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Authors

Adame-García, Sendi
Cervantes-Villagrana, Rodolfo
Orduna-Castillo, Lennis
et al.

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cAMP-dependent activation of the Rac guanine exchange factor P-REX1 by type I protein kinase A (PKA) regulatory subunits

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Sendi Rafael Adame-García^{‡1}, Rodolfo Daniel Cervantes-Villagrana^{§1}, Lennis Beatriz Orduña-Castillo^{‡1}, Jason C. del Rio[¶], J. Silvio Gutkind^{¶||2}, Guadalupe Reyes-Cruz[‡], Susan S. Taylor^{¶***‡}, and José Vázquez-Prado^{§3}

From the Departments of [‡]Cell Biology and [§]Pharmacology, Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV-IPN), 07360 Mexico City, Mexico and [¶]Moore's Cancer Center and the Departments of [¶]Pharmacology, ^{**}Chemistry and ^{‡‡}Biochemistry, University of California San Diego, La Jolla, California 92093

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Regulatory subunits of protein kinase A (PKA) inhibit its kinase subunits. Intriguingly, their potential as cAMP-dependent signal transducers remains uncharacterized. We recently reported that type I PKA regulatory subunits (RI α) interact with phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchange factor 1 (P-REX1), a chemotactic Rac guanine exchange factor (RacGEF). Because P-REX1 is known to be phosphorylated and inhibited by PKA, its interaction with RI α suggests that PKA regulatory and catalytic subunits may fine-tune P-REX1 activity or those of its target pools. Here, we tested whether RI α acts as a cAMP-dependent factor promoting P-REX1-mediated Rac activation and cell migration. We observed that G_s-coupled EP2 receptors indeed promote endothelial cell migration via RI α -activated P-REX1. Expression of the P-REX1-PDZ1 domain prevented RI α /P-REX1 interaction, P-REX1 activation, and EP2-dependent cell migration, and P-REX1 silencing abrogated RI α -dependent Rac activation. RI α -specific cAMP analogs activated P-REX1, but lost this activity in RI α -knockdown cells, and cAMP pulldown assays revealed that P-REX1 preferentially interacts with free RI α . Moreover, purified RI α directly activated P-REX1 *in vitro*. We also found that the RI α CNB-B domain is critical for the interaction with P-REX1, which was increased in RI α mutants, such as the acrodysostosis-associated mutant, that activate P-REX1 at basal cAMP levels. RI α and C α PKA subunits targeted distinct P-REX1 molecules, indicated by an absence of phosphorylation in the active fraction of P-REX1. This was in contrast to the inactive fraction in which phosphorylated P-REX1 was present, suggesting co-existence of dual stimula-

tory and inhibitory effects. We conclude that PKA's regulatory subunits are cAMP-dependent signal transducers.

The widely recognized role of cAMP as a second messenger controlling fundamental cellular processes, such as cell growth, adhesion, and polarized migration, has mainly been attributed to phosphorylation-dependent effects of the catalytic (C)⁴ subunits of cAMP-dependent protein kinase A (PKA) (1). The dimeric regulatory (R) subunits, containing two cAMP-binding domains in each monomer as well as a docking and dimerization domain, restrict the subcellular localization of the tetrameric holoenzyme, keeping the two C-subunits inhibited in the absence of cAMP. In response to increasing concentrations of cAMP, this second messenger is captured by the cAMP-binding domains, causing conformational changes that reduce the affinity of R-subunits for the C-subunits, thereby unleashing their catalytic activity and enabling them to phosphorylate diverse effectors that control a plethora of biological pathways (2).

PKA has served as prototype to understand the structural characteristics of the whole-human kinome (3). Remarkably, its multimeric organization, with independent regulatory cAMP-sensing domains as part of dissociable regulatory subunits (RI α , RI β , RII α , and RII β), is a unique feature among kinases (2). Studies on the regulatory subunits have focused on their role in controlling their kinase partners. By interacting with A kinase-anchoring proteins (AKAPs), the PKA R-subunits also localize PKA holoenzyme pools to precise subcellular compartments facilitating the phosphorylation of specific substrates, fre-

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³ To whom correspondence should be addressed: Dept. of Pharmacology, CINVESTAV-IPN, Av. Instituto Politécnico Nacional 2508, Col. San Pedro Zacatenco, 07360 Mexico City, Mexico. Tel.: 52-55-5747-3380; Fax: 52-555747-3394; E-mail: jvazquez@cinvestav.mx.

