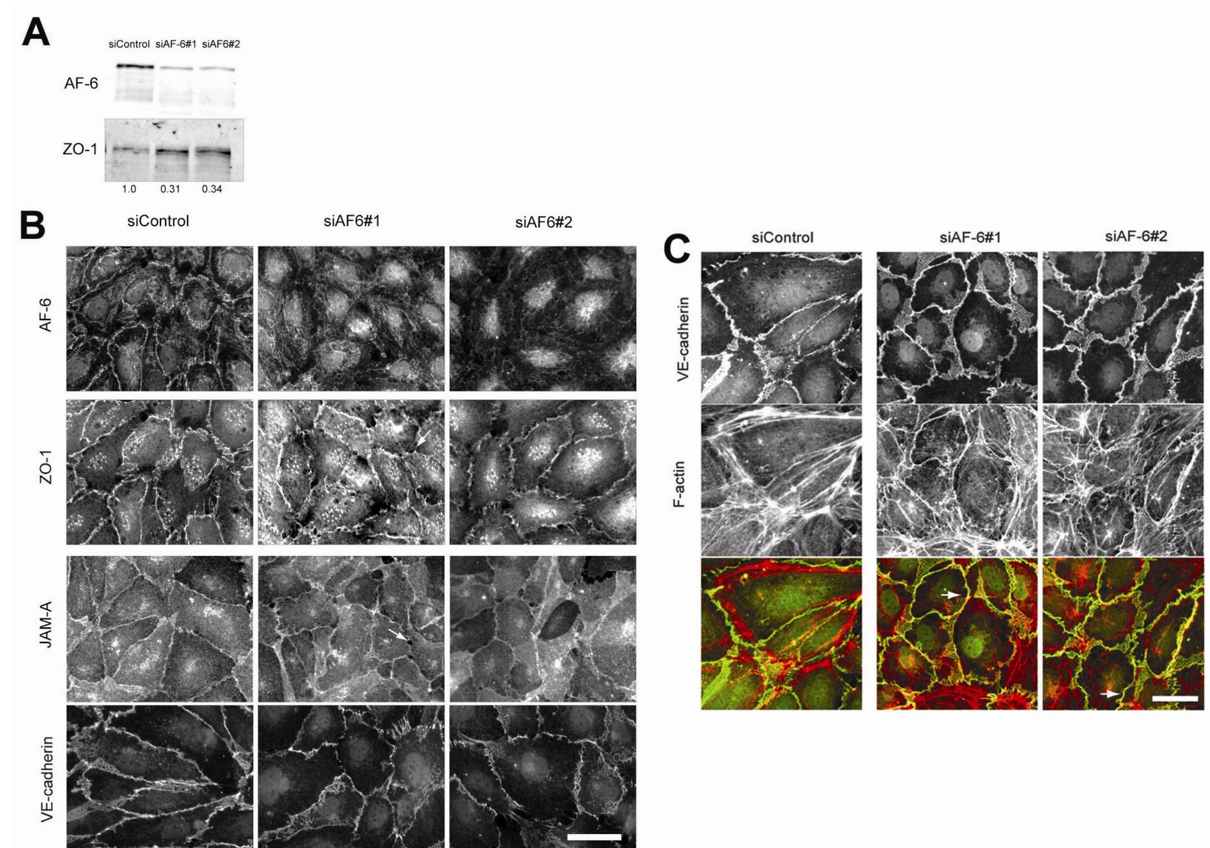

Figure 1. AF6 regulates endothelial integrity. (A) AF6 or ZO-1 expression levels after transfection of HUVEC with siRNA oligo's for AF6. Quantifications for AF6 are depicted relative to control; (B) HUVEC transfected with siRNA were immunostained as indicated. Bar = 25 μm; (C) Transfected cells were stained for VE-cadherin and F-actin. The merge is shown in the lower panel. Arrows indicate cortical actin; Bar = 25 μm.

With 2 different siRNAs targeting different regions of the protein, AF6 expression was reduced to approximately 30% as monitored by Western blotting. (Figure 1a). Also in immunofluorescence staining, AF6, particularly at the cell-cell junctions, was strongly reduced by both siRNAs. Importantly, knock-down of AF6 did not affect cell-cell junction formation and localization of junction proteins VE-cadherin, ZO-1, and JAM-A (Figure 1b). However, cells with reduced AF6 levels showed a reorganization of the actin cytoskeleton compared to the controls. There appeared to be a reduction in the amount of actin stress fibers and cellular contractility and an accumulation of cortical actin (Figure 1c, arrows). These observations suggest that AF6 is not required for the proper localization of junctional adhesion molecules, but appears to be involved in the organization of the actin cytoskeleton.

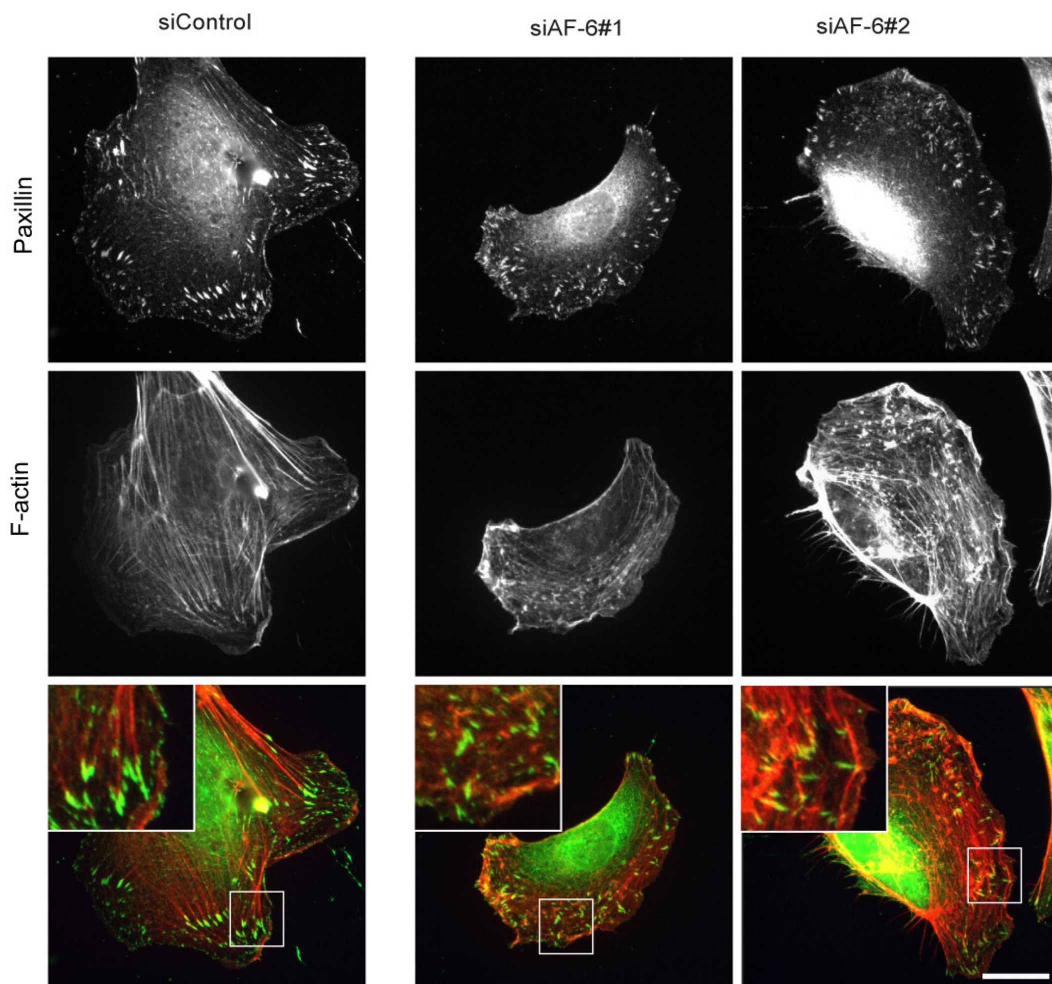

Figure 2. Organization of the actin cytoskeleton requires AF6. HUVEC were seeded sparsely for 16 hours, fixed and stained for paxillin and F-actin. Bar = 10 µm.

To exclude that this effect of AF6 knock-down on the actin cytoskeleton was dependent on cell-cell contact we analyzed the effect on single cells. We observed that similar to endothelial cells in a confluent layer knock-down of AF6 reduced the amount of stress

fibres and induced an accumulation of actin at the edges of the cells. When co-stained with paxilin to mark focal contacts, we found that in AF6 knock-down cells the focal contacts were smaller, but had increased F-actin content (Figure 2). From these results we conclude that the effect of AF6 knock-down on the actin cytoskeleton is independent of cell-cell junction formation.

Knock-down of AF6 reduces electrical resistance of junctions

Although knock-down of AF6 had no apparent effect on the formation of cell-cell junctions, it may affect their functionality as a barrier for trans-endothelial migration. This barrier function can be measured with the Electrical Cell Impedence System (ECIS) [23]. This system measures the electrical resistance of the endothelial layer at low frequency, which is a measure of the integrity of the junctions. We observed that knock-down of AF6 in a confluent monolayer of endothelial cells with two different siRNAs reduced the electrical resistance by 40% (Figure 3). This indicates that AF6 plays a role in the integrity of cell junctions.

