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Rap1 GTPases: An Emerging Role in the Cardiovasculature

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Abstract

The Ras related GTPase Rap has been implicated in multiple cellular functions. A vital role for Rap GTPase in the cardiovascular system is emerging from recent studies. These small monomeric G proteins act as molecular switches, coupling extracellular stimulation to intracellular signaling through second messengers. This member of the Ras superfamily was once described as the transformation suppressor with the ability to ameliorate the Ras transformed phenotype; however, further studies uncovered a unique set of guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and effector proteins for Rap suggesting a more sophisticated role for this small GTPase. At least three different second messengers can activate Rap, namely cyclic AMP (cAMP), calcium and diacylglycerol. More recently, investigation of Rap in the cardiovascular system has revealed multiple pathways of regulation involving Rap in this system. Two closely related isoforms of Rap1 exist, 1A and 1B. Murine genetic models exist for both and have been described. Although thought at first to be functionally redundant, these isoforms have differing roles in the cardiovascular system. Activation of Rap1a and 1b in various cell types of the cardiovascular system lead to alterations in cell attachment, migration and cell junction formation. This review will focus on the role of these Rap1 GTPases in hematopoietic, endothelial, smooth muscle, and cardiac myocyte function, and conclude with their potential role in human disease.

Keywords

Rap1; small GTPase; cAMP; Epac

Introduction

Rap1 is a member of the Ras superfamily of GTPases. It was described in 1989 by Kitayama et al as Krev-1, a 21 kDa protein with anti-oncogenic activity and subsequently identified by Bourne and colleagues as a Ras related protein. Rap1 has a critical role in development and morphogenesis in more genetically simpler organisms. For example, in the fly *Drosophila melanogaster* there lies a gain-of-function mutation in the only *rap1* gene that disrupts eye development, whereas the loss-of-function mutations are embryonic lethal. In the African frog *Xenopus laevis* and zebrafish, inactivation of Rap1 signaling leads to teratogenic

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developmental defects. However, in higher and more complex organisms there are multiple forms of the Rap protein, which are encoded by a variety of independent genes. These forms include Rap1a, 1b (referred to as Rap1) that share >90% sequence homology, and Rap2a, 2b, 2c (referred to as Rap2) that are 65–70% homologous to Rap1. Like other small GTPases, these monomeric proteins cycle in-between a GTP bound (active state) and a GDP bound (inactive state), thereby acting as a molecular on/off switch in their respective signaling pathways. Cycling between the active and inactive states is facilitated by GEFs which release GDP and allow binding of GTP as well as other GAPs which accelerate the GTP hydrolysis by several orders of magnitude. Earlier work focused on Rap1 as a direct inhibitor of Ras by which it was hypothesized that it was competing for the same effector proteins, however, further studies determined that both Rap1a and Rap1b act largely in entirely different signaling pathways than Ras, with a fundamentally different set of GAPs and GEFs. Although homologous, emerging evidence suggests that the Rap subtypes are not functionally redundant, and have a discrete cellular function that is only now being fully explored. For example, it is now well established that activated Rap1 is involved in inside-out signaling to integrins, focal adhesion formation, and in increasing cell adhesion. Subcellular localization of Rap1 is important for activation, although Rap is mainly localized to intracellular vesicles; it also has been localized to the plasma membrane. With the introduction of Rap1 as a regulator of cell adhesion, cell proliferation and cell junction formation, it is apparent that this small GTPase plays a significant role in the cardiovascular signaling pathways that are necessary for proper heart development and function. This review will focus on the role of Rap1 subtypes in the specialized cells of the cardiovascular.

