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Compartmentalisation of RAC1 Signalling

Aishwarya Payapilly and Angeliki Malliri

Cell Signalling Group, Cancer Research UK Manchester Institute, The University of Manchester, Alderley Park, SK10 4TG, UK

Correspondence: Angeliki.Malliri@cruk.manchester.ac.uk

Abstract

RAC1 signalling has been implicated in a variety of dynamic cell biological processes that are orchestrated through regulated localisation and activation of RAC1. As a small GTPase, RAC1 switches between active and inactive states at various subcellular locations that include the plasma membrane, nucleus and mitochondria. Once activated, RAC1 interacts with a range of effectors that then mediate various biological functions. RAC1 is regulated by a large number of proteins that can promote its recruitment, activation, deactivation, or stability. RAC1 and its regulators are subject to various post-translational modifications that further fine tune RAC1 localisation, levels and activity. Developments in technologies have enabled the accurate detection of activated RAC1 during processes such as cell migration, invasion and DNA damage. Here, we highlight recent advances in our understanding of RAC1 regulation and function at specific subcellular sites.

Introduction

Small GTPases are molecular switches, alternating between active GTP-bound and inactive GDP-bound states, that function to modulate almost every aspect of cell biology through a series of complex and dynamic biochemical signalling networks [1]. Large, multi-domain proteins called guanine nucleotide exchange factors (GEFs) activate small GTPases by promoting the release of GDP thus enabling GTP binding, whilst another class of proteins, the GTPase activating proteins (GAPs) inactivate small GTPases by enabling GTP hydrolysis [2]. In addition to GEFs and GAPs, guanine nucleotide dissociation inhibitors (GDIs) also regulate small GTPases by sequestering them away from activation by GEFs, and can additionally protect them from proteasomal degradation [2]. Once in the GTP-bound conformation, small GTPases bind a range of effectors, initiating downstream signalling cascades. In this review, we primarily focus on the regulation of the small GTPase RAC1.

RAC1 was first described to promote phagocytosis via NADPH activation and superoxide production [3] and growth factor-induced membrane ruffle formation via actin polymerisation [4]. Since then RAC1 has been found to be involved in a variety of cellular processes such as cell-cell or cell-substrate adhesion, apical polarity, migration, invasion, proliferation, transcription, vesicle formation, and apoptosis [1,5-7]. The ability of RAC1 to perform these functions is dependent on its tight spatio-temporal regulation. Therefore, multiple and separate pools of RAC1 present within the same cell might orchestrate different cellular processes. In the following sections, we summarise known targeting mechanisms, biochemical regulatory events and functions of RAC1 at distinct subcellular sites and highlight the impact of compartmentalised signalling on the versatility of RAC1 function.

Plasma membrane and cytoplasm

Since it was initially shown that RAC1 regulates plasma membrane-associated processes such as phagocytosis [3], lamellipodia formation and membrane ruffling [4,8], focal contact formation [8] and E-cadherin-mediated cell-to-cell contacts [9], several discoveries have been made regarding the recruitment of RAC1 to and its regulation at the plasma membrane.

Hydrophilic RAC1 does not possess a transmembrane domain therefore plasma membrane targeting is achieved, in part, via post-translational lipid covalent modification, i.e. geranylgeranylation of the cysteine-189 of the CLLL sequence at the extreme C-terminus of RAC1 [10]. This is followed by cleavage of the C-terminal tripeptide LLL, and carboxyl methylation, to increase its hydrophobicity [10]. Adjacent to the C-terminal CLLL sequence, the hypervariable polybasic and proline-rich regions are also required for RAC1 plasma membrane localisation [10]. Indeed, negatively charged phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂, PIP₂) and phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P₃, PIP₃) plasma membrane lipid molecules promote plasma membrane targeting of small GTPases by interacting with their positively charged polybasic sequence [11]. Interaction of RAC1 with PIP₃ at the plasma membrane is required for its localisation at the leading edge of migrating cells [12]. The polybasic region in RAC1 is also known to preferentially bind phosphatidylserine [13]. Moreover, clustering of RAC1 at the plasma membrane, via its polybasic sequence, may lead to increased protein-protein interactions or changes in protein conformation [14]. Indeed, a recent study confirmed that RAC1 forms nanoclusters of 50-100 molecules at the plasma membrane of migrating fibroblasts and that the polybasic region is sufficient for nanoclustering [15]••. This study also showed that RAC1 nanoclusters are increased upon RAC1 activation and through interaction with GEFs, GAPs and effectors [15].