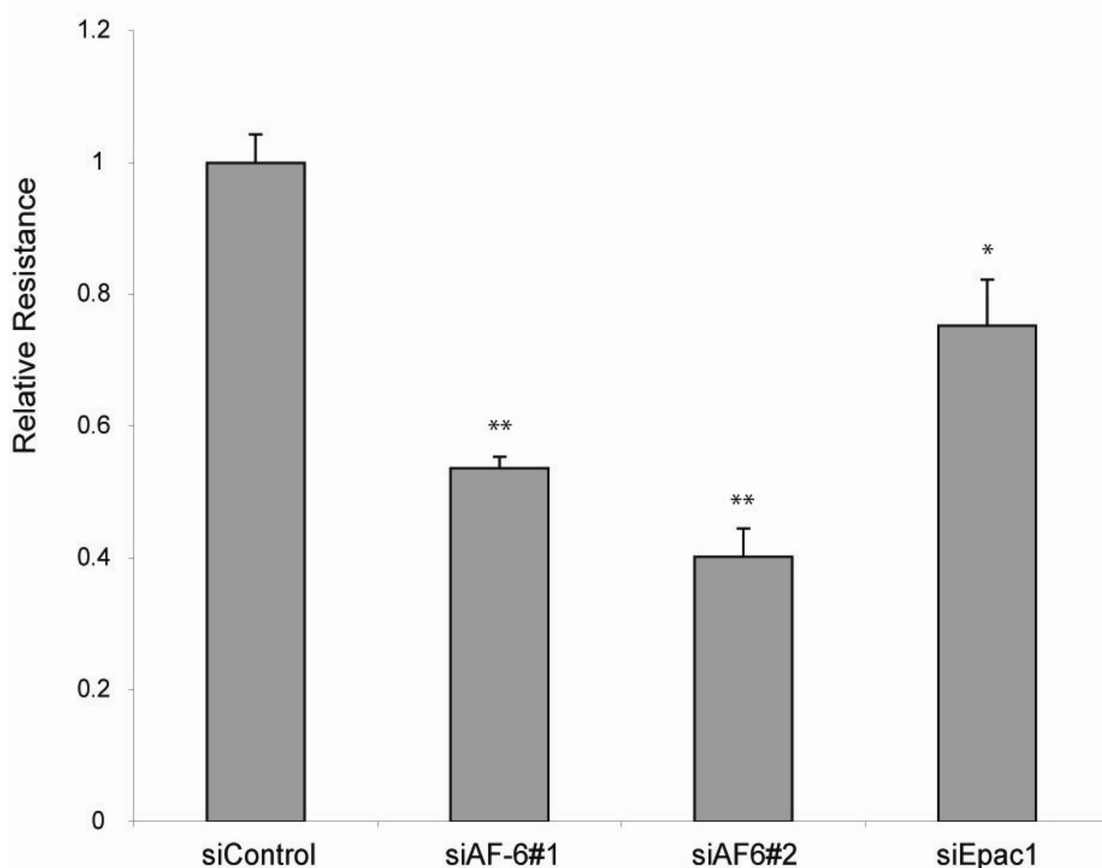

Figure 3. Knock-down of AF6 reduces endothelial resistance. HUVEC were transfected with siRNA oligos as indicated. Basal transendothelial resistance was measured at 4000Hz under confluent conditions 14 hours after plating. Significant differences from the control determined by Student's *t test* are indicated by a single asterisk ($p<0.01$) or double asterisk ($p<0.0001$).

Epac1 activation restores endothelial integrity in AF6 knock-down cells

cAMP is a well-known inducer of the integrity of endothelial cell-cell junctions, and recently a number of groups including ours, has shown that this cAMP effect is in part mediated by Epac1 and can be mimicked by the Epac1-selective analog of cAMP, 8pCPT-2'OMe-cAMP. This effect is mediated by Rap1, and we reasoned that if AF6 is indeed an effector of Rap1 in the induction of endothelial integrity, knock-down of AF6 should inhibit or at least reduce the 8pCPT-2'OMe-cAMP-induced increase in electrical resistance. Indeed, as shown previously (M.J. Lorenowicz et al., unpublished) 8pCPT-2'OMe-cAMP rapidly and strongly increased electrical resistance of the endothelial monolayer, which is sensitive to siRNA of Epac1 (Figure 4a). Importantly, although the electrical resistance of AF6-knock-down cells is reduced, the increase in electrical resistance by 8pCPT-2'OMe-cAMP was not affected (Figure 4a, lower panel). Similarly, 8pCPT-2'OMe-cAMP reduced irregularities in cell junctions as observed after staining for VE-cadherin (Figure 4b). This suggests that either AF6 is not involved in 8pCPT-2'OMe-cAMP-induced endothelial integrity, or at least that AF6 is not a rate-limiting step in this process, in contrast to the effect of AF6 knock-down on basal junction integrity.

Previously, we have also shown that 8pCPT-2'OMe-cAMP induces the formation of cortical actin, both in a monolayer of endothelial cells and in single cells. Knock-down of AF6 did not alter this effect of 8pCPT-2'OMe-cAMP (Figure 4b). From these results we conclude that activation of Epac1 by 8pCPT-2'OMe-cAMP still induces formation of cortical actin in AF6-knockdown cells.

Discussion

We have shown that knock-down of AF6 to about 30% of its original level in endothelial cells has no effect on the formation of cell-cell junctions, as demonstrated by the normal staining of VE-cadherin, ZO-1 and JAM-A, but clearly affects the integrity of the junctions as demonstrated by the reduced electrical resistance of the endothelial monolayer. AF6 knock down also clearly affects the actin cytoskeleton, by reducing the formation of thick stress fibers and the induction of cortical actin formation. However, AF6 knock down did not affect the ability of the Epac1 agonist 8pCPT-2'OMe-cAMP to increase endothelial resistance and to increase cortical actin formation.

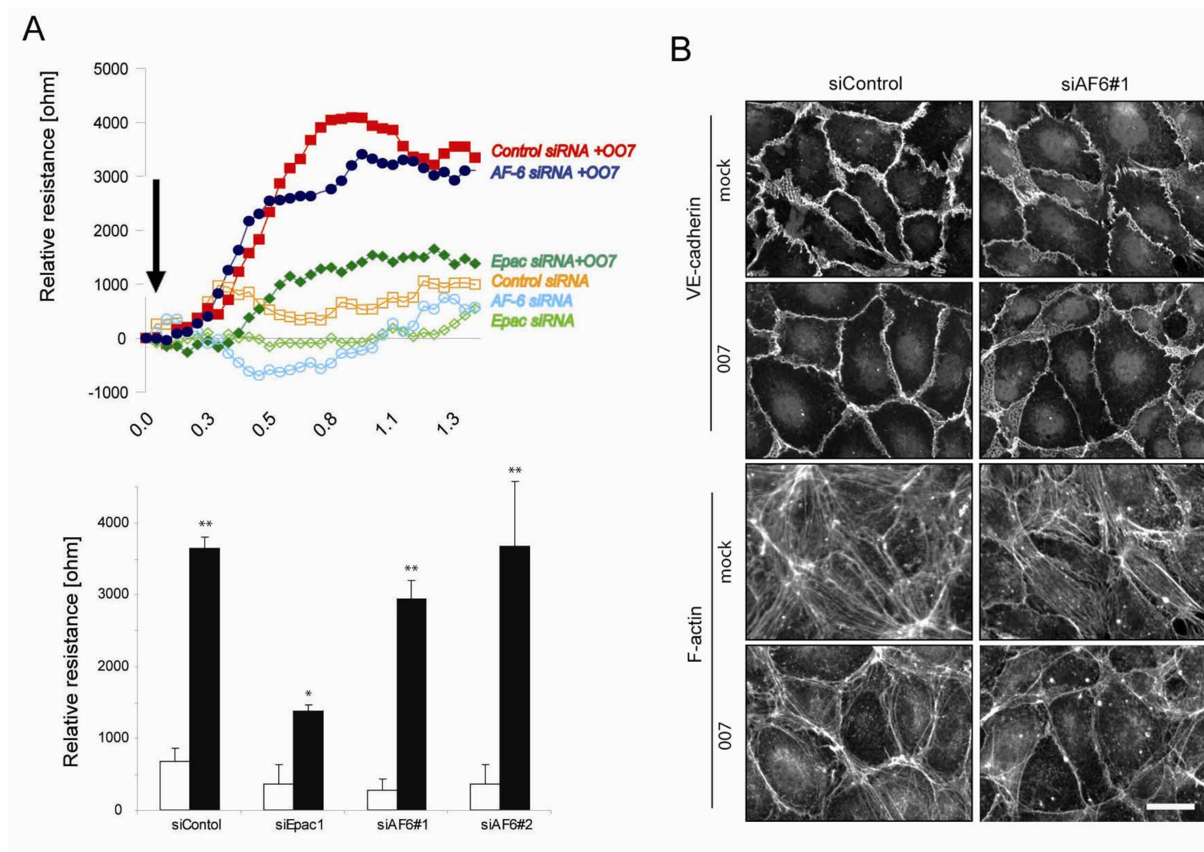

Figure 4. Activation of Rap1 increases endothelial integrity independent of AF6. (A) Transendothelial resistance of HUVEC monolayers on fibronectin-coated gold electrodes was measured. Cells were stimulated with vehicle (open symbols) of 100 µM 8pCPT-2'OMe-cAMP (007; closed symbols) at the indicated timepoint (arrow); ■- siControl; ▲- siEpac1; ●- siAF6#1; Bar-graph (lower panel) represents the average of three independent experiments at 1.5 hours after treatment with vehicle (white bars) or 007 (closed bars). *p<0.05, **p< 0.01; (B) HUVEC were transfected with AF6 or control siRNA and 48 hours later treated with or without 007 for 30 minutes, fixed and stained for VE-cadherin and F-actin. Bar = 25 µm.

These results clearly establish a role for AF6 in the control of endothelial cell-cell junctions. Previously, such a role had been shown for epithelial cells, both in the intestine of mice and in polarized MDCK cells. In MDCK cells, knock-down of AF6 affects the localization of tight junction proteins like JAM-A. We did not observe this effect in endothelial cells, but that may be caused by insufficient knock-down of AF6. The most striking effect of knock-down of AF6 was on the actin cytoskeleton and endothelial resistance. Since endothelial integrity is regulated by the actin cytoskeleton [24], it is plausible to assume that the effect of AF6-knock-down on the actin cytoskeleton is causative to the reduction of electrical resistance. However, we cannot exclude that AF6 knock down affects both processes independently.