cAMP signaling

In the cardiovascular a central role for Rap1 GTPase is evident from recent studies. Relaying extracellular signals to activation of intracellular signaling pathways is the function of these small G proteins. The intracellular second messenger cAMP is coupled to A-kinase activation and other downstream signaling events in the cardiovascular. cAMP controls many cellular processes and has been well associated with the PKA pathway. In addition to this canonical A-kinase signaling, cAMP has the potential to activate other signaling pathways in the cell, including the Ras GTP superfamily binding proteins Rap1 and Rap2. The GTP-bound Rap mediates signaling by associating with, and activating effector proteins. The adenylyl cyclase activator forskolin strongly and specifically activates Rap1, but not Rap2 in human microvascular smooth muscle cells. The Rap1 protein is activated by GEFs including C3G, CalDAG-GEF, RasGRP2, PDZ-GEF1, PDZ-GEF2 and PLC ϵ , however in the cardiovascular the most well described are referred to as exchange proteins directly activated by cAMP, or Epac 1 and 2. Epac is activated by the binding of cAMP which causes a gross conformational change, therefore allowing it to catalyze nucleotide exchange of Rap1. Rap1Gap catalyzes the hydrolysis of GTP by its asparagine side chain rendering Rap1 inactive. The cycling of Rap between its inactive and active states provides a mechanism to regulate the binding to effector proteins. These Rap effector proteins in the cardiovascular fall within pathways that regulate cell adhesion, proliferation, as well as cell migration leading to the regulation in hematopoietic, endothelial, smooth muscle and cardiac myocyte cells (Fig. 1).

Hematopoietic Cells

Formerly considered functionally redundant, murine models that are null for either Rap1a or Rap1b have provided phenotypic evidence that these two molecules have unique functions. In Rap1a null mice, analysis of blood cells determined that this isoform is not necessary for development, migration and for many of the mature functions of B, T and myeloid cells.

However, macrophages from these mice exhibited increased migration due to less matrix adhesion, as well as reduced chemotaxis. Reduced chemotaxis was also observed in B and T cells. Rap1a is highly expressed in neutrophils and co-purifies with the p22 subunit of the NADPH oxidase. So, not surprisingly, these null neutrophils (bone marrow derived) also have reduced superoxide production in response to fMLP (f-MetLeuPhe) stimulation. Rap1b is more highly expressed in B cells than Rap1a. Rap1b null mice were used to decipher the role of this isoform in this cell type. Rap1b is necessary for proper development, homing and T cell dependant immunity. The loss of Rap1b leads to deficiencies both in B cell migration and in adhesion.

In contrast to the Rap1a null model, the Rap1b null mouse has been further studied for alterations in platelet aggregation. Although both are ubiquitously expressed, Rap1a is highly expressed in neutrophils, whereas Rap1b is more highly expressed in platelets. Previous studies on isolated platelets from patients with congestive heart failure suggest a role for Rap1 in Ca^{2+} transport and homeostasis, necessary for platelet function. Later it was determined that it is the SERCA 3b isoform, which pumps Ca^{2+} into the endoplasmic reticulum and decreases cytosolic Ca^{2+} concentrations, which is a target for Rap 1b and that these two proteins have a physical interaction. Platelet inhibition occurring upon an increase in cAMP concentration leads to Rap 1b phosphorylation and its dissociation from the SERCA 3b protein. The overall results suggest that Rap 1b protein regulates the SERCA 3b-associated Ca^{2+} pool through its cAMP-dependent phosphorylation, and therefore plays a role in the transition between platelet activation and inhibition. Indeed, platelets from Rap1b null mice exhibit defective aggregation in response to integrin stimulation. These mice exhibit protection from arterial thrombosis and provide an independent role for this Rap1 isoform. (Fig. 2)

Endothelial cells

Whereas Rap1a and 1b appear to be key regulators in differing cell types of the blood, neutrophils and platelets, both appear to contribute to the normal function of endothelial cells and to angiogenesis. The endothelium of the vascular system is responsible for maintaining a barrier between the blood and extravascular space. This barrier is essential for maintaining the highly stringent blood-brain barrier of the cerebral vasculature, as well as, regulating permeability on the peripheral vasculature to allow for wound healing and infection resistance. Endothelial cell junctional proteins are involved in regulating vascular permeability. This dynamic regulation is regulated in part by Rap1 in response to cAMP activation through Epac1. In cultured human umbilical vein endothelial cells activation of Epac1-Rap1 by cAMP enhances endothelial barrier function by altering actin cytoskeleton organization, activating microtubule growth and results in a redistribution of adherens junctional proteins. This occurs through the scaffolding protein MAGI-1 and leads to adherens junction formation. In cultured human microvascular endothelial cells, knock down of either Rap1a or 1b appears to diminish adhesion to the extracellular matrix and impair cell migration, and also increases permeability.

