Activated G protein-coupled receptors (GPCRs) and receptor tyrosine kinases relay extracellular signals through spatial and temporal controlled kinase and GTPase entities. These enzymes are coordinated by multifunctional scaffolding proteins for precise intracellular signal processing. The cAMP-dependent protein kinase A (PKA) is the prime example for compartmentalized signal transmission downstream of distinct GPCRs. A-kinase anchoring proteins tether PKA to specific intracellular sites to ensure precision and directionality of PKA phosphorylation events. Here, we show that the Rho-GTPase Rac contains A-kinase anchoring protein properties and forms a dynamic cellular protein complex with PKA. The formation of this transient core complex depends on binary interactions with PKA subunits, cAMP levels and cellular GTP-loading accounting for bidirectional consequences on PKA and Rac downstream signaling. We show that GTP-Rac stabilizes the inactive PKA holoenzyme. However, β-adrenergic receptor-mediated activation of GTP-Rac-bound PKA routes signals to the Raf-Mek-Erk cascade, which is critically implicated in cell proliferation. We describe a further mechanism of how CAMP enhances nuclear Erk1/2 signaling. It emanates from transphosphorylation of p21-activated kinases in their evolutionary conserved kinase-activation loop through GTP-Rac compartmentalized PKA activities. Sole transphosphorylation of p21-activated kinases is not sufficient to activate Erk1/2. It requires complex formation of both kinases with GTP-Rac1 to unleash cAMP-PKA-boosted activation of Raf-Mek-Erk. Consequently GTP-Rac functions as a dual kinase-tuning scaffold that favors the PKA holoenzyme and contributes to potentiate Erk1/2 signaling. Our findings offer additional mechanistic insights how β-adrenergic receptor-controlled PKA activities enhance GTP-PKA-mediated activation of nuclear Erk1/2 signaling.

signal transduction | cross-talk

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ignal transduction cascades coordinate the plethora of extracellular stimuli into biological responses within cells. The specificity of receptor-initiated signaling responses is encoded by spatial and temporal dynamics of downstream signaling networks (1). These networks, initiating from e.g., the G protein-coupled receptor (GPCR) superfamily and receptor tyrosine kinases (RTK), tightly regulate signaling pathways at several critical points via feedback loops and cross-talk among other pathways (2–5). A large number of GPCR signaling cascades uses cAMP as an intracellular second messenger (3, 6). In response to hormone binding to distinct GPCRs, cAMP is produced and binds to its canonical effector, the cAMP-dependent protein kinase A (PKA). PKA binding to the PKA regulatory subunits (R) induces dissociation of the tetrameric PKA holoenzyme, resulting in active PKA catalytic subunits (PKAc; Fig. 1C) (7, 8). To ensure substrate specificity, PKA is tethered to distinct subcellular compartments through physical interaction with A-kinase anchoring proteins (AKAPs; refs. 9 and 10). It has been long regarded that the GPCR-cAMP-PKA signaling axis participates among others, in the regulation of cell growth, differentiation, and motility (9–13). Such fundamental cellular functions are controlled by mitogenic signals that are transmitted through cascades involving crucially regulated mitogen-activated protein kinases (MAPK) like Erk1/2 (14). It has been described that the Rho GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1) participates in the regulation of transformation, growth, and survival of tumor cells at least partially by controlling extracellular signal-regulated kinase (Erk)1/2 activation (15, 16). So far, several functionally diverse means have been illustrated how hormone-triggered cAMP pulses participate in the regulation and transmission of downstream signals originating from RTK and GPCR cascades (18). Moreover, the mentioned β2AR pathway (3, 6) leading to cAMP-PKA activation has been implicated in malignant cell growth in a mouse model of ovarian carcinoma (24). Here, we examine a unique mechanism how cAMP-activated PKA is involved in the regulation of Erk1/2 activities. We report that Rac1, a member of the Rho GTPase family (25), contains AKAP properties and, thus, show direct interactions with PKA R subunit type II (RIIβ) in vitro and in vivo. Our cell-based studies demonstrate that complex formation of active GTP-Rac1 and PKA increase inactive PKA complexes but does not directly affect Rac1 GTPase activity. However, we unveil that β2AR-activated PKA phosphorylates the main Rac effectors, p21-activated kinases (PAKs), which leads to GTP-Rac1-dependent elevation of downstream signaling to Erk1/2. We describe a mechanism through which PKA, a key component of cAMP-GPCR cascades, participates in the regulation of RTK-activated Rac-PKA-Erk1/2 signaling to nuclear transcription factors.