Previously it has been shown that cAMP reduces the permeability of an endothelial cell layer in part through Epac1, and that this effect can be mimicked by the Epac1-selective analog of cAMP, 8pCPT-2'OMe-cAMP. This effect is mediated by Rap1, but the effectors of Rap1 for this effect are currently elusive. However, several observations suggest that AF6 may be involved in this process. First, AF6 has an RA domain, which can bind Rap1 in vitro and in vivo. Secondly, in *Drosophila melanogaster*, both Rap1 and the AF6 homolog, canoe, are required for dorsal closure of the embryo. Furthermore, active Rap1 could not rescue the phenotype of a canoe loss-of-function, suggesting that canoe is located downstream of Rap1 [15]. Thirdly, inhibition of endocytosis of E-cadherin *in vitro* by dominant-negative Rap1 can be rescued by a mutant of AF6 lacking the Rap1-binding domains, but not by full length AF6 [16]. Our results now show that knock-down of AF6 does not abolish the induction of electrical resistance by 8pCPT-2'OMe-cAMP nor does it inhibit the effect of 8pCPT-2'OMe-cAMP on the formation of cortical actin. However, the level of electrical resistance of 8pCPT-2'OMe-cAMP-treated cells was reduced in AF6 kncopk-down cells compared to control cells, suggesting that AF6 and Rap1 regulate independent additive effects on cell-cell junction integrity. Our observations suggest that AF6 does not function as an effector of Rap1 in endothelial junction formation, but operates in parallel. However, we can not exclude that the reduced level of AF6 in the knock-down cells is sufficient to mediate the effect of 8pCPT-2'OMe-cAMP.

Rap1 could act at various stages during junction formation. A first step may be the transient binding of a rap GEF, C3G, to cadherins during junction formation [3]. Similarly, Sakurai and colleagues have shown that Rap1 is activated during junction formation in a MAGI-1 dependent-manner in endothelial cells [25]. MAGI-1 is a scaffold protein that directly binds to PDZ-GEF. Finally, Epac1 can increase the junctional integrity of confluent endothelial monolayers [4, 7]. Thus AF6 is clearly involved in the regulation of junctional integrity, most likely in a pathway parallel to Rap1.

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CHAPTER V

MICROTUBULE DYNAMICS AND RAC1 SIGNALING INDEPENDENTLY REGULATE BARRIER FUNCTION IN LUNG EPITHELIAL CELLS

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Abstract

Cadherin-mediated cell-cell adhesion controls the morphology and function of epithelial cells and is a critical component of the pathology of chronic inflammatory disorders. Dynamic interactions between cadherins and the actin cytoskeleton are required for stable cell-cell contact. Besides actin, also microtubules target intercellular, cadherin-based junctions and contribute to their formation and stability. Here, we studied the role of microtubules in conjunction with Rho-like GTPases in the regulation of lung epithelial barrier function using real-time monitoring of transepithelial electrical resistance. Unexpectedly, we found that disruption of microtubules promotes epithelial cell-cell adhesion. This increase in epithelial barrier function is accompanied by the accumulation of β-catenin at cell-cell junctions. Moreover, we found that the increase in cell-cell contact, induced by microtubule depolymerization, requires signaling through a RhoA/Rho kinase pathway. The Rac1 GTPase counteracts this pathway, because inhibition of Rac1 signaling rapidly promotes epithelial barrier function, in a microtubule- and RhoA-independent fashion. Together, our data suggest that microtubule-RhoA-mediated signaling and Rac1 control lung epithelial integrity through counteracting, independent pathways.

Introduction

Epithelia form tightly regulated barriers that protect the body from the external environment. Loss of epithelial integrity greatly increases the risk of microbial infection and is often a hallmark of chronic (lung) inflammatory disorders such as asthma or COPD (Chronic Obstructive Pulmonary Disease). In polarized epithelium, adhesion between adjacent cells is mediated by various types of intercellular junctions, such as tight junctions (TJs), adherens junctions (AJs) and desmosomes [1]. AJs are characterized by the presence of E-cadherin, a transmembrane adhesion molecule that mediates Ca^{2+}-dependent homophilic binding through its extracellular domain. The intracellular domain of E-cadherin interacts with β/γ- and α-catenins, that link E-cadherin with the actin cytoskeleton [2,3].

The members of the Rho family of small GTPases are key regulators of the actin cytoskeleton and are involved in the formation, maintenance and breakdown of AJs. Activation of Rac1 is associated with increased cell-cell adhesion, and a GEF for Rac1, Tiam1, promotes cadherin-based cell-cell adhesion [4-7]. In contrast, activation of Rac1 is also associated with disassembly of cell-cell contacts [8-10]. Similar to Rac1, RhoA both promotes and inhibits cell-cell contact. Inhibition of RhoA with C3 toxin dislocalizes E-cadherin from

cell-cell junctions [11], and several effectors of activated RhoA, e.g. Dia1 and Dia2, induce epithelial cell-cell adhesion [12]. In contrast, overexpression of constitutively active RhoA or Rho kinase (a RhoA effector kinase) stimulates stress fiber formation and contractility, resulting in loss of cell-cell contact [13,14]. Thus, Rho-like GTPases play divergent roles in the control of cadherin-mediated intercellular contacts.

Microtubule (MT) dynamics regulates intercellular, cadherin-mediated adhesion in endothelial and epithelial cells [15-17]. MTs are polarized polymers of α- and β- tubulin dimers, which show fast growth at their plus ends and slow growth at their minus ends [18]. In polarized epithelial cells, MT minus ends orient toward the apical part of the cell and the plus ends to the basal part of the cell [19]. MT disassembly disrupts TJs in thyroid epithelial cells and decreases endothelial barrier function [20-22]. Conversely, MT stabilization counteracts thrombin-induced endothelial permeability [23], whereas formation of AJ by expression of E- and N-cadherin in fibroblasts results in the stabilization of microtubule minus ends [24]. Thus, the role of MTs in the control of cell-cell adhesion differs, possibly as a function of cell type.

Here, we studied the role of MT dynamics in conjunction with the Rho GTPases Rac1 and RhoA in the regulation of lung epithelial barrier function. We show that MT depolymerization promotes transepithelial resistance in a RhoA-dependent fashion in lung epithelial cells. In addition, we found that inhibition of Rac1 rapidly promotes transepithelial resistance, through a MT- and RhoA-independent pathway. Our findings show that lung epithelial barrier function is controlled by microtubule dynamics in conjunction with counteracting Rac1 and RhoA-driven signaling.

Materials and Methods

Reagents

Nocodazole, taxol and cytochalasin B were purchased from Sigma. Y27632 and ML-7 were from Biomol (UK). Tat-ctrl, Tat-Rac1,Tat-Rac2, Tat-CDC42 and Tat-RhoA C-terminal peptides as well as TAT-CRIB and Tat-17-32 have been described elsewhere [25-28]. Mouse monoclonal antibodies to tyrosinated- (clone TUB-1A2) and acetylated- (clone 6-11B-1) tubulin were from Sigma. Mouse monoclonal antibody to β-catenin was purchased from Transduction Laboratories.

Cell culture

All cell lines were purchased from ATCC (Manassas, VA, USA) and were cultured at 37ºC and 5% CO_2. H292 cells (bronchial epithelial cell line) and A549 cells (alveolar epithelial cell line) were maintained in RPMI 1640 medium (GIBCO) containing 10% heat-inactivated FCS (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. MDCK (Madin Darby Canine Kidney) cells were cultured in IMDM medium (BioWhittaker, Brussels, Belgium) containing 10% heat-inactivated FCS (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Transepithelial electrical resistance measurement

Cells were seeded at $3x10^5$ cells per well (0.8 cm^2) on fibronectin-coated electrode arrays (Applied Biophysics Inc) and grown to confluency. Measurements of transepithelial electrical resistance were performed in real time by means of an electrical cell-substrate impedance sensing system (ECIS, Applied Biophysics Inc.) at 37ºC, 5% CO_2 [29]. Briefly, the small measuring electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier, and a 1 V, 4000 Hz signal was supplied by a 1-MΩ resistor to approximate a constant current source of 1μA. The in-phase and out-phase voltages between the electrodes were monitored in real-time, followed by conversion to scalar measurements of transepithelial impedance. The indicated relative electrical resistance values were obtained by subtracting the resistance at t=0 from every resistance value in the successive time points.

Immunofluorescence

Cells were cultured on glass coverslips. After treatment, cells were fixed with 3.7% (w/v) formaldehyde for 10 minutes at room temperature and permeabilized with 0.1% (w/v) Triton X-100 for 5 minutes. Thereafter, cells were incubated with the indicated primary antibodies, followed by incubation with a goat-anti-mouse-Ig antibody conjugated to Alexa 488 (Molecular Probes, Leiden, The Netherlands). F-actin was visualized with Texas Red-labelled phalloidin (Molecular Probes, Leiden, The Netherlands). Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope. Fluorescence distribution profiles were created with Image-Pro Plus 6.0 software (Media Cybernetics).

RhoA activation assay

RhoA pull-down experiments were performed as previously described [30]. In brief, after stimulation, cells were lysed in buffer containing 50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1% (w/v) NP-40, 10% (w/v) glycerol, 1 mM NaF, 2 mM $NaVO_3$ and protease inhibitor cocktail (Roche) for 10 minutes on ice. Lysates were cleared by centrifugation at 10,000x*g* for 10 minutes at 4ºC. GST-Rhotekin fusion protein, coupled to glutathione-Sepharose beads (Amersham Biosciences), was added to the supernatants and incubated for 1 hour at 4ºC. Beads were washed three times in lysis buffer and bound proteins were eluted with Laemmli sample buffer. Total cell lysates and precipitates were analyzed by Western blotting with a mouse monoclonal antibody anti-RhoA (Santa Cruz Biotechnology), anti-mouse IgG antibodies coupled to horseradish peroxidase and enhanced chemiluminescence (Amersham Biosciences).