The growth of new vessels from existing vascular beds, angiogenesis, occurs late in development and in response to organ growth and repair. The deregulation of this process leads to the metastatic, inflammatory, immune and ischemic disorders. Therefore proper regulation of both pro- and anti- angiogenic factors is essential. Loss of Rap1a or 1b unexpectedly blocked angiogenesis by abolishing the angiogenic response to FGF2 or to VEGF, leading to an inability for these cells to form tubular structures. Ablation of either isoforms leads to decrease in FGF-2 mediated ERK, p38 and Rac activation which are all important angiogenesis signaling molecules. In addition, Rap1a and b are involved in the activation of $\beta 1$ -integrins in endothelial cells and play a key role in integrin dependent

angiogenic functions such as sprouting, tube formation, migration and adhesion. In contrast, recent studies overexpressing activated Rap 1a in dermal microvascular endothelial cells show defective angiogenesis through regulation of thrombospondin-1. Although counter intuitive, these results suggest that an imbalance of angiogenic factors in either direction can lead to deleterious effects in angiogenesis. Together both Rap1a and 1b appear to play an important role in angiogenesis signaling and the proper regulation of pro- and anti-angiogenic factors (Fig. 3). Further, activation of Rap1 by Epac1 leads to increased integrin activity and adhesion of endothelial progenitor cells (EPC), CD34+ hematopoietic progenitor cells and mesenchymal stem cells (MSC). Following hind limb ischemia these Rap1 activated EPCs have increased homing and neovascularization capabilities. Although the role of each specific subtype is yet to be determined, this mechanism provides insight into enhanced therapeutics to improve progenitor cell homing.

Smooth muscle cells

The smooth muscle cells of the vasculature make up the majority of cells in the blood vessel wall. These cells contract and relax in response to stimuli allowing for the redistribution of blood throughout the body and regulating blood pressure. In human microvascular smooth muscle cells explanted from dermal arterioles, Rap1 activation is coupled to selective α_2C -adrenoceptor expression (Chotani et al., 2005; Eid et al., 2008). This vascular bed-specific role of Rap1 allows α_2C -adrenoceptors to modulate peripheral vascular resistance (blood flow and pressure) physiologically. In these cells cAMP-Rap1 signaling increases cell adhesion, activates Rho-ROCK-stress-fibers, activates c-Jun N-terminal kinase (JNK) and extracellular regulated kinase ERK1/2 (p44/42 mitogen-activated protein kinase), in addition to increasing de-novo synthesis of α_2C -adrenoceptors, and receptor deployment to the cell surface (Chotani et al., unpublished observations and manuscript in preparation). However, when these cells are stimulated with the hormone estrogen, Rap2 is activated. Thus, Rap subtypes couple distinct extracellular stimuli to intracellular signaling in the vasculature, potentially by microdomains that result in compartmentalized cAMP signaling. In the microvasculature, therefore, Rap1 may elicit a protective response to maintain vessel wall integrity in response to cellular stress (Fig. 4).

Cardiac myocytes

The myocardium is composed primarily of a collection of specialized muscle cells called cardiac myocytes. Found throughout the myocardium, myocytes function to pump blood to the systemic circulation. This coordinated contraction requires proper electrical excitation mediated by gap junction formation. Six connexins form one connexon in a cell and together with the connexon of the adjoining cell form a gap junction. In the cardiac myocyte the most abundant of these is connexin 43. The importance of this connexin is evident since the loss of its expression results in cardiac arrhythmias. Although gap junction gating function is regulated by A-kinase signaling, cAMP also activates Epac-Rap1 signaling which redistributes connexin 43, increasing accumulation at cell-cell contacts, and leads to formation of new gap junctions. This increase in gap junction formation is due to an increase in adherens junction, the integrin mediated cell junctions that connect the cytoskeleton on neighboring cells, formation of which is also accelerated by Rap1 signaling.

