

Interplay of PKA and Rac

Fine-tuning of Rac localization and signaling

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Cellular membrane receptors sense environmental changes and relay the reshaped signal through spatially and temporally organized protein-protein interactions (PPI). Many of such PPI are transient and occur in a certain cell-dependent context. Molecular switches such as kinases and GTPases are engaged in versatile PPI. Recently, we have identified dynamic interaction and reciprocal regulation of cAMP-dependent protein kinase A (PKA) and Rho-GTPase Rac signaling. We demonstrated that GTP-activated Rac acts as a dual kinase-tuning scaffold for p21-activated kinase (PAK) and PKA activities. We showed that receptor-triggered PKA trans-phosphorylation of GTP-Rac-organized PAK contributes to elevations of nuclear Erk1/2 signaling and proliferation. We discuss these recent observations and we provide additional insights how the cAMP-PKA axis might also participate in the regulation of Rac localization.

Extracellular cues are sensed by membrane-localized receptors and transmitted through a cascade of intracellular signaling events. These signaling circuits are based on defined protein complexes, a mandatory requirement for exact signal processing. Signaling cascades communicate and integrate the signaling input spatially and temporally via alterations of post-translational modifications, binding of diverse small molecules (like cAMP and GTP), and formation of dynamic protein complexes. Paradigms for dynamic protein-protein interactions (PPI) are interactions emanating from molecular switches and flexible scaffolding platforms.^{1,2} Prominent examples are interactions:

(1) between kinases, (2) between kinase subunits, (3) of kinases with diverse scaffolds and (4) of small GTPases with their multifaceted regulators and effectors.²⁻⁷ Central regulators of diverse cellular activities are Rho family GTPases.⁸ Members of this family have been classically linked to actin dynamics, cell polarity, and migration. However, they have also been implicated in several aspects of cancer progression and tumorigenesis.⁹ Rho GTPases like Rac, Cdc42 and RhoA, exist either in a GDP-bound inactive state or in a critically controlled active GTP-loaded state. In the GTP-bound conformation these molecular switches interact with distinct effector molecules to execute their cellular function.^{4,8}

Recently, we have identified dynamic associations of cellular GTP-Rac1 with the cAMP-dependent protein kinase A (PKA). We explained the functional relevance of this kinase:GTPase interplay in our recent work on reciprocal regulation of PKA and Rac signaling.¹⁰ We illustrated that GTP-activated Rac1 acts as bivalent signal integrator. GTP-Rac1 functions as activator and as scaffolding complex for distinct kinase activities without being a direct target for PKA phosphorylation. It underlines a dynamic crossroad between 2 critical receptor pathways, G protein coupled receptors (GPCR) and receptor tyrosine kinases (RTK), respectively (Fig. 1). We reported the finding that Rac1 shows binary interaction with PKA regulatory subunits (R). We have shown that the first 45 amino acids of RII β (the core sequence is conserved and almost identical in RII α) have affinities for Rac1 in RII overlay assays. Our studies revealed

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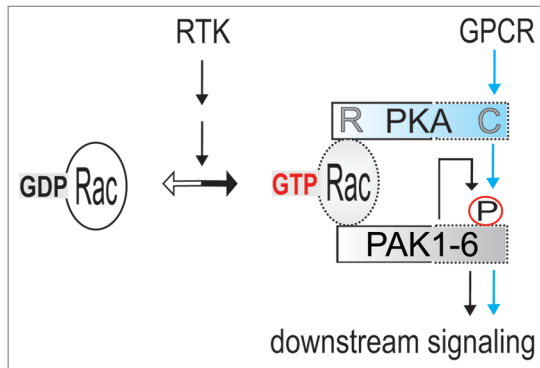


Figure 1. Reciprocal regulation of PKA and Rac signaling. As examples signals from RTK and GPCR cascades converge on the Rac:PKA complex leading to modulation of GTP-Rac downstream signaling. GTP-Rac interacts and activates its main effectors p21-activated kinases (PAK1–6). Furthermore, GTP-Rac stabilizes the inactive PKA-holoenzyme. GPCR-triggered cAMP-elevation promotes PKA dissociation and PKAc phosphorylation of GTP-Rac1-bound and GTP-Rac1-activated PAK which contributes to elevations of downstream signaling.

that complex formation of GTP-Rac1 and PKA subunits increase the occurrence of inactive PKA complexes. At the same time, we observed no direct impact of PKA activity on GTP loading of Rac1 in HEK293 cells. However, we showed that GPCR-activated PKA phosphorylates the main Rac-effectors, p21-activated kinases (PAK). Elevation of PAK phosphorylation enhances GTP-Rac1 mediated downstream signaling to Erk1/2. Overall we illustrated a novel mechanism how GTP-Rac1-bound and GPCR-controlled PKA activities participate in the regulation of Rac-PAK-Erk1/2 downstream signaling to nuclear transcription factors which are critically implicated in cellular proliferation (Fig. 1).