⁴ The abbreviations used are: C, catalytic; R, regulatory; PKA, protein kinase A; RacGEF, Rac guanine exchange factor; P-REX1, phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchange factor 1; AKAP, A kinase-anchoring protein; ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PAE, porcine aortic endothelial cell; IBMX, isobutylmethylxanthine; siEGFP, short-interfering enhanced green fluorescent protein; CNB-B, cyclic nucleotide-binding domain B; PGE₂, prostaglandin E₂; S1P, sphingosine 1-phosphate; CREB, cAMP-response element-binding protein; ACRO, acrodysostosis; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PKAS, phospho-PKA substrate antibody; FBS, fetal bovine serum; DAPI, 4,6-diamidino-2-phenylindole; GST, glutathione S-transferase; DEP, Dishevelled, EGL-10, and Pleckstrin domain; DH, Dbl homology domain; PH, pleckstrin homology domain; esiRNA, endoribonuclease-prepared siRNA; 6-Bnz, N⁶-benzoyladenine 3',5'-cyclic monophosphate; 8-AHA, 8-(6-aminohexyl)aminoadenosine-3',5'-cyclic monophosphate.

quently including AKAPs themselves as part of regulatory feedback loops (4). AKAP-Lbc and $\alpha 4$ integrins are examples of those interacting proteins involved in cell migration (5, 6).

Strikingly, the kinase-independent signaling potential of cAMP-bound PKA regulatory subunits remains poorly understood. Recently, we reported that type I α PKA regulatory subunit (RI α) interacts with P-REX1, a chemotactic RacGEF, via the cyclic nucleotide-binding domain B (CNB-B) of RI α that establishes noncanonical interactions with P-REX1–PDZ domains. Signaling via G_i-coupled CXCR4 receptors promotes P-REX1 translocation to the plasma membrane that mobilizes RI α in the process. The direct regulatory role that PKA catalytic subunits exert on P-REX1 occurs by phosphorylation of P-REX1 at its DEP1 domain, on serine 436, promoting intramolecular inhibitory interactions between the phosphorylated DEP domain and the catalytic DH-PH cassette (7). Here, we expand our understanding of P-REX1 regulation by PKA by providing evidence that supports a novel mechanism of cAMP-dependent P-REX1 activation via RI α . The emerging model indicates a dual ability of PKA to regulate separated and independent P-REX1 protein pools, stimulating a fraction of this chemotactic RacGEF via direct interaction with regulatory subunits and restricting other fractions by phosphorylation-dependent inhibition. Moreover our results reveal novel signaling features of PKA regulatory subunits, beyond their regulatory role on their kinase counterparts, directly acting as cAMP-dependent transducers.

Results

EP2 prostaglandin receptors promote interaction of endogenous PKA–RI α with active P-REX1 and endothelial cell migration