Statistical analysis

All results were expressed as the mean ± SEM of at least three independent experiments. Where applicable, values were compared in a paired two-tailed Student t-test. A *p* value lower than 0.05 was considered significant.

Results

Depolymerization of microtubules increases lung epithelial barrier function

To establish the role of microtubules (MTs) in epithelial barrier function we monitored transepithelial resistance (TER) of monolayers of H292 cells in real-time. Cells were treated with nocodazole, a microtubule-disrupting agent, or with taxol, a microtubule-stabilizing agent. Depolymerization of MTs by nocodazole resulted in a fast and pronounced increase in TER, which leveled off after 30 minutes, and remained stable over the three-hour period of measurement. Taxol had a minor, lowering effect on bronchial epithelial permeability (Figure 1A). As expected, disruption of the epithelial actin cytoskeleton with cytochalasin B induced a dramatic decrease in TER (Figure 1A).

Disruption of MTs by nocodazole also increased the TER of monolayers of the alveolar epithelial A549 cells (Figure 1B), albeit to a lower extent as observed with the bronchial H292 cells. As in H292 cells, treatment of A549 cells with taxol induced a small decrease in TER, while disruption of the actin cytoskeleton with cytochalasin B caused a pronounced decrease in the TER (Figure 1B). These data show that MT disassembly promotes

epithelial barrier function in lung epithelial cells. In contrast to the findings in lung epithelial cells, depolymerization of MTs in MDCK cells (kidney epithelium) resulted in a decrease in TER (M. Lorenowicz, unpublished observations).

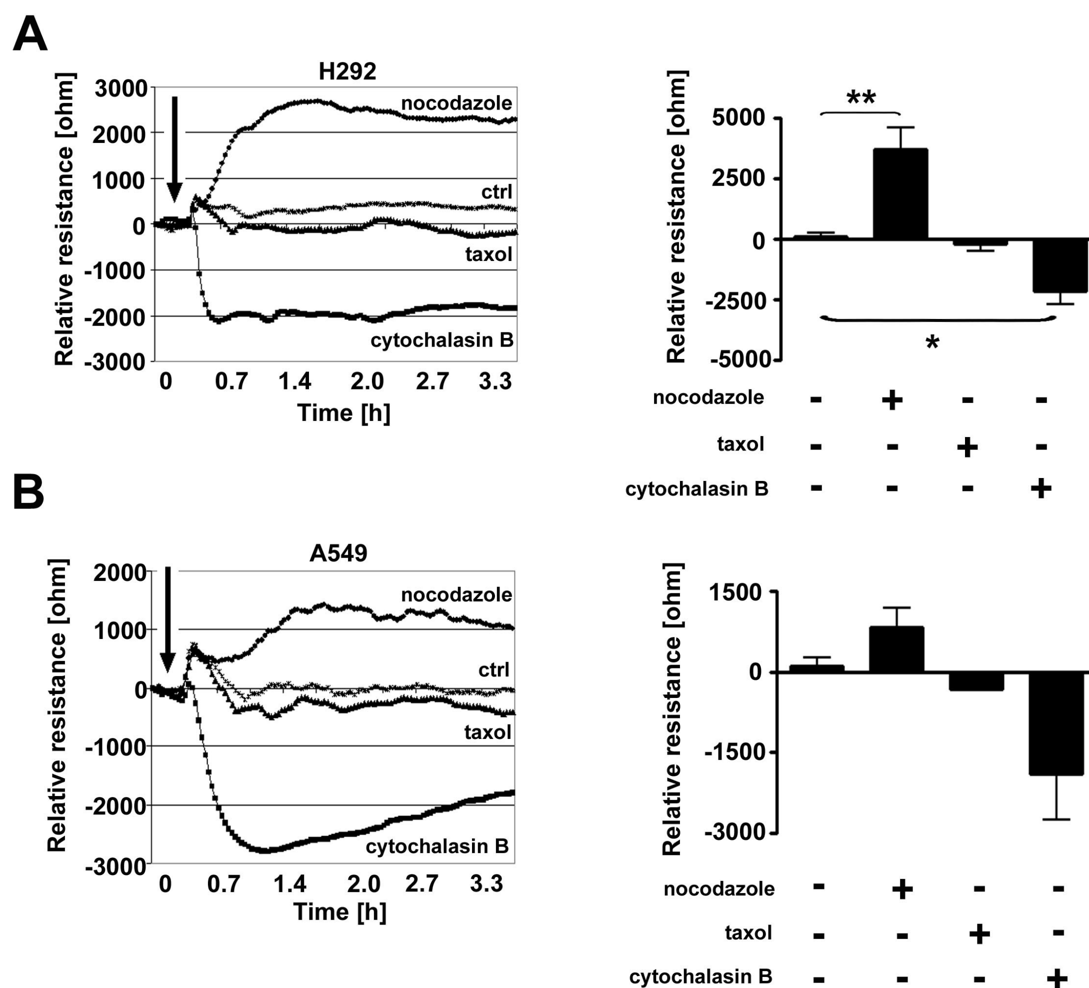

Figure 1. Depolymerization of MT promotes lung epithelial cell barrier function. Cells were plated on fibronectin-coated gold electrodes and grown to confluency. TER was measured in real time as described in Materials and Methods. **(A)** H292 cells were treated with 8 µM nocodazole, 10 µM taxol or 25 µg/ml cytochalasin B (arrow indicates time of addition). *Crosses* - control; *circles* - nocodazole; *triangles* - taxol; *squares* - cytochalasin B. Representative results from at least 5 independent experiments are shown. Bar graph (right panel) represents the relative resistance at the 2 hour time point when resistance values plateaud. Data are mean (+/- SEM) of 5 independent experiments performed in duplicate. $^{*}p<0.05$; $^{**}p<0.005$. **(B)** A549 cells were treated with 8 µM nocodazole, 10 µM taxol or 25 µg/ml cytochalasin B (arrow indicates time of addition). *Crosses* - control; *circle*- nocodazole; *triangle*- taxol; *square*- cytochalasin B. Representative results from 2 independent experiments are shown. Bar graph (right panel) represents the relative resistance at the 2 hour time point when resistance values plateaud. Data are mean (+/- SEM) of 2 independent experiments performed in duplicate.

A common modification of MT is the enzymatic addition of a tyrosine residue at the C-terminal end of α-tubulin, resulting in increased dynamic behavior (high polymerization/depolymerization rates). Since nocodazole is known to act primarily on dynamic, tyrosinated microtubules [31] we performed immunofluorescence labeling of tyrosinated MTs on nocodazole-treated H292 cells. Indeed, nocodazole induced an almost complete loss of tyrosinated MT in the lung epithelial cells (Figure 2A).

Epithelial integrity depends on junctional E-cadherin-β/γ-catenin complexes and on their interaction with the actin cytoskeleton. Since MT dynamics regulate the actin cytoskeleton (refs), we investigated whether the increase in TER, induced by MT disassembly, was accompanied by changes in the organization of the actin cytoskeleton and/or of intercellular junctions. MT depolymerization caused an increase in the levels of β-catenin at cell-cell junctions and at the apical side of the cells (Figure 2B). This suggests that MT depolymerization induces accumulation of E-cadherin-β-catenin complexes at the plasma membrane, possibly through an effect on protein trafficking. The F-actin levels at the cell-cell junctions did not change significantly in cells treated with nocodazole (Figure 2C, left panel). However, MT disassembly induced distinct F-actin bundling at the bottom side of bronchial epithelial cells, generating long actin spikes (Figure 2C, right panel).

Microtubule disassembly activates RhoA

Rho-like GTPases are key regulators of cytoskeletal dynamics, and act as signaling intermediates between MTs and F-actin [32-34] [35]. Depolymerization of MTs resulted in a transient activation of RhoA with a maximum at 5 minutes and returning to basal levels within 30-60 minutes (Figure 3A). To test whether RhoA was required for the microtubule disassembly-induced increase in TER we blocked RhoA signaling by pre-incubation of epithelial monolayers with a cell-permeable peptide corresponding to the hypervariable C-terminus of RhoA (Tat-RhoA, [25]). Subsequently, cells were treated with nocodazole, followed by real-time recording of TER. Inhibition of RhoA attenuated partially, but significantly, the increase in TER induced by MT depolymerization. The peptide by itself did not affect basal TER (Figure 4A). To analyze this in more detail, we tested whether the RhoA effector Rho kinase (ROCK), known to induce myosin phosphorylation and actin bundling, was required for this effect. The ROCK inhibitor Y27632 significantly blocked the nocodazole-induced increase in TER (Figure 3B). In contrast, inhibition of myosin light chain kinase (MLCK),

which directly phosphorylates myosin and induces actin bundling, did not affect the increase in TER induced by microtubule disassembly (Figure 3C). These data show that the increase in lung epithelial barrier function induced by microtubule breakdown is, in part, mediated by a RhoA-ROCK pathway and does not require MLCK activity.