RAC1 can also be targeted to the plasma membrane via interaction with membrane-localised GEFs, adhesion molecules, and receptor tyrosine kinases (RTKs) and scaffold proteins [16]. For example, the transmembrane protein LGR5, expressed in adult intestinal crypt cells and colon cancer cells, recruits IQGAP together with RAC1 to the plasma membrane and thereby increases levels of cortical F-actin and enhances cell-cell adhesion [17].

Post-translational, geranylgeranyl lipid-modified RAC1 is often held in its inactive, GDP-bound form in the cytoplasm by RhoGDIs. However, extracellular and intracellular signals enable RhoGDI dissociation and promote RAC1 membrane targeting and activation [18]. In addition to the geranylgeranyl type of prenylation, RAC1 also undergoes reversible palmitoylation at cysteine-178, which stabilises RAC1 at actin cytoskeleton-linked membrane regions, to enable functional membrane dynamics during spreading and migration [19].

Advances in microscopy techniques such as Fluorescence Resonance Energy Transfer (FRET), has enabled the simultaneous assessment of localisation and activation of Rho-GTPases in real-time in living cells [20]. This has refined our understanding of RAC1 function during lamellipodia formation and migration. FRET-based studies indicated that active RAC1 forms a broad gradient just behind a narrow region of active RHOA that is located at the leading edge of migrating fibroblasts [21,22]. Active RAC1 antagonises RHOA activity and by restricting RHOA activity to the leading edge reinforces newly formed protrusions [22]. FRET-based studies have also shed light on the role of RAC1 during various cellular processes that involve changes in plasma membrane dynamics. For example, the use of a single-chain RAC1 FRET biosensor showed that increased RAC1 activity is required for invadopodia disassembly in invading tumour cells [23]. Another FRET-based study found that RAC1-activity peaked immediately before membrane closure during macropinocytosis, the formation of endocytic vesicles during plasma membrane ruffling [24]. Reversible photo-switching of RAC1 together with FRET imaging of RAC1 activity, during receptor-mediated phagocytosis of erythrocytes, demonstrated that RAC1 ON and OFF states are required for lamellipodial extension and cup-constriction respectively [25]•. Super resolution microscopy and live-cell imaging FRET-based biosensors for RAC1 also demonstrated that the activity and differential localisation of RAC1 is required for the formation of macrophage tunnelling nanotubes (TNTs), which are membranous channels that connect cells to enable the transfer of signalling material that can include vesicles and organelles [26]••. Finally, FRET-based conformational changes demonstrated that constitutively active RAC1 together with CDC42 in exosomal multivesicular endosomes (MVEs) are required for the formation of actin filaments during cargo sorting into intraluminal vesicles of MVEs [27]•. In conclusion, membrane localisation of RAC1 is regulated via its intrinsic biochemical properties that enable interaction with phospholipids and various membrane-attached proteins, which may in turn serve to positively or negatively regulate RAC1 activity and levels during various cellular processes involving changes in plasma membrane structure and function.

Nucleus

Most studied RAC1 functions are mediated at or around the plasma membrane. However, RAC1 activity was recently demonstrated at the outer envelope of the nuclear membrane, stimulated by the RAC1 GEF, STEF/TIAM2, where it regulates the formation