Rap has also been implicated in calcium signaling, again leading to a role in cardiac excitation-contraction. Rap 1 when bound to PLC ϵ , which acts as a Rap-GEF and is activated by isoproterenol, appears to increase calcium induced calcium release. This mechanism appears to act by increasing calmodulin-dependent protein kinase phosphorylation of the ryanodine receptor and phospholamban. However, these studies on gap junction formation and PLC ϵ -coupled calcium signaling were performed on Rap1Gap

transduced isolated and cultured neonatal rat cardiac myocytes or adult mouse ventricular cardiac myocytes. These studies therefore, do not differentiate which subtypes are responsible, since Rap1Gap can act on both Rap1a and Rap1b. Certainly use of the Rap1 subtype specific knockout models would distinguish the specific *in vivo* roles of each GTPase in live animals, in addition to the pathways identified in isolated myocytes. Indeed, preliminary examination of Rap1a-deficient mice in our laboratory shows that this subtype has a novel role in the heart and is vital for maintaining normal heart structure and electrophysiology (Jeyaraj, Chotani, Quilliam, unpublished observations).

Aside from excitation-contraction, Rap1 has been suggested to have a role in cardiac hypertrophy. Rap has been shown to mediate activation of ERK1/2, which has been linked downstream to hypertrophy, ischemia-reperfusion injury and heart failure. It has been shown in neonatal rat ventricular cardiac myocytes that activation of Rap by PGE₂ through the G-protein coupled receptor EP4, leads to ERK1/2 activation and suggests that it may affect myocyte growth. In contrast, studies in isolated neonatal rat cardiac myocytes suggest that activation of Rap1 blocks ERK 5 activation and leads to suppression of ERK 5 mediated myocyte growth. Similar studies in isolated adult rat ventricular myocytes suggests Epac1 signaling results in cardiac myocyte hypertrophy, however Rap1 signaling is not the effector molecule that regulates the downstream signaling. Further, it has been determined in H9c2 cells and neonatal cardiac myocytes that activation of Rap1 has a protective effect on NO-induced apoptosis in cardiac myocytes, suggesting multiple roles for Rap1 in the heart (Fig. 5). These conflicting reports not only suggest differing roles for Rap1 during varying stages of myocyte development, but also highlight the need for *in vivo* studies to understand the discrete roles that each subtype play in the intact heart.

Rap1 has been implicated in adrenergic signaling in cardiac myocytes, with a proposed function in cAMP metabolism, enhancement of intracellular Ca²⁺ release from the sarcoplasmic reticulum, and in maintaining homeostasis. However, the mechanistic impact of Rap1 deficiency in normal heart structure and function remains unknown. It is possible that Rap1 subtypes have a discrete function in cardiac myocytes due to compartmentalized signaling, similar to the function in vascular smooth muscle cells.

Conclusion and Future Perspective

Results from studies in varied cell types of the cardiovascular system, as well as, from animal models and knockout mice have served to determine an important role for Rap1 in the cardiovascular system. Rap1 subtypes are emerging as novel regulators of significant processes in the cardiovascular system ranging from blood vessel formation and permeability, platelet aggregation, to cardiac myocyte growth and survival. Preliminary gene expression results in our laboratory suggest a potential pathway for cardiovascular aberrations due to alterations in Rap1a (unpublished data). An intriguing observation includes potential autoregulation of Rap1a by miRNAs, which is currently under investigation. This suggests that alterations not only in Rap1a itself, but a misregulation of certain miRNA's could also lead to an imbalance of Rap activity resulting in physiological aberrations. Although preliminary and requiring validation, these studies not only link Rap1a to changes in (coding) mRNA, but also (non-coding) miRNA gene expression.

In human microvascular smooth muscle cells cAMP-Rap1- α_2C -adrenoceptors are coupled to vascular stress (vasospasm triggered inflammation and injury, or vibration-associated mechanical, chemotherapy-associated or autoimmune-disease related injury and inflammation), and under pathological conditions this signaling pathway may contribute to augmented vasoconstriction associated with peripheral vascular disorders.