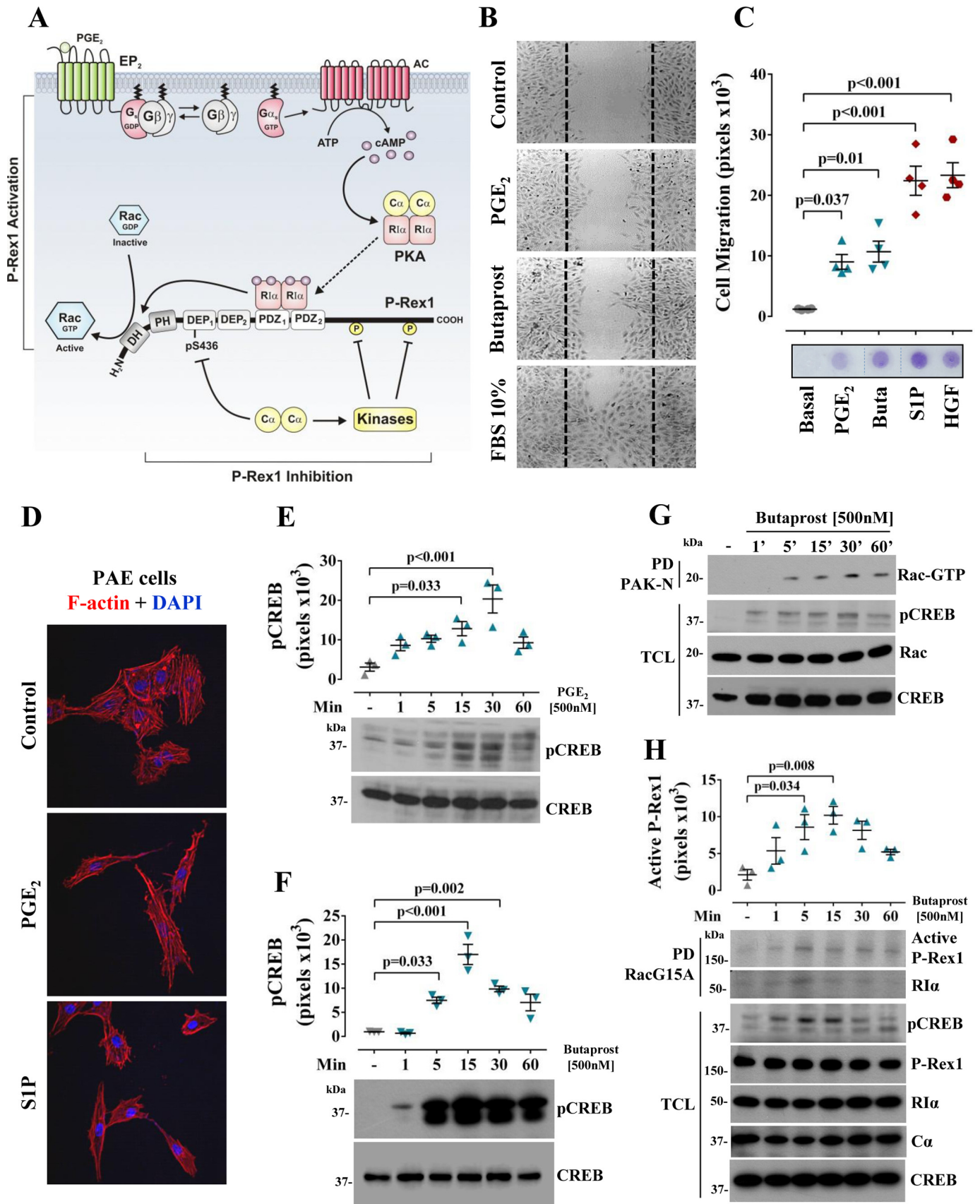
We have previously demonstrated that PKA phosphorylates P-REX1 at Ser-436 promoting inhibitory intramolecular interactions (7). In addition, we reported that the regulatory subunits of type I PKA directly interact with P-REX1 increasing the potential mechanisms by which PKA might regulate this multidomain RacGEF. Specifically, RI α –cAMP-binding domains, particularly CNB-B, establish noncanonical interactions with P-REX1, raising the possibility that RI α might play a direct cAMP-dependent and kinase-independent effect on P-REX1. Thus, hypothetically, PKA catalytic and regulatory subunits might fine-tune P-REX1 activity by a dual-regulatory input (Fig. 1A) or target different P-REX1 molecules. To start addressing these possibilities, with emphasis on the potential positive role of RI α on P-REX1, we studied the effects of endogenous EP2 receptors in endothelial cells. EP2 is a prostaglandin E₂ (PGE₂) receptor described as a G_s-coupled angiogenic receptor (8). We addressed EP2-dependent effects on cell migration, actin cytoskeleton reorganization, P-REX1 and Rac activation, and the interaction of RI α with active P-REX1. As control, we confirmed the effect of the EP2-signaling pathway on PKA activity as evidenced by the phosphorylation of CREB, a prototypic PKA substrate. We initially validated the functional and signaling properties of EP2 in porcine aortic endothelial cells (PAE). We found that PGE₂ and butaprost, an EP2-specific agonist, stimulated PAE cells to migrate in two different experimental

systems, wound-healing assays and chemotactic assays in Boyden chambers (Fig. 1, B and C, respectively). These results correlated with PGE₂-induced actin cytoskeleton remodeling promoting endothelial cell extension (Fig. 1D), similar to the effect observed in response to sphingosine 1-phosphate (S1P) (Fig. 1D), suggesting the activation of the P-REX1/Rac signaling axis. In cell migration experiments, serum (Fig. 1B), sphingosine 1-phosphate, and hepatocyte growth factor (Fig. 1C) were used as positive controls. In these cells, PGE₂ and butaprost also promoted CREB phosphorylation on serine 133, a residue known to be phosphorylated by PKA (Fig. 1, E and F), suggesting the coupling of this receptor to G_s and the cAMP–PKA signaling pathway. Next, we directly tested the effect of EP2 activation on the P-REX1/Rac signaling axis. Butaprost promoted Rac (Fig. 1G) and P-REX1 activation (Fig. 1H), in very similar kinetics. As we previously reported an interaction and reciprocal regulation between P-REX1 and PKA, we had particular interest on the dynamics of P-REX1 and PKA interaction. Then, we looked for the association of PKA subunits with the fraction of active P-REX1, isolated by pulldown with recombinant nucleotide-free Rac. Interestingly, we found endogenous RI α PKA-regulatory subunit interacting with active P-REX1 (Fig. 1H, 2nd blot, PD), whereas the catalytic C α subunit was undetectable. Thus, we hypothesized that PKA might have a positive role on P-REX1 signaling, mechanistically explained by the effect of cAMP-promoted direct interaction between RI α and P-REX1, leading to P-REX1 activation (Fig. 1A).