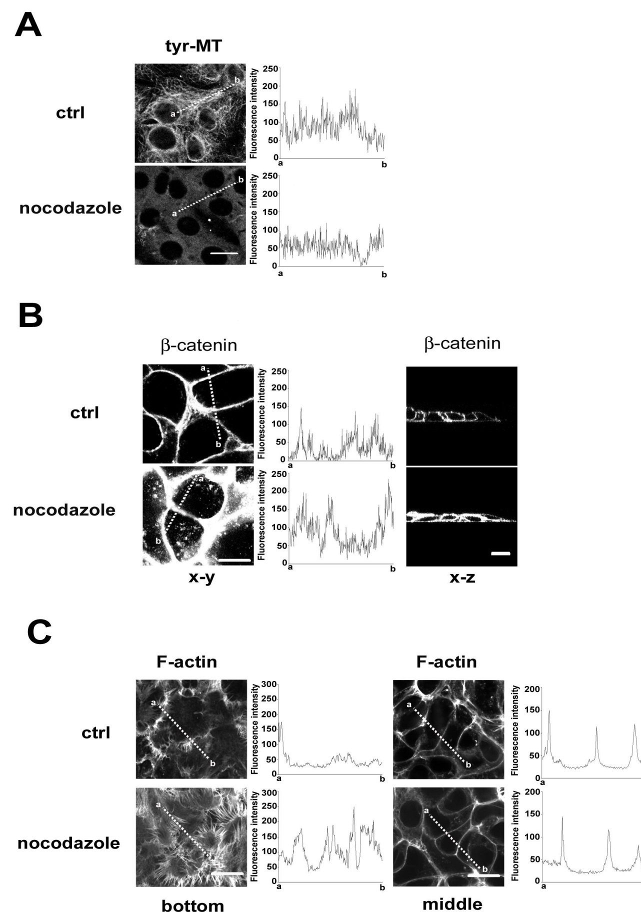

Figure 2. Disruption of microtubules results in actin bundling and increased levels of β-catenin at cell-cell junctions. (**A**) H292 cells, grown on glass coverslips were treated or not with 8 μM nocodazole for 1 hour, fixed and stained for tyrosinated microtubules (tyr-MT). Bars, 20 μm. Images are representative of at least 3 independent experiments. Fluorescence intensity profiles along the indicated dashed line between points indicated as **a** and **b** are shown. (**B**) H292 monolayers were treated or not with 8 μM nocodazole for 1 hour, fixed and stained for β-catenin. Images are representative of 4 independent experiments. Fluorescence intensity profiles along the indicated dashed line between points **a** and **b** are shown. (**C**) H292 cells, cultured and stimulated as in A,B were fixed and stained for F-actin. Confocal sections of the middle section of the cells (left)

and from the floor (bottom) of the cells are shown. Bars, 10 μm. Fluorescence intensity profiles along the indicated dashed line between points **a** and **b** are shown.

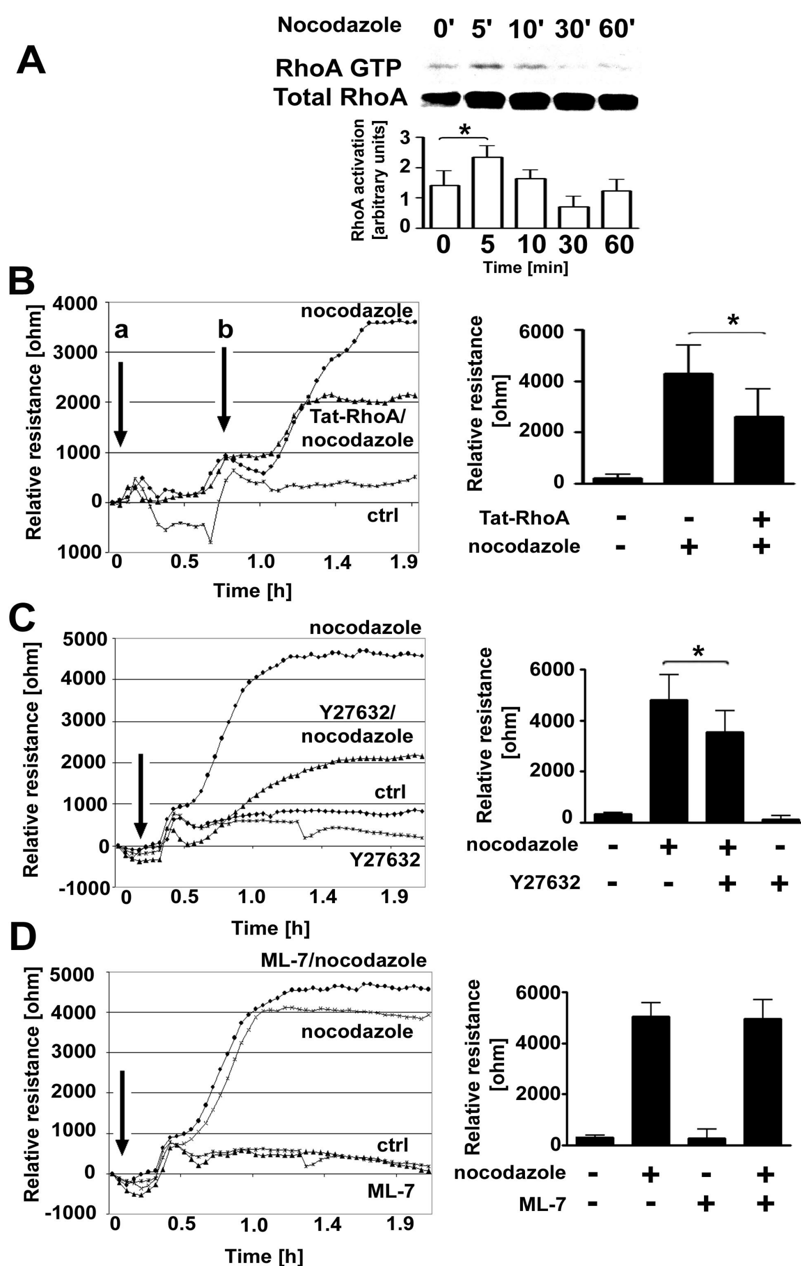

Figure 3. Role for RhoA in MT-regulated TER. **(A)** H292 cells were treated with 8 μM nocodazole for the indicated time periods and RhoA GTP-loading was assayed by a pull-down assay (upper panel). Total levels of RhoA in whole cell lysates are shown (lower panel). Representative results from 3 independent experiments are shown. Bar graph represents the densitometric analysis of RhoA activation. Data are mean (+/- SEM) of 3 independent experiments. *p<0.05. **(B-D)** H292 cells were plated on fibronectin-coated gold electrodes and grown to confluency. TER was measured in real time as described in Materials and Methods. **(B)** Cells were treated or not with 200 μg/ml Tat-RhoA peptide at the timepoint indicated as **a** and with 8 μM nocodazole at the time point indicated as **b** (left panel). *Asterisks* - control; *circles* - nocodazole; *triangles* -TAT-RhoA and

nocodazole. Representative results from 3 independent experiments are shown. Bar graph (right panel) represents the relative resistance at the 2-hour time point. Data are mean (+/- SEM) of 3 independent experiments performed in duplicate. *$p<0.05$. **(C)** Cells were pretreated or not for 30 minutes with 10 μM Y27632 and treated with 8 μM nocodazole (arrow). *Asterisks* - control; *circles* - nocodazole; *diamonds* - Y27632; *triangles* - Y27632 and nocodazole. Representative results from 3 independent experiments are shown. Bar graph (right panel) represents the relative resistance at the 2-hour time point. Data are mean (+/- SEM) of 3 independent experiments performed in duplicate. *$p<0.05$ **(D)** Cells were pretreated or not for 30 minutes with 10 μM ML-7 and treated with 8 μM nocodazole (arrow). *Asterisks* - control; *circles* - nocodazole; *triangles* - ML-7; *crosses* - ML-7 and nocodazole. Representative results from 3 independent experiments are shown. Bar graph (right panel) represents the relative resistance at the 2-hour time point when resistance values have reached a plateau in most experiments. Data are mean (+/- SEM) of 3 independent experiments performed in duplicate.

Inhibition of Rac1 increases lung epithelial barrier function

Rac1 is another Rho-like GTPase that has previously been clearly implicated in the control of E- and VE-cadherin-based cell-cell contact [5,6,9,11,36,37] Moreover, the balance between Rac1 and RhoA signalling regulates cell-cell adhesion [6,13,30]. Because RhoA activation is involved in the increase in TER of lung epithelial cells upon MT depolymerization (Fig. 3), we subsequently tested the relevance of Rac1 for this effect. To block Rac1 signaling, we pre-incubated H292 monolayers with a cell-permeable peptide corresponding to the hypervariable C-terminal domain of Rac1 (Tat-Rac1), previously shown to inhibit Rac function [25,26,38], and monitored changes in TER. This peptide blocks Rac1 signaling within minutes by competing with Rac1-targeting proteins. Unexpectedly, inhibition of Rac1 induced an immediate and dramatic increase in basal TER (Figure 4A). Inhibition of RhoA using the corresponding C-terminal peptide did not have any major effect on TER (Fig. 4A). Also a peptide encoding the C-terminal domain of Cdc42 did not affect TER (not shown), further supporting the specificity of the Tat-Rac1 effect.

To further establish the role of Rac1, we blocked Rac1 signaling in H292 cells with a different Rac1-inhibiting peptide, Tat-17-32, which encodes part of the effector domain of Rac1 and competes with Rac1-effector interactions [25,27]. This peptide also induced a rapid increase in TER. Finally, we blocked Rac1-effector interactions with a cell-permeable version of the PAK-CRIB domain, which specifically binds GTP-loaded Rac1 [28]. This CRIB domain peptide also enhanced TER, albeit but to a lesser extent (Figure 4B). Thus, inhibition of Rac1 signaling strongly promotes TER of monolayers of lung epithelial cells.

The Rac1 inhibiting C-terminal peptide did not induce a detectable increase in the levels of β-catenin at cell-cell junctions (Figure 4C), as was observed in nocodazole-treated cells (Figure 2B). However, Rac1 inhibition did induce the reorganization of the actin cytoskeleton, i.e. the induction of F-actin and formation of stress fibers, and markedly promoted cell spreading, particularly of cells at the periphery of small clusters of cells (Figure 4D). Inhibition of RhoA had little effect and did not induce an increase in actin stress fibers, as expected (Figure 4D).