Previously, we demonstrated that cellular PPI between PKA subunits and diverse GTPases is not static. For example, complex formation of RII with the ‘inhibitory’ trimeric G α protein G α i was observed in response to cAMP elevation. Indeed, mechanistically different, the cAMP-triggered formation of RII:G α i elevates G α i-protein coupled receptor signaling leading, among others, to Erk1/2 activation.¹¹ In case of Rac1, we observed that complex formation of Rac1:PKA depends on cellular GTP-loading of Rac1, bound Rac1 effectors, and cAMP levels. Unlike G α i, cAMP-elevation showed a decrease of affinity between recombinant RII and cellular GTP-Rac1.¹⁰ For that reason we assumed that the inactive PKA holoenzyme interacts with GTP-Rac1. To test

our assumption, it was necessary to analyze cellular interactions of at least 3 proteins (Rac1:RII β :PKAc), which we expect to depend on cellular cAMP-levels and GTP-loading. It is a technical challenge to determine cellular interactions of multimeric enzyme complexes. Detailed cellular analyses offer the possibility to explain spatially and temporally regulated functions related to binary interaction among them. Approaches to study multisided interactions have been described.^{12–16} We chose to adapt a strategy based on dynamic sentinels based on protein-fragment complementation assays (PCA).^{11,12,14} We took advantage of a previously described HEK293 cell line stably expressing the *Renilla* Luciferase (*Rluc*)-PCA based PKA reporter, for the analyses of interactions of cellular Rac1 with the PKA holoenzyme (Fig. 2A). The advantage of the PCA-based *Rluc* PKA reporter is that it can report absolute values of PPI in vivo.¹⁷ We immuno-precipitated endogenous Rac1 complexes from the stable HEK293 cell line expressing the RII β -F[1]:PKAc-F[2] sentinel and observed bioluminescence signals originating from Rac1-associated PKA holoenzyme complexes fused to the *Rluc*-PCA fragments. To verify that the bioluminescence signals originate from the PKA-biosensor, we added an excess of cAMP to trigger dissociation of Rac1 associated RII β :PKAc holoenzymes (Fig. 2B). We further extended this strategy of analyzing trimeric cellular protein complexes by isolation of the endogenously existing

subpopulation of GTP-activated Rac1. We applied GST hybrid proteins to isolate cellular GTP-loaded Rac1. It has been illustrated previously that the PAK binding domain (PBD) is the exclusive binding site for active GTP-Rac1.^{18,19} In pull-down assays, we confirmed our previous observations that GTP-Rac1 interacts with cellular PKA subunits by showing interaction with the PCA-tagged PKA holoenzyme. This experiment also illustrates that simultaneous interaction of PBD (part of PAK) and PKA with GTP-Rac1 is possible (Fig. 2C).¹⁰ We have proven that combining PCA technology and biochemical isolations is suitable to study trimeric PPI. Our data illustrate that a subpopulation of endogenous GTP-Rac1 is bound to cytoplasmatic PKA type II β holoenzymes. We assume that GTP-Rac1, bound to its main cellular effector PAK, has the highest affinity for PKA holoenzyme complexes. This is supported by observations by our group and others that PKAc forms complexes with PAK as well.^{10,20} The PKAc:PAK interaction might stabilize this multimeric conformation emanating from GTP-Rac with 2 distinct kinase complexes.

Upon cAMP-elevation, the R:PKAc holoenzyme complex dissociates, PKAc phosphorylates substrates and takes over functions in the nucleus. We have observed that activated and compartmentalized PKAc subunits contribute to the phosphorylation of PAK. PAK pursue their specific functions in the cytoplasm but also in the nucleus. Furthermore, populations of activated PKAc and Rac1 carry out functions in the nucleus. To test if cAMP levels affect Rac1 localization by disintegration of the macromolecular GTP-Rac:PKA complex, we performed subcellular fractionation experiments with HEK293 cells treated with the general cAMP-elevating agent Forskolin. We enriched cytoplasmatic and nuclear cell fractions of HEK293 cells using an optimized biochemical protocol. Under basal conditions we observed Rac1 in both subcellular compartments. Quantification of the immunoblot signal of Rac1 obtained from four independent experiments indicates that under basal conditions roughly 10% of Rac1 is located in the nucleus of HEK293 cells. However, upon cAMP elevation for 60 min we detected an