Direct stimulation of type I PKA promotes P-REX1 activation

Because we observed that G_s-coupled EP2 receptors simultaneously activated PKA and P-REX1 and coincidentally promoted the interaction between RI α and the active fraction of P-REX1, we decided to explore whether P-REX1 could be activated in response to direct stimulation of type I PKA. Thus, we used two cAMP analogs 6Bnz/8AHA-cAMP (9), which combined are specific for RI α (Fig. 2A), and we evaluated their effect as activators of endogenous Rac and P-REX1 in endothelial cells. We found that stimulation of type I PKA led to activation of endogenous Rac and P-REX1 (Fig. 2, B and C, respectively). Moreover, we detected that these cAMP analogs promoted interaction of endogenous RI α with active P-REX1 (Fig. 2C, PD, 2nd panel). As expected, direct activation of type I PKA led to CREB phosphorylation in endothelial cells (Fig. 2, B and C) as well as in HEK293T cells (Fig. 2D). Since we previously demonstrated a phosphorylation-dependent inhibitory effect of the P-REX1 C-region on P-REX1 activity (7), we assessed whether the positive effect of RI α -specific cAMP analogs on P-REX1 could be restricted to the N-region of P-REX1 containing the DH-PH cassette and the two DEP and two PDZ regulatory modules. We measured the effect of exclusive stimulation of type I PKA on P-REX1 activity in HEK293T cells expressing FLAG–P-REX1–DH–PDZ2. As in the case of endogenous P-REX1 activation in endothelial cells, we observed a similar increase in FLAG–P-REX1–DH–PDZ2 activity and detected endogenous RI α associated with the isolated fraction of active FLAG–P-REX1–DH–PDZ2 (Fig. 2D). Interestingly, although the absence of P-REX1 C-region led to a high basal amount of active P-REX1, the RI α -specific cAMP analogs still had a significant

cAMP activates P-REX1 via type I PKA regulatory subunits



effect that also coincided with an increased association of endogenous RI α (Fig. 2D, PD). These results support the existence of a cAMP \rightarrow PKA \rightarrow P-REX1 signaling axis in which RI α interaction with P-REX1 contributes to the activation of this RacGEF.

P-REX1 activation by type I PKA is linked to its interaction with RI α

Previously, using the yeast two-hybrid system, we identified a fraction of RI α , corresponding to its second cAMP-binding domain, as a specific interactor of P-REX1-PDZ domains (7). Thus, the complex of P-REX1 with RI α is reminiscent of guanine exchange factors directly activated by cAMP, which are structurally characterized by the presence of DEP and cAMP-binding domains (10). Because our previous results indicated that in endothelial cells endogenous G_s-coupled EP2 receptors that stimulate type I PKA lead to P-REX1 activation coincidently with its interaction with RI α , we hypothesized that RI α -P-REX1 interaction could directly be stimulated by cAMP. Consistent with this possibility, pharmacological activation of adenylate cyclase (with forskolin) promoted the interaction between endogenous RI α and P-REX1 in PAE cells (Fig. 3A, upper panel, IP-RI α), coincident with the activation of PKA detected by the phosphorylation of CREB (Fig. 3A, pCREB in TCL). Similarly, in HEK293T cells, adenylate cyclase stimulation with forskolin and RI α -specific cAMP analogs led to a robust interaction between endogenous RI α and P-REX1-DH-PDZ2, expressed as a GST construct lacking the inhibitory C-region, detected by pulldown experiments (Fig. 3, B and C, respectively). Remarkably, the Z6 construct, the original clone identified as a P-REX1 interactor in the yeast two-hybrid system, having an isolated RI α CNB-B domain, increased its binding to P-REX1 PDZ-PDZ tandem in response to forskolin stimulation (Fig. 3D, upper panel), indicating that the CNB-B domain is a structural determinant for the cAMP-dependent binding of RI α to P-REX1. To gain further insight into the role of cAMP-binding domains on the interaction between RI α and P-REX1, we analyzed the potential of RI α CNB-B mutants (Fig. 3E) as P-REX1 interactors and activators. In HEK293T cells, we expressed wildtype RI α (WT) or RI α mutants, R335K, and acrodysostosis (ACRO(1-365)) characterized by perturbations on the CNB-B domain (cAMP-resistant), together with GST-P-REX1-PDZ-PDZ domains, and we assessed their interac-