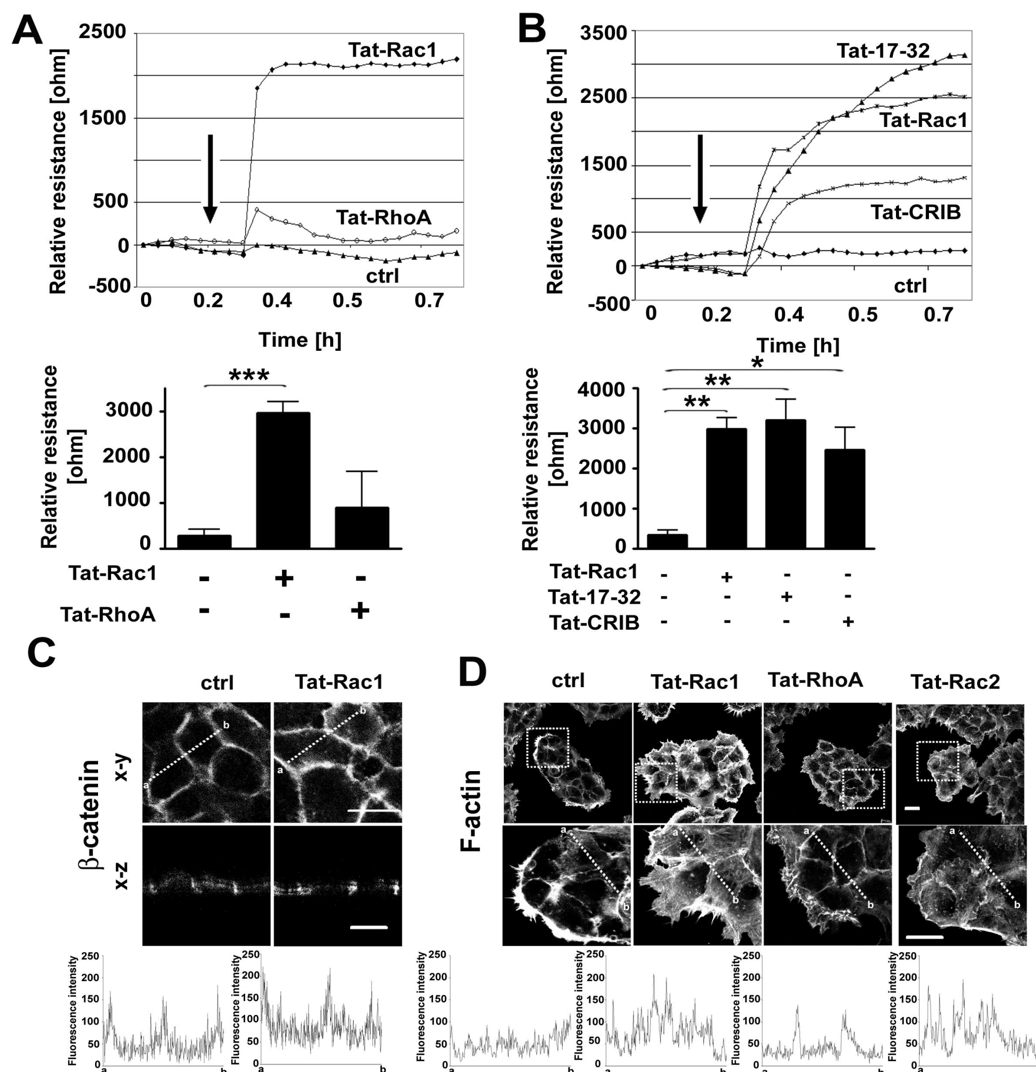

Figure 4. Inhibition of Rac1 increases lung epithelial barrier function. **(A,B)** Cells were plated on fibronectin-coated gold electrodes and grown to confluency. TER was measured in real time as described in Materials and Methods. **(A)** Cells were treated or not with 200 µg/ml Tat-Rac1 or Tat-RhoA peptides at the time indicated by the arrow (upper panel). *Triangles* – ctrl; *open circles* - TAT-RhoA and *diamonds* - TAT-Rac1. Representative results from at least 5 independent experiments are shown. Bar graph (lower panel) represents the relative

resistance at the 0.5-hour time point. Data are mean (+/- SEM) of 5 independent experiments performed in duplicate. ***p<0.0005. **(B)** Cells were treated or not with 200 µg/ml Tat-Rac1, TAT-17-32, Tat-CRIB peptides at the time indicated by the arrow (upper panel). *Diamonds* - control; *asterisks* - TAT-CRIB; *circles* - TAT-Rac1; *triangles* - TAT-17-32. Representative results from 4 independent experiments are shown. Bar graph (lower panel) represents the relative resistance at the 0.5-hour time point. Data are mean (+/- SEM) of 4 independent experiments performed in duplicate. *p<0.05; **p<0.005. **(C)** H292 cells were plated on glass coverslips and were treated or not for 2 hours with 200 µg/ml Tat-Rac1. Thereafter, cells were fixed and stained for β-catenin. Images are representative of 3 independent experiments. Bars: 20 µm **(D)** H292 cells were plated on glass coverslips and treated or not with 200 µg/ml Tat-Rac1, Tat-Rac1 or TAT-RhoA peptides for 2 hours. Next, cells were fixed and stained for F-actin. Magnifications of the insets (indicated by the dashed boxes) are shown below and some were rotated to facilitate comparison. Images are representative of 3 independent experiments. Bars, 20 µm. Fluorescence intensity profiles along the indicated dashed line between points **a** and **b** are shown.

The nocodazole-induced increase in lung epithelial barrier function is, at least in part, mediated by RhoA-ROCK signaling (Fig. 3). Since Rac1 inhibition also promoted barrier function and F-actin formation (Figure 4D), we tested whether the effect of Rac1 inhibition is mediated by RhoA-ROCK signaling. Inhibition of ROCK with Y27632 did not change the effect of the Tat-Rac1 peptide on TER (Figure 5A). Similarly, addition of Y27632 following incubation of cells with the Rac1 C-terminal peptide did not abrogate the barrier-enhancing effect of Rac1 inhibition (Figure 5B). Moreover, direct inhibition of RhoA by pre-incubation of H292 monolayers with the RhoA inhibitory peptide or C3 toxin (ref of C3 toxin) also failed to attenuate the increase in TER induced by inhibition of Rac1 (ML, unpublished observations). These data suggest that RhoA/ROCK signaling is not required for the increase in TER that follows the inhibition of Rac1.

Microtubule disassembly promotes barrier function independently of Rac1

To further investigate the relation between Rac1 and microtubule-mediated regulation of epithelial barrier function we monitored the TER of H292 monolayers that were treated with nocodazole prior to the addition of the Rac1-inhibiting peptide. Interestingly, inhibition of Rac1 in nocodazole-treated cells further enhanced TER (Figure 6A), suggesting that the pathways that regulate barrier function downstream of Rac1 inhibition and MT depolymerization are separate and not redundant. Stabilization of microtubules with taxol, prior to inhibition of Rac1, did not have any effect on the increase in TER (Figure 6B). This further indicates that Rac1 inhibition does not increase TER through the regulation of MT dynamics. These data, together with the differential requirement for RhoA in MT- and Rac1–

mediated signaling, suggest that MT-dynamics and Rac1 control lung epithelial cell-cell adhesion through independent pathways.

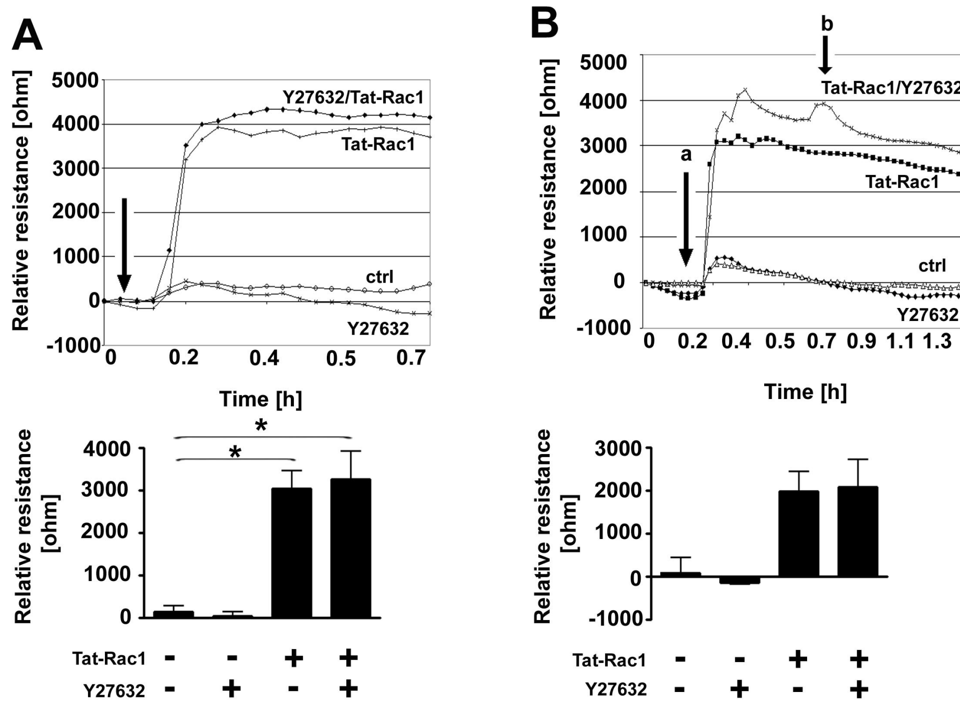

Figure 5. RhoA/ROCK signaling does not mediate the increase in epithelial TER induced by inhibition of Rac1. **(A)** and **(B)** Cells were plated on fibronectin-coated gold electrodes and grown to confluency. TER was measured in real-time as described in Materials and Methods **(A)** Cells were pretreated or not for 30 minutes with 10µM Y27632 and treated or not with 200 µg/ml TAT-Rac1 (arrow). *Open circles* - control; *asterisks* - Y27632; *crosses* - Tat-Rac1; *diamonds* - Tat-Rac1 and Y27632. Representative results from 3 independent experiments are shown. Bar graph (lower panel) represents the relative resistance at the 0.5hour time point. Data are mean (+/- SEM) of 3 independent experiments performed in duplicate. *p<0.05. **(B)** Cells were treated or not with 200 µg/ml Tat-Rac1 at the time point indicated as **a** and subsequently with 10 µM Y27632 or carrier at the time point indicated as **b** (upper panel). *Open triangles* - control; *diamonds* - Y27632; *squares* - Tat-Rac1; *asterisks* - Tat-Rac1 and Y27632. Representative results from 3 independent experiments are shown. Bar graph (lower panel) represents the relative resistance at the 0.5-hour time point. Data are mean (+/- SEM) of 3 independent experiments performed in duplicate.