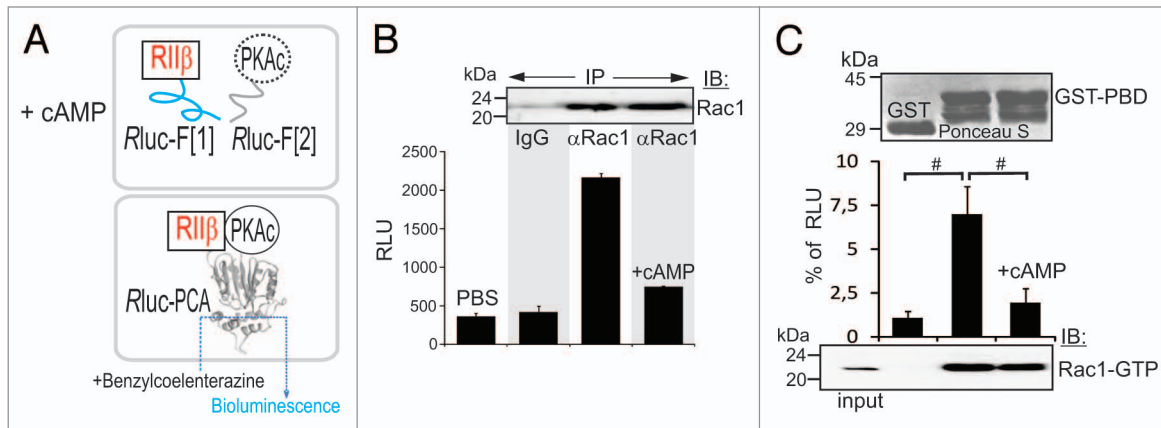


Figure 2. Rac1 forms cellular complexes with the PKA holoenzyme. **(A)** Schematic view of the principle of the Rluc-PCA based PKA reporter to quantify dynamics of PKA holoenzyme formation. cAMP-elevation triggers RIIβ:PKAc complex dissociation which is accompanied by a decrease of bioluminescence originating from complemented PCA-Rluc fragments (Rluc-F1 and Rluc-F2). **(B)** Co-immunoprecipitation (IP) of endogenous Rac1 from HEK293 cells stably expressing the Rluc based PKA-PCA sensor followed by bioluminescence analyses (representative of $n = 3$, \pm SEM). **(C)** GST-PBD pulldown of GTP-Rac1 from HEK293 cells stably expressing the Rluc based PKA-PCA sensor followed by bioluminescence analysis (RLU, relative light units, average of $n = 4$ independent experiments; \pm SEM). Indicated is the percentage of RLU compared with bioluminescence signals obtained in GST-RIIβ pulldowns, performed in parallel.

approximately 2-fold increase of Rac1 in the nuclear fraction (Fig. 3). An explicit elevation of the nuclear PKAc- α signal was not detectable in this time frame. This extends our previous findings of reciprocal regulation of cAMP-PKA and Rac signaling.¹⁰ In addition to the involvement of cAMP/PKA dependent phosphorylation of GTP-Rac1 controlled PAK, cAMP-elevations seem to participate in controlling Rac1 localization. Several components of this macromolecular GTP-Rac1:kinases complex pursue nuclear functions. The versatility of PAK1–6 activities relies partially on its subcellular localization. Activated PAKs are found in the nucleus where they directly affect gene transcription.^{21–23} Expression profiles and nuclear localizations of phosphorylated PAK4 are discussed to be prognostic markers for ovarian cancer.²¹ Also cAMP-activated PKAc subunits translocate into the nucleus where they phosphorylate their substrates with impact on the transcriptional output. PKAc can be exported from nuclei by a mechanism involving the protein kinase inhibitor (PKI). Binding of PKI causes inactivation of PKAc and export of the complex out of the nucleus.^{3,24–27} Furthermore, it has been postulated that Rac1 accumulation in the nucleus promotes cell division. The C-terminally located interaction site for PKA R subunits in Rac1 is flanked at

the C-terminus with a polybasic sequence which contains a nuclear localization signal which is responsible for targeting Rac1 into the nucleus. Furthermore, this C-terminal sequence participates in proteosomal degradation.^{28–30} It has been described that transcription factors like STAT3 directly interact with Rac1. Binding of GDP or GTP-bound Rac1 either inhibited or activated STAT3 activities. Overall, interactions and localizations of Rac and its interaction partners STAT and PAK are critically implicated in cancerogenesis.^{22,31–34} The binding of distinct hormones to GPCRs enhances cAMP-dependent PKA activities which regulate cell growth by various means. It is a hallmark of cAMP to either boost proliferation in many cell types or inhibit cell growth using distinct mechanisms in others.^{10,35–37} We hypothesize that physical interactions of PKA with the GTP-Rac machinery accounts for an enhancement of proliferative signaling in a cell/tissue type specific way. Previously, we have confirmed an involvement of PKA:Rac:PAK4 signaling in proliferative responses in an ovarian cancer cell system (OVCAR3).¹⁰ However, additional investigations need to be performed to understand the mechanism how the cAMP-PKA signaling axis affects Rac localization and the details of nuclear signaling leading to enhanced proliferation.