Increased actin polymerization and stress fiber formation in the blood vessel medial layer can increase internal mechanical stress and potentially lead to vessel stiffness or culminate in vascular pathologies of hypertrophy and hypertension. Thus, regulation of actin is vital to vessel physiology. In this setting, the link between Rap1 and the actin cytoskeleton in the microcirculation is intriguing. Perturbation in Rap1 activity can therefore be associated with activation of hypertrophic pathways including integrin activation and increased ERK, JNK, Rho-ROCK activity, contributing to vascular pathologies. In support of this possibility, recent studies by Panchatcharam et al. show elevation of Rap1 biological activity in smooth muscle cells under hyperglycemic conditions, implicating Rap1 in diabetes associated vessel remodeling.

Further, Rap has been implicated in regulation of extracellular matrix, cellular proliferation and migration in certain fibroblast cells. This suggests a role for Rap1a in cardiac fibroblasts with potential implications in cardiac fibrosis. Therefore, the Rap1 signaling cascade acts at multiple levels of gene regulation to alter the cellular transcriptome and is poised to regulate both disease and developmental states. Taken together, these studies suggest an integral role of Rap1 in the cells of the cardiovascular system.

Recently, the identification of Rap1a and Rap1b retrogenes in both human and mice has suggested not only a new pathway to malignancy, but potentially a new mechanism for cardiovascular disease. These retrogenes have altered GTP binding affinities that would push them to a greater Rap1 activated state, thereby altering this signaling pathway and downstream events. This discovery, taken together with known functions of Rap1 suggests a potential pathway for cardiovascular aberrations due to alterations in Rap1. Further studies of the regulation and the downstream targets of Rap1 GTPases are warranted. The development of knock out murine models of both Rap1a and b subtypes should prove invaluable tools to decipher the redundant and exclusive roles of these two small G protein subtypes. As with many other small G-proteins that regulate a plethora of cellular events and signaling cascades, Rap1 may not be a suitable target for therapeutic intervention. However, further understanding of the downstream targets that are regulated by these small proteins could provide insight into signaling pathways of cardiovascular disease and provide more specific, novel therapeutic targets.

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Fig. 1. Regulation of Rap1 biological activity

Rap1 GTPases, like other small GTPases, cycle between a GTP bound (active state) and a GDP bound (inactive state), thereby acting as a molecular on/off switch in their respective signaling pathways. Cycling between the active and inactive states is facilitated by guanine nucleotide exchange factors (GEFs) which release GDP and allow binding of GTP, as well as other GTPase activation proteins (GAPs) which accelerate the GTP hydrolysis by several orders of magnitude. Activation of cell surface stimulatory G protein (G_s) coupled β_2 -adrenoceptors by the endogenous agonist norepinephrine (NE) or by the synthetic catecholamine isoproterenol (ISO) leads to increased intracellular production of the second messenger cyclic AMP by the enzyme adenylyl cyclase and activation of Rap1 by the exchange protein activated by cAMP (Epac), a GEF for Rap1. Activated Rap1 elicits biological responses via downstream effectors. Arrowhead (green, up) denotes increased interactions.

Fig. 2. Hematopoietic Cells

(A) In Rap1a null ($-/-$) mice, analysis of macrophages showed increased random non-directional movement (haptotaxis), due to less matrix adhesion, as well as reduced ability for directed migration toward chemokines (reduced chemotaxis). Reduced chemotaxis was also observed in B and T lymphoid cells (Li et al. 2007). (B) Rap1 is highly expressed in neutrophils and co-purifies with the p22 subunit of NADPH oxidase. Neutrophils from Rap1a null ($-/-$) mice therefore, have reduced superoxide production. (C) Rap1b is highly expressed in platelets, and reduced sarcoplasmic endoplasmic reticulum Ca^{+2} ATPase (SERCA3b) activity in platelets has been attributed to decreased Rap1b phosphorylation. The two proteins interact; phospho-Rap1b dissociation from SERCA3b stimulates its activity, increasing Ca^{2+} re-uptake, restoring basal Ca^{2+} level and inhibiting platelet function. Indeed, Rap1b null ($-/-$) mice exhibit defective platelet aggregation in response to integrin stimulation and show protection from arterial thrombosis. Arrowhead (green, up) denotes increase, or (red, down) denotes decrease or reduction.