Results

PKA RII Subunits Form Protein Complexes with Rac1 in Vitro and in Vivo. We performed a systematic screen using a “Venus” yellow fluorescent protein (YFP) protein-fragment complementation assay (PCA) in mammalian cells to identify transient protein–protein interactions emanating from PKA R subunits with downstream components of RTK and GPCR cascades (18). We identified interaction of PKA RIIβ with the small Rho family GTPase Rac1 (Fig. 1D). The PKA homodimer RIIβ:RIIβ was restricted to the cytosol, but both the Rac1:Rac1 homodimer and the identified Rac1:RIIβ complex were primarily localized to the plasma membrane (Fig. 1B). This observation highlights physical connection between PKA and Rac1, which are key effectors of canonical receptor cascades, e.g., of the GPCR and RTK family, respectively (Fig. 1C). We confirmed direct protein-protein interaction of Rac1:RIIβ by using two independent in vitro tests: First, we confirmed...
complexes, suggesting that Rac1 interacts preferentially with the inactive PKA holoenzyme (Fig. 1G and Fig. S1E).

**Analyses of Binding Regions of RIIβ on Rac1 and Related GTPases.** To map the interaction sites on Rac1, we performed a peptide spotting experiment of Rac1b to confirm interaction in vitro and to determine specific amino acids required for RIIβ binding (18). We identified two potential binding sites located at the C terminus of Rac1, in regions distinct from GTPase-activity sites (“switch regions”; Fig. 2A and Fig. S1F). In the Rac1 structure, we highlight two binding sites (BD1 and BD2) located in close vicinity to permit protein:protein interaction with RIIβ (Fig. 2B, Left). We allocated an amphipathic helix motif (BD2) almost matching with a described consensus site for RII-binding domains (RIBD) found in other AKAPs (Fig. 2B, Right). Red brackets and red F (Phenylalanine) indicate the difference; ref. 29. Structural and detailed biochemical analyses have specified that amino acids 1–45 of RIIβ cover the primary determinants for binary protein:protein interaction of R subunits with AKAPs (30, 31). We confirmed that the first 45 amino acids of RIIβ are sufficient to interact with full-length Rac1 (Fig. 2C). Next, in a GST-pulldown experiment with GST and GST-BD2 hybrid proteins, we precipitated endogenous RIIβ from HEK293 cells independent from altering cAMP levels (Fig. 2D). Next, we tested conserved members of the Rho GTPase family for AKAP properties (Fig. S2A) (32). We observed that substitutions of amino acids in the nonpolar region of BD2 are sufficient to decrease the affinity between GST-RIIβ and peptide mutants of Rac1/2/3. Surprisingly, we detected interaction of human RIIβ at least with the *Saccharomyces cerevisiae* Rho GTPase Rho1 (Fig. 2E). To further characterize the RII binding sites in Rac1, we performed alanine substitution scanning of BD1 and BD2 to identify key amino acids responsible for the interaction (Fig. S2B and C). Following structural examination of BD1 in Rac1 and based on the alamine substitution experiment, we generated the GTP-Rac1 mutants Q61L-A1 (H104A, P106A) and Q61L-A2 (T108A, P109A). Moreover, we disrupted the C-terminal amphipathic helix of BD2 of GTP-Rac1 and generated the Q61L-P mutant (I173P). Exchange of I173P abolished binding of Q61L-P to both GST-RIIβ and GST-PBD. In contrast, A1 mutations of BD1 in GTP-Rac1 (Q61L-A1) had no impact on PBD binding but showed a slight reducing effect on complex formation of GTP-Rac1:RIIβ (Fig. 2F). These results support the notion that both binding domains of Rac1 are involved in the formation of cellular complexes with RIIβ.