Material and Methods

Rluc-PCA based assays for analyses of trimeric protein complexes

We have generated stable HEK293 cell lines co-expressing the PCA hybrid proteins RIIβ-Rluc-F[1] and PKAc-RlucF[2], as previously described.¹⁷ We performed either immuno-precipitations (IP) of Rac1 with polyclonal anti-Rac1 (Santa Cruz; sc95) antibodies or pull-downs of GTP-Rac1 with GST-PBD (three hours). Following 3 washing steps with lysis buffer and 2 subsequent washing steps with PBS, we subjected the probes to bioluminescence analyses using the LMax-TM-II-384 luminometer (Molecular Devices). Rluc bioluminescence signals were integrated for 10 s following addition of the Rluc substrate benzyl-coelenterazine (5 μ M; Nanolight, #301). We confirmed precipitation of Rac1 and GTP-Rac1 using monoclonal anti-Rac1 (Millipore, #05–389) antibodies in immuno-blotting analyses.

Subcellular fractionation assay

HEK293 cells grown in 5 x 100 mm cell culture dishes were washed and precipitated in ice-cold PBS buffer following Forskolin exposure (60 min, 50 μ M). After resuspension in Extraction Buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 1 mM PMSF, 2 μ g/mL aprotinin, 1 μ g/mL leupeptin,

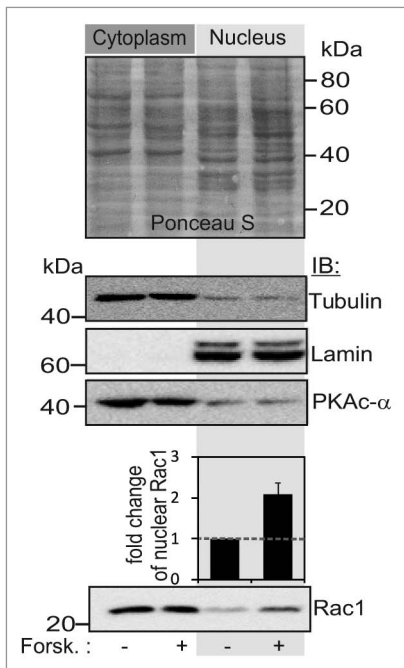


Figure 3. Biochemical localization studies of Rac1. Following general cAMP-elevation with 50 μ M Forskolin for 60 min, we performed subcellular fractionations of untreated and treated HEK293 cells. Ponceau S staining indicates similar amounts of proteins used for the immuno-blotting analyses (monoclonal antibodies vs. Rac1 and PKAc have been used). Tubulin is the marker for cytoplasmic fractions, lamin A/C acts as marker for the nuclear fraction. Quantification of four independent experiments is shown in the bar graph (\pm SEM), values have been normalized on the loading control lamin A/C.

1 μ g/mL pepstatin), cells were lysed with 25 strokes using a Potter S (B. Braun Biotech International). To precipitate the nuclei, the lysed cells were centrifuged at 1,200 \times g (HB-6 rotor, Sorvall). The supernatant corresponds to the cytoplasmic fraction. Additional centrifugation steps (25,000 \times g, SS-34 rotor, Sorvall) were performed to remove residual cytoplasmic material. The crude nuclei fraction was resuspended in Extraction Buffer C (20 mM HEPES pH 7.9, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 μ g/mL pepstatin) and homogenized with 30 strokes. The lysate was clarified (25,000 \times g, HFA 15.2 rotor, Heraeus Megafuge 1.0R) and the resulting supernatant was dialyzed for 5 h against Extraction Buffer D (20 mM HEPES pH

7.9, 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF). The nuclear fraction was frozen in liquid nitrogen and stored at -80°C . In immunoblotting analyses we used anti- α -Tubulin (Sigma Aldrich, #T5168) and anti-Lamin A/C (Cell Signaling, #4777) antibodies as markers for cytoplasmic and nuclear fractions, respectively; in addition we used anti-PKAc antibodies (BD Bioscience, # 610981).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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