or assembly of apical actin filaments required to orientate the nucleus during migration [28]. Furthermore, several studies have identified the presence of nuclear RAC1 and described the mechanisms promoting nuclear localisation of RAC1 and its functional implications. For example, nuclear localisation signals identified in the C-terminal polybasic region of RAC1 were shown to promote its nuclear accumulation [29]. Nuclear localisation of RAC1 is cell cycle-dependent, with increased nuclear localisation during late G2 phase and nuclear exclusion during early G1 [30]. RAC1 is also found to localise to centrosomes during G2, prophase and early prometaphase where it regulates centrosome separation and mitotic entry [31,32]. Additionally, nuclear import of bacterial toxin-activated RAC1 is mediated by its direct interaction with nuclear import receptor karyopherin alpha2 (KPNA2) [33]. Phosphorylation of threonine-108 in RAC1 by activated ERK, upon epidermal growth factor (EGF) treatment, also leads to increased nuclear RAC1 [34]. Furthermore, spatiotemporal resolution of fluorescence lifetime imaging microscopy (FLIM)-FRET demonstrated that DNA damage induced active, monomeric RAC1 segregation into the nucleus from inactive, dimeric RAC1 in the cytoplasm [35].

Various nuclear RAC1 functions have been identified. For example, RAC1 directly interacts with nuclear proteins such as the transcription factor STAT3 to regulate its activity by promoting its phosphorylation [36] and with BCA3 to promote nuclear NF-kB signalling [37]. Moreover, nuclear RAC1 directly interacts with nucleophosmin 1 (NPM1), that limits the GTP-loading of RAC1 and reduces cell spreading [38]. The interaction of RAC1 with NPM1 was also recently confirmed via a mass spectrometry screen [39]••. In this study it was demonstrated that RAC1 localises to both nucleus and nucleolus and that NPM1 acts as a chaperone to promote the nuclear export of RAC1. The authors detected two functional nuclear export signals (NES) in RAC1. Mutation of the NES sequences increased RAC1 nuclear accumulation, resulting in altered lamin A/C and emerin localisation, nuclear morphology deformation and increased invasiveness [39]. Besides acting as a RAC1 chaperone, NPM1 also forms a nuclear protein complex with RAC1, its GEF, ECT2, and the nucleolar transcription factor, upstream binding factor 1 (UBF1), to promote ribosomal DNA transcription essential for KRAS-Trp53-induced lung tumorigenesis [40]••. Recently, TIAM1, another RAC1 GEF, has also been found to negatively regulate YAP/TAZ transcriptional activity in the nucleus of colorectal cancer cells suppressing their invasiveness [41]•. Moreover, RAC1 nuclear accumulation in complex with β -catenin occurs following Wnt or RAC1 activation, resulting in RAC1-dependent phosphorylation of β -catenin on serines 161 and 605,

promoting the formation of nuclear β -catenin- lymphoid enhancer factor (LEF-1) complexes, necessary for transactivation of Wnt-dependent genes [42]. Identification of these various nuclear RAC1 functions and the mechanistic implications of nuclear import and export sequences in RAC1, will undoubtedly expand our understanding of RAC1 signalling.

Mitochondria

The mitochondrial localisation of RAC1 is the most recent and novel subcellular localisation of RAC1. Mitochondrial membrane localisation of RAC1 was first discovered through the interaction of RAC1 with BCL2 in BCL2-overexpressing B-cell lymphoma cells [43]. This interaction stabilises the anti-apoptotic, BCL2-mediated superoxide production required to maintain a mild pro-oxidant intracellular milieu. The role of RAC1 in superoxide production through NADPH oxidase activation is well documented [7]. Inhibiting the BCL2-RAC1 interaction or blocking superoxide production, decreased mitochondrial O_2^- levels and sensitised lymphoma cells to apoptosis [43]. In support of this finding, formation of a mitochondrial membrane complex between RAC1, sigma-1 receptor (Sig1 R), 1,4,5-triphosphate receptor and BCL2, also regulates neuroplasticity and prevents apoptosis and autophagy by maintaining mild oxidative stress [44]. Interestingly, in contrast, loss of mitochondrial RAC1-mediated superoxide production promoted cell survival under certain conditions. For example, the inhibition of RAC1-BCL2 complex formation in the mitochondria, by siRNA-mediated RAC1 reduction, had a protective effect in both an in vivo cerebral ischaemic reperfusion injury rat model and neurotoxicity induced by high glucose in PC-12 cells [45]. Inhibition of RAC1 activity also protected retinal bovine cells exposed to high glucose from ROS production, mitochondrial DNA damage and apoptosis [46]. Therefore, mitochondrial RAC1-mediated superoxide production can have either cyto-protective or cytotoxic effects, depending on cell type and environmental conditions such as glucose levels.