Discussion

One of the main findings of this work is that the loss of dynamic MT promotes cell-cell adhesion in lung epithelial cells. This finding is in line with data from Kee et al. who showed that MT dynamics negatively regulate cell-cell contacts in keratinocytes and in

intestinal epithelium [15,16]. In contrast, however, MT dynamics are required for proper barrier function of thyroid epithelial cells [20], and MT depolymerization induces loss of cell-cell adhesion in newt lung epithelial cells [39]. Similarly, we found that in kidney epithelial cells, MT disassembly reduces barrier function. Together, these data suggest that the regulation of epithelial cell-cell contact by MT is cell-type specific.

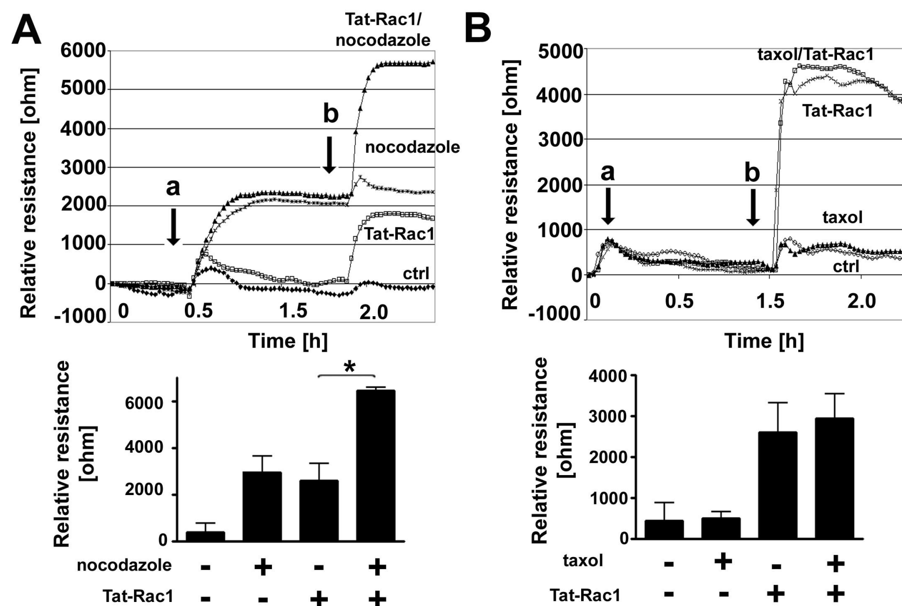

Figure 6. Microtubule disassembly promotes barrier function independently of Rac1. Cells were plated on fibronectin-coated gold electrodes and grown to confluency. TER was measured in real time as described in Materials and Methods. **(A)** Cells were treated with 8 µM nocodazole at the time indicated as **a** and with 200 µg/ml Tat-Rac1 peptide at the time indicated as **b** (upper panel). *Diamonds* - control; *asterisks* - nocodazole; *open squares* - Tat-Rac1; *triangles* - Tat-Rac1 and nocodazole. Representative results from 3 independent experiments are shown. Bar graph (lower panel) represents the relative resistance at the 2.5-hour time point. Data are mean (+/- SEM) of 3 independent experiments performed in duplicate. *$p<0.05$. **(B)** Cells were treated with 10 µM taxol at the time point indicated as **a** and with 200 µg/ml Tat-Rac1 peptide at the time point indicated as **b** (upper panel). *Circles* - control; *triangles* - taxol; *asterisks* - Tat-Rac1; *open square*s - Tat-Rac1 and taxol. Representative results from 3 independent experiments are shown. Bar graph (lower panel) represents the relative resistance at the 2.5-hour time point. Data are mean (+/- SEM) of 3 independent experiments performed in duplicate.

The observed increase in TER upon MT disassembly in lung epithelial cells was accompanied by increased levels of β-catenin at cell-cell junctions. This indicates that MTs regulate trafficking of E-cadherin-β-catenin complexes to and from cell-cell junctions, leading to an internalization defect in nocodazole-treated cells. Accordingly, Ivanov et al. reported

that inhibition of the microtubule motor kinesin attenuated junction disassembly in intestine epithelium [16]. Similarly, N-cadherin-positive vesicles were found to move in a microtubule-dependent, kinesin-driven fashion in fibroblasts [40], and junctional complex proteins such as p120-catenin and β-catenin were shown to interact with microtubules and with the microtubule-associated motors kinesin and dynein [41-44].

Depolymerization of MT in keratinocytes not only promotes recruitment of E-cadherin to the sites of cell-cell contact but also induces an increase in F-actin at cell-cell junctions [15]. Although we did not find such an increase of F-actin at cell-cell junctions, MT disassembly induced pronounced actin rearrangements at the basal side of the cells. F-actin accumulation induced by MT disassembly is a RhoA-dependent phenomenon in fibroblasts as well as in endothelial cells [21,45]. In line with this notion, we found that, also in lung epithelial cells, MT disassembly stimulates RhoA activation. More importantly, we show that the increase in lung epithelial barrier function, stimulated by MT depolymerization, is partially mediated through RhoA and ROCK signaling. This finding is in good agreement with data from others, implicating RhoA in the stimulation of cell-cell adhesion. Inhibition of RhoA signaling by the C3 toxin or using a dominant-negative mutant of RhoA in epithelial cells was found to disrupt junctional localization of E-cadherin [11,36,46]. However, constitutively active RhoA failed to increase epithelial cell-cell contacts [13], indicating that RhoA by itself is not sufficient to enhance epithelial cell-cell adhesion. Interestingly, in keratinocytes, MT depolymerization-induced cell-cell adhesion appears to be dissociated from MT disassembly-induced RhoA-driven cytoskeletal dynamics, further indicating that, in addition to RhoA, other factors are involved in the regulation of epithelial barrier function [47].

Our data demonstrate that inhibition of Rac1 signaling, by means of cell-permeable peptides, rapidly and dramatically improves lung epithelial barrier function. Accordingly, activation of Rac1 in epithelial cells and various carcinoma cell lines induces disassembly of cell-cell contact [8,48,49]. In contrast, several studies have shown that Rac1 activity is also required for the formation of cadherin-based adhesion [5,11,36]. This discrepancy may relate to differences in experimental conditions, cell type, and state of maturation of the junction or the type of extracellular matrix used [10].

Activation of Rac1 promotes MT growth into lamellipodia of migrating kidney epithelial cells, while a dominant-negative mutant of Rac1 inhibits MT polarization toward the leading edge of migrating cells and blocks lamellipodia formation [39,50]. Conversely, MT growth activates Rac1 in migrating fibroblasts, whereas MT depolymerization inhibits Rac1 activation [50]. We found that, although both MT disassembly and inhibition of Rac1 induced

actin polymerization, the consequent increase in lung epithelial barrier function occurs through independent pathways. In contrast to MT disassembly, the increase in TER induced by the inhibition of Rac1 signaling did not induce the accumulation of E-cadherin/β-catenin complexes at cell-cell junctions and did not require RhoA/ROCK signaling. Moreover, inhibition of Rac1 and MT depolymerization were additive in the enhancement of lung epithelial TER. Our data suggest that, in contrast to their functional coupling in cell migration, Rac1 and MT act independently in the control of lung epithelial barrier function

Together, the current findings provide new and unexpected insights in the control of lung epithelial intercellular contacts by MT and RhoGTPases. In these cells, MT dynamics regulate cell-cell adhesion in a RhoA-dependent, but Rac1-independent fashion, presumably through actin cytoskeletal dynamics. In contrast, Rac1 negatively controls epithelial cell-cell contact, acting through a RhoA-independent pathway. Finally, our data identify dynamic MT, RhoA and Rac1 as new and attractive therapeutic targets in the treatment of (chronic) lung inflammatory disorders.

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CHAPTER VI

SUMMARY AND CONCLUSIONS

Migration of leukocytes from the blood to the underlying tissues is critical for tissue homeostasis. This is not only relevant in the defense of the organism against invading pathogens or tissue damage but is also essential for immunosurveillance and stem cell homing. Understanding the molecular mechanisms that control leukocyte migration is of key importance for the development of anti-inflammatory therapies that target (subsets of) leukocytes and molecules that control disease. Ideally, this should be achieved without affecting the migration and function of leukocytes required for a proper protective immune response. In addition to the migratory potential of the leukocytes, the adhesion to and migration across endothelial and epithelial barriers are also important determinants of the inflammatory response.

In this thesis we provide new insights into the signaling that governs leukocyte migration as well as signaling involved in endothelial and epithelial integrity. Each of these pathways is equally relevant for the process of inflammatory cells diapedesis.