**GTP-Rac1 Stabilizes the PKA Holoenzyme.** To get insights whether PKA RIIβ subunits have different affinities for the mentioned Rac1-YFP variants, we performed GST-pulldown experiments. We observed preferential binding of GST-RIIβ to cellularly expressed GTP-Rac1 (Q61L) (Fig. 3A and Fig. S3A). This result underlines that PKA preferentially binds to cellular GTP-Rac1 complexes that exist bound to downstream effectors like PAKs. First, we evaluated the effect of GTP-Rac1 overexpression on PKA activity. We transiently overexpressed indicated variants of Rac1 in the osteosarcoma cell line U2 (U2OS) and in HEK293 cells, which both stably express the Rac1-YFP (phospho) based PKA reporter (Fig. 3B; ref. 33). Exclusively, the overexpression of GTP-Rac1 (Q61L) increased the inactive complex of RIIβ:PKAc in transient transfections in both cell lines significantly approximately 50% (Fig. 3B). Following the analyses of the impact of GTP-Rac1 on PKA signaling, we set the focus on investigating the influence of PKA activities on Rac1 downstream signaling to the nucleus.

**CAMP-Dependent Regulation of GTP-Rac1 Signaling to Erk1/2.** It has been reported that Rac1 participates to promote the Raf-Mek-Erk cascade (16, 34–36). To investigate the role of GTP-Rac1 in Erk1/2 activation, we used HEK293 cells, which show no increase of Erk1/2 phosphorylation in response to Forskolin alone (18). We confirmed observations that exclusively the overexpression of the constitutive active GTP-Rac1 variant activates Erk1/2 (16) (Fig. 3C). Activation of Erk1/2 causes activation
of nuclear transcription factors like Elk1. We observed that ectopic expression of GTP-Rac1 further enhances activation of gene transcription by Erk1/2-mediated Elk1 phosphorylation (Fig. 3D). Next, we tested the impact of cAMP elevation on Erk1/2 activity. We observed that cAMP elevation enhances Erk1/2 activation exclusively in the presence of GTP-Rac1 to above threefold (Fig. 3E). We tested the GTP-Rac1 mutants presented in Fig. 2F for interference with signaling to Erk1/2. We observed that the GTP-Rac1 mutant Q61L-A1 prevented Forskolin-mediated Erk1/2 activation. This observation underlines that BD1 in Rac1 participates to permit cAMP-PKA-mediated Erk1/2 activation (Fig. 3F). However, to confirm that PKA kinase activity accounts for potentiation of Erk1/2 phosphorylation in the presence of GTP-Rac1, we pretreated cells with the selective PKA inhibitor KT5720. PKA inhibition prevents the Forskolin-mediated potentiation of Erk1/2 phosphorylation (Fig. S3C). To analyze whetherGPCR cascades linked to cAMP production participate in the regulation of GTP-Rac1 signaling to Erk1/2, we tested the involvement of the β2AR that, among other vital cellular functions, has also been linked to aberrant proliferation (3, 6, 24). As already known, this notion that β-adrenergic agonist Isoproterenol (Iso) induces a transient increase of Erk1/2 phosphorylation in HEK293 cells stably expressing the β2AR. Isoproterenol-triggered activation of Erk1/2 is mediated via pathways that are sensitive to both the PKA inhibitor H89 and Gai inhibitor pertussis toxin (37). In addition, it has been described that GPCR-bound β-arrestin participates in signaling to Erk1/2 (38). Isoproterenol treatment of HEK293-β2AR cells transiently overexpressing GTP-Rac1 further potentiated Erk1/2 phosphorylation (Fig. 3G). These data support the notion that β2AR provoked cAMP release and subsequent activation of GTP-Rac1–bound PKA further promotes activation of Erk1/2 as illustrated with the blue arrows in the scheme.