Other mitochondrial functions of RAC1 include its contribution to the formation of pulmonary fibrosis that occurs due to increased H_2O_2 production in alveolar macrophages dependent on the direct transfer of an electron to Cys-178 of RAC1 from cytochrome c [47]. The same study also showed that Cys-189 of RAC1 was required for its mitochondrial import. Activation of mitochondrial STAT3 by BCL2 is also dependent on RAC1-GTP-induced superoxide production [48]. Therefore, with the establishment of the mitochondrial localisation of RAC1, several studies now link this localisation to

inhibition or promotion of cytotoxicity and to pathologies. Future characterisation of the mechanisms that lead to the mitochondrial localisation of RAC1 and the molecular pathways by which mitochondrial RAC1 regulates survival would help define its impact on various diseases.

Localisation of GEFs and GAPs as a mode of regulation

The ~20 members of the Rho-GTPase family are greatly outnumbered by their regulators, with ~80 mammalian GEFs and ~70 mammalian GAPs [2]. RAC1 alone can be activated by over 30 different GEFs and inactivated by several GAPs. This large number of regulators spatially and temporally coordinates the molecular switching of Rho-GTPases. Moreover, besides activating Rho molecules, GEF proteins control the repertoire of effector proteins they interact with. Thus, the RAC1 GEF, PREX1, functions as a scaffold to enhance the interaction of active RAC1 with a distinct group of effectors, such as FLII, an actin remodelling protein, thereby regulating cell contraction and migration [49].

Most GEFs possess lipid-binding domains, such as pleckstrin homology (PH) and dock homology region-1 (DHR-1) domains with varying affinities for phosphoinositides, such as PIP2 and PIP3 [50], which promote the membrane targeting of GEFs. Bin-Amphiphysin-Rvs (BAR) domains in GEFs and GAPs can also target the proteins to the plasma membrane based on specific membrane curvature [51]. Localisation of the F-BAR domain containing RhoGAP protein, srGAP2, to contact protrusions is required to halt extensions during contact inhibition of locomotion by inhibiting RAC1 at these specific regions [52]. The GEF, TRIO, primarily acts as a scaffold to recruit and anchor RAC1 along the surface of cells facing flow, promoting endothelial cell alignment [53]•.

GEF localisation in response to various extracellular and intracellular stimuli can result in recruitment and complex formation of Rho-GTPases and their effectors at specific subcellular sites. In particular, activation of RTK by growth factors or ligands can contribute to the spatio-temporal regulation of RAC1 activity through recruitment or phosphorylation of various GEFs. EGFR signalling in lung adenocarcinoma promotes accumulation of the RAC1-specific GEF TIAM1 through phosphorylation by AKT that promotes its interaction with the 14-3-3 scaffold protein, leading to increased TIAM1 stability [54]•. Consequently, increased TIAM1 stability promotes RAC1-mediated tumorigenesis [54]. Additionally, HGF-induced accumulation of the functional complex

between the CDC42/RAC1-specific GEF, Asef, and IQGAP1 at the cell cortex is essential for endothelial cell barrier function through RAC1 activation, leading to reorganisation of cortical actin filaments by the interaction of IQGAP1 with Arp3 and cortactin [55]. Plasma membrane lipid PIP3 and G β γ are secondary messengers generated downstream of RTK and G-protein coupled receptor (GPCR) activation respectively, and can interact with and activate GEFs such as PREX1 [56], PREX2 [57], VAV and SOS1 [58,59] to subsequently induce RAC1 activity.