Fig. 3. Endothelial cells

(A) Knock down (KD) of either Rap1a or 1b in human microvascular endothelial cells appears to diminish adhesion to the extracellular matrix (collagen or fibronectin) and slows fibroblast growth factor (FGF-2) mediated cell migration and proliferation, and also increases permeability. Rap1a or Rap1b knocked-down cells are unable to form tubular structures in response to FGF-2 stimulation. These cells have decreased ERK, p38 and Rac1 activation which are all important angiogenesis signaling molecules. In addition, Rap1a and Rap1b play a key role in β 1-integrin dependent angiogenic functions such as sprouting, tube formation, migration and adhesion. Indeed, knockout (KO) of Rap1a or Rap1b impairs the angiogenic response to FGF-2 or vascular endothelial growth factor (VEGF) determined by Matrigel plugs injected subcutaneously in wild-type or KO mice. (B) Cyclic AMP triggered increased Rap1 activity in human umbilical vein endothelial cells or in human pulmonary artery endothelial cells increases cortical actin and VE-cadherin cell surface redistribution leading to increased cell-cell contacts, tight junctions between cells, and reduces permeability. There is also change in microtubule dynamics and growth, which is Rap1-independent but Epac-dependent, required for cortical actin formation. (C) Recent studies in cultured human dermal microvascular endothelial cells activating endogenous Rap1 or using constitutively active Rap1a have shown that this leads to defective angiogenesis through regulation of thrombospondin-1 (TSP1). Inhibitor of differentiation 1 (ID1) is an inhibitor of transcription which can reduce the expression of TSP1. Activation of Rap1 can down-regulate ID1 and therefore allow expression of TSP1, causing inhibition of angiogenesis. Together both Rap1a and 1b appear to play an important role in angiogenesis signaling and the proper regulation of pro- and anti-angiogenic factors. Arrowhead (green, up) denotes increase or overexpression, and (red, down) denotes decrease or reduction.

Fig. 4. Smooth muscle cells

In human peripheral circulation microvascular smooth muscle activation of adenylyl cyclase by Gs-coupled receptors (cell surface estrogen ER α/β , β_2 -ARs, prostacyclin, E-prostanoid), by cholera toxin, or directly by forskolin increases intracellular cAMP, activates Rap, and leads to de novo synthesis of α_{2C} -adrenoceptors (α_{2C} -ARS). The response to Rap1 signaling is mediated by c-Jun NH2-terminal kinase (JNK)-c-jun signaling targeting the promoter AP-1 cis element, and estrogen to Rap2 signaling (Eid et al. 2007, 2008). Increased expression of α_{2C} -ARs by these mechanisms contributes to increased intracellular pool of receptors available for cell surface deployment, and in augmenting vasoconstriction. In these cells Rap1 signaling also activates ERK1/2 and increases cell adhesion, Rho-ROCK coupled stress-fibers. Arrowhead (green, up) denotes activation or increase.

Fig. 5. Cardiac myocytes

(A) In rat neonatal ventricular cardiac myocytes, activation of Rap1 by Epac1, results in attenuation of ERK5 activation thereby impeding ERK5 induced myocyte growth and hypertrophy. (B) Rap1 is involved in gap junction formation, examined in neonatal rat cardiac myocytes. Activation of the Epac-Rap1 pathway by cAMP shows increased accumulation of connexin-43 at cell-cell contacts, and together with cAMP activated A-kinase signaling, enhances gap junction mediated intercellular communication. (C) Rap1 isoforms are also involved in regulation of electrically evoked calcium induced calcium release, determined in adult mouse ventricular cardiac myocyte. In these cells, the Epac-Rap1 pathway is parallel to the A-kinase pathway, and is important for optimum β -adrenoceptor regulation of cardiac function. Arrowhead (green, up) denotes activation or increase, and (red, down) denotes decreased activity.