**PKA Activities Affect p21-Activated Kinase Signaling to Erk1/2.** First, we confirmed other studies that Rac1 is not a direct target for PKAc phosphorylation, using the PKA substrate RhoA as positive control (39) (Fig. 4A). We aimed to identify the target of PKA phosphorylation, which links GTP-Rac1 to Erk1/2 activities. In Fig. 4, we demonstrate that cellular GTP-Rac1 complexes have the highest affinity for the PKA holoenzyme. The key effector and conserved interacting partner of active GTP-Rac1 are PAKs: PAK1–6 (26, 27, 40). It has been described that PAK1 is phosphorylated by PKA (41). Sequence alignment and phosphorylation prediction highlight the existence of a PKA consensus site in the evolutionary conserved activation loop of PAKs (Fig. 4B). This highly conserved site can be found in the activation loop of PKAc subunit as well (Thr197). In both cases, autophosphorylation has been confirmed upon kinase activation (42, 43). In Fig. 4B, we highlight structural conservation. Just recently Park et al. (44) showed that PKAc subunits form protein complexes with its substrate PAK4. That is why we hypothesized that physical association of GTP-Rac1 with both kinases, PKA and PAKs, accounts for the observed GTP-Rac1 and PKA-dependent Erk1/2 activation. First, we confirmed the possibility of physical interaction of PKAc subunits with PAK1 in dot blot analyses. We identified two preferential stretches of interaction close to the PKA consensus site for phosphorylation in the ultimate C terminus of PAK1 (Fig. S3 D and E). These results indicate that interaction of PKAc:PAK might be involved in stabilizing the interaction of the PKA holoenzyme with Rac1 variants (Figs. 1G, 2F, and 3 A and B). Next, in two independent cell systems, we tested whether PKA activation causes PAK phosphorylation, which has been described to modulate Raf-Mek-Erk signaling (16, 34–36): First, we show that both overexpression of PKAc and Forskolin treatment elevates PAK1-Thr423 and PAK2-Thr402 phosphorylation (Fig. S4A). Next, we analyzed whether type II PAKs (PAK4–6) are targets of PKA as well. Upon general cAMP elevation and following activation of β2AR (Iso), we observed significant elevations of PAK4-Ser474 phosphorylation in the absence and presence of indicated Rac1 versions. Overexpression of GTP-Rac1 causes basal levels of PAK4-Ser474 phosphorylation, which can be further raised by PKA activation (Fig. 4C). However, PKA-mediated PAK4 phosphorylation is not sufficient to promote downstream Erk1/2 activation. These data highlight that PKA activities in the...
presence of GTP-Rac1 further enhance PAK phosphorylation, which elevates GTP-Rac:PAK signaling to Erk1/2 (Fig. 4B). Next, we tested GTP-Rac1 mutants for interference with signaling to PAK4 and Erk1/2. We detected that basal enhancements of Erk1/2 and PAK4-Ser474 phosphorylations are comparable in the presence of Q61L or Q61L-A1. However, we observed that compared with GTP-Rac1 (Q61L), the mutant Q61L-A1 significantly reduced Isoproterenol-mediated Erk1/2 and PAK4-Ser474 phosphorylation. This observation underlines that BD1 of GTP-Rac1 participates to boost PAK phosphorylation. This observation underlines that BD1 of GTP-Rac1 participates to boost PAK phosphorylation. This observation underlines that BD1 of GTP-Rac1 participates to boost PAK phosphorylation.