Epac1-Rap1 signaling in leukocytes

Cyclic AMP is a well established second messenger known to regulate a large variety of cellular processes, including cell proliferation, differentiation, apoptosis, gene transcription, cell adhesion and migration. Until the discovery of Epac1 in 1998 [1,2], cAMP-mediated cellular functions were attributed to its canonical target PKA. However, increasing evidence has implicated Epac1 in the regulation of several cAMP-dependent processes, such as cell-cell or cell-matrix adhesion, Fcγ–receptor-mediated phagocytosis or Ca^{++}-induced exocytosis [3-6]. In **chapter II** we studied the role of Epac1-Rap1 signaling in the control of leukocyte adhesion and chemotaxis. In contrast to previous reports, we were able to demonstrate that Epac1 protein is expressed in human hematopoietic progenitor cells and primary leukocytes, with the exception of neutrophils [6,7]. This differential pattern of Epac1 expression in hematopoietic cells may reflect the functional differences between various subsets of leukocytes and their different functions in the body. We found that activation of Epac1 in monocytes, which show a high Epac1 expression, resulted in increased adhesion and chemotaxis. In contrast, there is evidence that raising the level of cAMP in neutrophils inhibits their adhesion and directional movement [8,9]. Given the absence of Epac1 protein in neutrophils, PKA may be responsible for this inhibitory effect on cell migration. It will be interesting to test whether in leukocytes that also express Epac1, PKA and Epac1 counteract each other, with Epac1 being stimulatory and PKA inhibitory for cell migration, depending on the type or stimulus, compartmentalization of the cAMP or some other factor.

The data presented in chapter II demonstrate that Epac1-Rap1 signaling is important in the control of monocyte polarization. In line with this notion, we show that Epac1 is distributed in a polarized fashion, accumulating in the uropod of a migrating cell. It is unknown how Epac1 regulates leukocyte polarization and what the significance of its localization to the back of the cell is. Interestingly, the Rap1 effector RAPL has been shown to redistribute from a perinuclear location to the leading edge membrane upon lymphocyte stimulation [10]. Thus, one explanation for the localization of Epac1 to the back of a migrating leukocyte is that it activates Rap1 at a perinuclear location followed by subsequent trafficking of activated Rap1-RAPL complexes to the leading edge, where activated Rap1 has been detected [11,12]. In the light of recent findings in neutrophils, in which Rac1 was shown to regulate RhoA-mediated contraction at the cell trailing edge, and given our own unpublished data that Epac1 activates Rac1 in monocytes, it is possible that Epac1 controls monocyte polarization, a.o., through Rac1-mediated cytoskeletal rearrangements [13].

Another important finding reported in chapter II is that serotonin, a well-known neurotransmitter implicated in inflammation [14], induces Epac1-Rap1-mediated monocyte adhesion, polarization and chemotaxis. Since serotonin has been found in atherosclerotic lesions and asthmatic lungs, the Epac1-Rap1 signaling pathway may well represent a promising target for therapy of inflammatory disorders such as atherosclerosis and asthma [15,16].

Epac1 and PKA signaling in endothelium

It is generally accepted that cAMP promotes endothelial barrier function. Many studies have implicated PKA in the cAMP-mediated regulation of endothelial cell-cell adhesion. The proposed mechanisms that control endothelial integrity downstream of PKA involve inhibition of RhoGTPase-mediated cytoskeletal contractility, vasodilator-stimulated phosphoprotein-mediated cytoskeletal changes, reduction of myosin light-chain phosphorylation and regulation of Rac1 GTPase activation [17-20]. Recently, also Epac1 was reported to be required for cAMP-induced endothelial barrier function [4,21-23]. In **chapter III** we present the first comparative studies on the interdependence between PKA and Epac1-induced signaling in the regulation of endothelial barrier function and motility. We found that specific activation of either PKA or Epac1 increased endothelial barrier function and promoted endothelial cell migration. In addition, using an Epac1 knock-down strategy, we demonstrated that Epac1 and PKA control the endothelial integrity by two independent signaling pathways. The PKA signaling seems to be more dependent on integrin engagement

than Epac1 signaling in the regulation of endothelial cell-cell contact. Both PKA and Epac1 induce cytoskeletal rearrangements. However, PKA promotes stress fiber formation while Epac stimulates an increase in cortical actin. Control of endothelial cell migration by both Epac and PKA requires integrins. However, Epac1 and PKA signaling has different effects on focal adhesions. Epac1 appears to be important for targeting of focal adhesions to the leading edge of migrating cells, while PKA is necessary for organization of such focal complexes. Thus, endothelial cells use two independent cAMP-induced pathways for control of cell-cell adhesion and migration. The first question that arises from this study is, why two pathways are required to regulate one process in the endothelium? The possible explanation could be that Epac1 and PKA, due to their different sensitivity for cAMP, may respond to a broad range of cAMP concentrations, generated by different stimuli [24]. It is also likely that differentially localized Epac1 and PKA signaling is regulated by local generation of cAMP and that cellular responsiveness is further controlled by proximity of downstream targets [25,26].

Our data suggest that both Epac1 and PKA control endothelial integrity and migration through changes in the organization of the actin cytoskeleton. However, because both Epac1 and PKA have also been implicated in the regulation of vesicle trafficking, they may control junction assembly through regulation of junctional protein trafficking [27,28].

It is still an open issue which targets are downstream of Epac1 in the control of endothelial cell migration and integrity. We approached this question in **chapter IV**, by investigating the role of AF-6, one of the possible effectors of Epac1-Rap1 signaling in the regulation of endothelial barrier function. Studies involving downregulation of AF-6 expression demonstrated that AF-6 is necessary for endothelial integrity and is important for Rap1 activation. However, AF-6 does not appear to act downstream of Epac1, suggesting that other targets are more relevant for Epac1-Rap1-driven regulation of cell-cell contact. Another potentially relevant Epac1 effector could be RIAM, which has been implicated in Rap1-dependent and integrin-mediated adhesion of Jurkat T cells and is known to bind proteins such as Ena/Vasp, well-established modulators of the actin cytoskeleton [29]. Vav2 and Tiam1, guanine nucleotide exchange factors (GEFs) for the Rho-like GTPase Rac1, localize to sites of cell-matrix contacts following Rap1 activation. Since Rac1 is an important regulator of endothelial cell-cell contact, these GEFs are interesting Epac1 targets as well [30,31]. Another candidate, relevant in the context of cytoskeletal rearrangements, is ARAP3, a GEF for RhoA shown to be regulated by Rap1 [32]. Further studies will be required to

establish whether any and if so which of the above-mentioned proteins is/are the downstream effector(s) of Epac1 in the control of endothelial integrity.

Microtubules and small GTPases in the regulation of epithelial barrier function

In chronic inflammatory disorders such as in the lung, leukocytes not only extravasate from the vasculature across the endothelium, but also continue their journey and pass polarized epithelium to reach the inflammatory site. Understanding the regulation of epithelial integrity is therefore essential to gain more insight into the pathology of many inflammatory disorders. Similar to endothelium, epithelial barrier function is largely dependent on cytoskeletal dynamics. It is well accepted that the actin cytoskeleton plays an important role in the maintenance of epithelial integrity. However, as shown in **chapter V** and some earlier reports, also microtubules contribute to the stability of epithelial cell-cell contact [33,34]. We have demonstrated that disassembly of microtubules in lung epithelial cells promotes cell-cell adhesion. This is in agreement with earlier studies in keratinocytes and intestinal epithelium, showing that MT depolymerization induces cell-cell contact and attenuates junction disassembly, respectively [35,36]. However, work in other types of epithelia and in endothelial cells showed that MT depolymerization can also negatively regulate cell-cell adhesion [33,37,38]. This suggests that the role of MT in the control of cell-cell contact is very much dependent on cellular context.

MT disassembly in lung epithelium resulted in an increase in the levels of E-cadherin-β-catenin complexes at cell-cell junctions. Thus, MT may regulate trafficking of E-cadherin-β-catenin complexes to and from cell-cell junctions whereas in the absence of MT, the internalization of junctional complexes is inhibited. This model is supported by earlier observations showing that cadherins are internalized in a microtubule-dependent and kinesin-driven fashion [36,39]. In addition, it was reported that junctional complex components such as p120-catenin, β-catenin and N-cadherin interact with microtubules and with the microtubule associated motors kinesin and dynein [40-43].

Our findings indicate that epithelial cell-cell adhesion induced by MT disassembly is partially mediated through RhoA and Rho kinase (ROCK) signaling. This is in line with studies that reported that MT depolymerization induced RhoA activation as well as RhoA-dependent actin polymerization [38,44]. Moreover, RhoA has been implicated in a positive regulation of epithelial barrier function [45-47]. RhoA, activated by MT depolymerization,

may control epithelial integrity through reorganization of the actin cytoskeleton or through effects on MT-dependent, E-cadherin complex trafficking.

In our search for additional factors that are required for MT disassembly-induced epithelial barrier function we found that, similar to MT depolymerization, inhibition of Rac1 GTPase promotes epithelial cell-cell contacts. The balance between Rac1 and RhoA activities is known to control many cellular functions, including cell-cell adhesion [48]. Moreover, Rac1 was previously implicated in the regulation of epithelial integrity [45,49]. However, in contrast to MT disassembly, the effect of Rac1 inhibition on epithelial cell-cell adhesion does not appear to depend on RhoA/ROCK signaling. In addition, inhibition of Rac1 in cells with disrupted MT further promotes epithelial barrier function. These two observations indicate that two different mechanisms control the increase in epithelial cell-cell adhesion induced by MT disassembly and Rac1 inhibition. Further studies are necessary to define the downstream factors, regulated by MT depolymerization and Rac1 inhibition that mediate an increase in epithelial barrier function.

Together, the findings described in this thesis provide additional insight into the complex signaling that controls leukocyte chemotaxis as well as endothelial and epithelial cell-cell adhesion. Moreover, the data presented here raise new and intriguing questions to be addressed in future studies. Finally, the research reported in this thesis points to new molecules from signaling cascades controlling leukocyte extravasation that may provide attractive targets for anti-inflammatory therapies.

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