Recruitment of GEFs such as β PIX, DOCK180, TRIO, VAV1, VAV3, and TIAM1 to nascent focal adhesions occurs due to the interaction of GEFs with components of the adhesion complex formed in response to integrin clustering [60]. The recruitment of the DOCK1 GEF by ELMO to cell-cell adhesion sites in MDCKII cells is required for establishment of strong cell-cell adhesions through localised RAC1 activity, actin remodelling and E-cadherin spreading [61]. Conversely, DOCK5 recruitment to focal adhesions is inhibited by GIT2. Depletion of GIT2 or inhibition of GIT2 focal adhesion targeting leads to recruitment and interaction of DOCK5 with Crk at membrane protrusions and nascent focal adhesions promoting activation of the Crk-p130Cas pathway, RAC1-mediated lamellipodial extensions and focal adhesion turnover [62]•.

GEFs and GAPs can also form regulatory complexes themselves, to precisely modulate Rho-GTPase activity [18]. Specifically, the modulation of RAC1 activity during excitatory synaptogenesis is achieved by the complex formation between the RAC-GAP, BCR with the RAC-GEF TIAM1 at synapses [63]. BCR inactivates TIAM1-activated RAC1 by promoting GTP to GDP hydrolysis of active, GTP-bound RAC1, thus preventing excessive synaptic growth [63]. Additionally, TIAM1 and BCR activities can be co-ordinately regulated by the EphB receptor during excitatory synaptogenesis, most likely via promoting TIAM1 phosphorylation/ GEF activation and BCR dephosphorylation/ GAP inactivation [63]. Therefore, a balance between the opposing activities of TIAM1 and BCR, achieved via their interactions, is essential for normal dendritic spine formation.

Spatially restricted control of RAC1 activity can also be modulated through regulation of GEF abundance. TIAM1 is degraded at cell-cell junctions following ubiquitylation by HUWE1 in response to HGF stimulation and following its phosphorylation by Src. This loss of TIAM1 specifically from cell-cell adhesions facilitates cell-cell junction destabilisation, leading to increased migration and invasion [64,65]. TIAM1 could also be degraded by the Skp Cullin F-box-containing β -TrCP ubiquitin ligase, following its phosphorylation by casein kinase-1 [66]. Therefore, the localisation and regulation of

RAC1-interacting proteins contributes an additional level of complexity to the modulation of RAC1 function.

Conclusions

While significant progress has been made in uncovering the mechanisms of RAC1 function and regulation at various subcellular sites, there is a need to integrate these various signalling modules to better understand the role of RAC in cellular function as a whole. Indeed RAC1 deregulation and dysfunction is implicated in a variety of diseases, including atherosclerosis, diabetes and cancer [67]. The knowledge of how different subcellular pools of RAC1 impact its function will enable the design of chemical inhibitors or regulators that can inhibit or enhance specific RAC1 functions, as required.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure Legend

Figure 1. Compartmentalised RAC1 signalling: Summary of known subcellular RAC1 localisations or recruitments due to post-translational modifications or various biochemical interactions. (1) RAC1 is post-translationally modified at the ER. (2) Rho-GDIs hold RAC1 in its inactive GTP-bound state in the cytoplasm. (3) Both active and inactive RAC1 can form oligomers at the plasma membrane. (4) Nano-clusters of RAC1 lead to the clustering of GEFs and effector proteins. (5) During migration on an extracellular matrix, a gradient of active RAC1 is formed just behind the leading edge of the cell. (6) Activation of RTKs or GPCRs may lead to the recruitment of GEFs and result in RAC1 activation. (7) Scaffold proteins present at focal adhesions and cell-cell junctions can lead to the recruitment of GEFs and the activation of RAC1. (8) RAC1 is involved in macropinocytosis, an actin-dependent endocytic process. (9) RAC1 activity is required during maturation of MVEs. (10) At the mitochondria, RAC1 interacts with BCL2 and can also be imported into the mitochondria. (11) Active RAC1 has been detected in the nucleus. Additionally, inside the nucleus, NPM1 acts as a chaperone for RAC1 to promote its nuclear-cytoplasmic shuttling. (12) RAC1 is also present in the nucleolus and forms a complex with its GEF, ECT2, UBF1 and NPM1 to promote ribosomal DNA synthesis. (13) RAC1 localises to centrosomes and regulates centrosome separation.