Next, we performed cellular proliferation assays (18 h) to test how far βAR activities and scaffolding complexes like RII: Rac1 are relevant for proliferation. Activation of βAR cascades showed a significant enhancement of proliferation of OVCAR3 cells (Fig. 4F, doubling time of 4 d; ref. 46). In addition, we tested membrane permeable AKAP:PKA disrupting peptides (Fig. 4D), which significantly reduced proliferation (Fig. 4F). The peptides (in the used concentration of 10 μM) showed no impact on the formation of the PKA-holoenzyme complex (Fig. 4E). These results support the notion that βAR-controlled PKA activities contribute to proliferative effects by active participation in the GTP-Rac:PKA signaling axis leading to Erk1/2 phosphorylation and activation of the nuclear transcription factor Elk1. Overall, our observations highlight a bidirectional function of the Rac1:PKA complex. GTP-Rac1 enriches and compartmentalizes inactive PKA complexes through binary interaction with RII subunits. However, the three-part complex of PAK:GTP-Rac1:PAK acts as compartmentalized modulator of Erk1/2 activities that is controlled first by GTP loading, second through physical interaction with both kinases (PKA and PAK), third through PKA activities, and last but not least through βAR-triggered cAMP:PKA activities (Fig. 4G).

Discussion
Rac1 belongs to the Rho GTPase family of small GTP-binding proteins. Prominent members of this family Rho, Cdc42, and Rac emerge to regulate a diverse array of cellular events, including control of the reorganization of the cytoskeleton, cell growth, and activation of diverse protein kinases (25). We now report that Rac1 contains AKAP properties and directly interacts with PKA RII subunits. Complex formation of RII:Rac1 is not static; it depends on cellular GTP loading, bound Rac effectors, and cAMP elevation, and it accounts for bidirectional consequences on signal transmission. We describe a mode of regulation that is complementary to the regulation by guanine exchange factors (GEFs). GTPase activating proteins (GAPs), and guanine dissociation inhibitors. We show that cAMP elevation has no direct impact on Rac1 GTPase activities and scaffolding complexes like RII: Rac1 are relevant for proliferation. Activation of βAR cascades showed a significant enhancement of proliferation of OVCAR3 cells (Fig. 4F, doubling time of 4 d; ref. 46). In addition, we tested membrane permeable AKAP:PKA disrupting peptides (Fig. 4D), which significantly reduced proliferation (Fig. 4F). The peptides (in the used concentration of 10 μM) showed no impact on the formation of the PKA-holoenzyme complex (Fig. 4E). These results support the notion that βAR-controlled PKA activities contribute to proliferative effects by active participation in the GTP-Rac:PKA signaling axis leading to Erk1/2 phosphorylation and activation of the nuclear transcription factor Elk1. Overall, our observations highlight a bidirectional function of the Rac1:PKA complex. GTP-Rac1 enriches and compartmentalizes inactive PKA complexes through binary interaction with RII subunits. However, the three-part complex of PAK:GTP-Rac1:PAK acts as compartmentalized modulator of Erk1/2 activities that is controlled first by GTP loading, second through physical interaction with both kinases (PKA and PAK), third through PKA activities, and last but not least through βAR-triggered cAMP:PKA activities (Fig. 4G).
that active GTP-Rac1 augments Erk1/2 phosphorylation, which causes subsequent activation of the transcription factor Elk1. Third, we demonstrate that the activity of the GTP-Rac1:PAK signaling axis leading to activation of Erk1/2 can be directly controlled through GTP-Rac1–compartmentalized PAKA activities. Fourth, we have revealed that in the ovarian cancer cell line OVCAR3, the proliferation-relevant βAR-PKA signaling axis (24, 47) is linked to Rac-PAK-Erk1/2-mediated activation of the transcription factor Elk1. Overall our findings disclose a unique crossroad of frequently targeted receptor cascades (RTK, βAR) that integrates cAMP responses and GTPase activities spatially and temporally, leading to modulation of the crucial Raf-Mek-Erk signaling axis (11–16). It has been described that PAKA activities regulate GEFs and GAPs that change the GTP loading of Rac1, resulting in changes of signaling related to morphological alterations of the cytoskeleton (48–50). These processes are distinct from the mechanism we describe here, where Rac-anchored PAKA activities directly contribute to the activation of downstream effectors of GTP-Rac1 signaling leading to Erk1/2 activation. Another GTPase, which we have identified in the same screen to be a binary interaction partner of PAKA RI subunits, is a component of the trimeric G protein complex Gai, although without AKAP features (18). In contrast to Rac1:PKA complex formation of RII subunits with Gai was observed in response to cAMP elevation. Indeed, mechanistically different, the appearance of cAMP-RII:Gai elevates GPCR-mediated downstream signaling leading, among others, to Erk1/2 activation. Rac1 is not the first small GTPase with AKAP properties. The GTPase Rab32 is classified as an AKAP and targets PKA activities to the mitochondrion. Interestingly, another scaffolding protein with AKAP properties, WAVE-1, directs actin reorganization by relaying signals from the GTPase Rac to downstream effectors. Overall several AKAPs (like AKAP16/22 or AKAP220) group PAKA, other Rho GTPases, and their regulator molecules together, thereby regulating small GTPase activities affecting cytoskeleton reorganizations (9, 10, 48, 51). Several means have been described how the second messenger cAMP alters signaling through the Ras-Raf-Erk cascade positively or negatively. However, it is still a controversy how cell type-dependent components of the cAMP machinery (PKA, AKAPs, Epac, PDEs) contribute to these opposed consequences on Erk1/2 activation that lead to cell growth and/or aberrant proliferation (11–13, 17–23, 52, 53). Here, we present a mechanism how GPCR and cAMP-mediated PKA activation regulates GTP-Rac1 signaling via Erk1/2 to nuclear transcription factors. The detailed mechanism of Rac1-mediated Erk1/2 activation has been described: GTP-Rac1 endorses Raf-Mek-Erk signaling by PAK-mediated phosphorylation of Raf at Ser338 or of Mek at Ser298, which promotes interaction between Erk and Mek (16, 34–36). A link between PAKA activities and RhO GTPase-PAK1 signaling has been discovered more than a decade ago (41). Just recently, it has been depicted that PAKAc subunits form a protein complex with PAK4 (44), which we confirmed with PAK1 (Fig. S3 D and E). First, this observation is one possible explanation how cellular GTP-Rac1 stabilizes the PAK holoenzyme by improved affinities of cellular GTP-Rac:PAKs for RIIβ/PAKAc (Fig. 3 A and B). Second, this data underlines the concept of compartmentalization of PAKA through interaction with the GTP-Rac1-effector complex. In agreement with Park et al. (44), we show that PAK4 is a target of PKA activity by phosphatase the conserved kinase activation loop (Fig. 4). We link GTP-Rac1–compartmentalized PAKA activities, initiated directly by adenylyl cyclase or βAR activation, to the phosphorylation of PAK isoforms. This PKA phosphorylation event contributes to sustained elevations of Erk1/2 activities that implicate enhanced proliferation. Of note in this work is that sole transphosphorylation of PAK4 in its activation loop is not sufficient to promote Erk1/2 activation (Fig. S4B). It requires the complex formation of compartmentalized and PAK-phosphorylated PAK4 with GTP-Rac1, which acts as an active kinase-tuning scaffold [in positive (PAKs) or negative manner (PKA)] to unleash cAMP-PKA–controlled Erk1/2 activation (Fig. 4G), which can be prevented by introducing mutations into BD1 of GTP-Rac1 (Fig. 4D). PAKs are key effectors central to numerous
physiological processes whereby PAK deregulation has been implicated in oncogenesis (26, 44, 45). It has been described that PAK4 activities regulate cancer cell proliferation, migration, and invasion. Besides the abundance of PAK4, the phosphorylation of PAK4-Ser 192/193 has been determined as a crucial factor in cancer progression (45). In this context, our research highlights a unique route how βAR-provoked cAMP fluxes might participate, besides PAKA-mediated Erk1/2 modulation, in further diverse functions of the miscellaneous Rac-effectors PAKs. Given that PAKs activities are considered as marker for the diagnosis of different types of cancer, we would like to note that besides PAKs abundance and its phosphorylation status, the GTP-Rac/PKA complex and βAR activities need to be considered (16, 44, 45, 54). The disclosure of the involvement of the βAR-PAK cascade in regulation of Rac-PAK–mediated Erk1/2 activities offers an explanation how cAMP fluxes contribute to cell growth in a cell-dependent manner.