tion by pulldown. Highlighting the importance of CNB-B in the interaction between RI α and P-REX1 PDZ domains, we found that both RI α mutants, R335K and especially the ACRO, exhibited a better interaction with P-REX1 PDZ domains than WT RI α (Fig. 3F); however only the ACRO mutant had a discrete but consistent effect on P-REX1 activation (Fig. 3G). The coincident effect of the ACRO mutant as a better interactor and activator of P-REX1 suggested that P-REX1 activation indeed depended on its interaction with RI α , but also a particular conformation at the CNB-B domain seems relevant.

P-REX1 activation by type I PKA depends on regulatory but not catalytic subunit expression

Because P-REX1 activation correlated with its interaction with RI α , stimulated by cAMP, we assessed whether knockdown of type I PKA regulatory or catalytic subunits had an effect on P-REX1 activation. Using esiRNAs (a mixture of siRNAs targeting a fraction of 436 nucleotides within the 6633-nucleotide length of P-REX1 mRNA), we decreased P-REX1 expression in MCF7 cells and observed that P-REX1 knockdown prevented the effect of 6Bnz/8AHA-cAMP, type I PKA-specific analogs, as promoters of Rac activation (Fig. 4A). These results indicated that stimulation of type I PKA requires P-REX1 to activate Rac. In contrast, the catalytic kinase activity of type I PKA was not influenced by a reduced expression of P-REX1, as demonstrated by the phosphorylation of CREB in response to 6Bnz/8AHA-cAMP in P-REX1 knockdown cells (Fig. 4A). Similarly, using RI α - or C α -specific esiRNAs, we observed that decreased expression of RI α but not C α prevented P-REX1 activation in response to type I PKA stimulation (Fig. 4C). As expected, C α knockdown prevented the phosphorylation of CREB (Fig. 4C, 2nd panel). Consistent with previous reports, we observed that PKA C α knockdown also decreased RI α expression (11). Furthermore, to confirm the specific effect of esiRNAs, we transfected individual siRNAs that target P-REX1 (3625 and 3809) or RI α (740 and 175). EGFP and β -Gal siRNAs were used as controls. These P-REX1 and RI α siRNAs efficiently decreased the expression of their targets and similarly abrogated their respective contribution on PKA-dependent Rac and P-REX1 activation (Fig. 4, B and D, respectively) without significant alteration on CREB phosphorylation by the PKA C α subunits. These results further support the idea that

Figure 1. EP2 prostaglandin receptors promote endothelial cell migration, P-REX1/Rac activation, and interaction of endogenous PKA-RI α with active P-REX1. A, working hypothesis postulating that P-REX1 activity is fine-tuned by independent actions of type I PKA regulatory and catalytic subunits. Accordingly, G_s-coupled EP2 receptor activates P-REX1 via cAMP-dependent direct interaction with regulatory subunits and eventually, as we previously described (7), inhibited by phosphorylation leading to intramolecular inhibitory interactions. B, PGE₂ (1 μ M) and butaprost (1 μ M) (a specific EP2 agonist) promote migration of PAE cells in wound-healing assays. The pictures represent the wound closure of PAE cells after 16 h of stimulation, and S1P (1 μ M) and 10% FBS were used as positive controls. Three independent experiments were performed ($n = 3$). C, PGE₂ (1 μ M) and butaprost (1 μ M) promote chemotactic migration of PAE cells in Boyden Chamber Chemotaxis assays; S1P (1 μ M) and HGF (10 ng/ml) were used as positive controls. Graph shows the densitometric analysis of four independent chemotaxis assays ($n = 4$). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test, and p value is indicated. D, effect of EP2 activation on actin cytoskeleton reorganization in PAE cells was assessed with phalloidin staining. PAE cells, starved for 16 h, were stimulated with PGE₂ (1 μ M) and S1P (1 μ M) for 15 min, and DAPI was used to show the nuclei. Images are representative of 10 different fields. E and F, PGE₂ (500 nM) and butaprost (500 nM) promote phosphorylation of PKA substrate CREB at serine 133. Serum-starved PAE cells were stimulated with PGE₂ or butaprost at the indicated times and then lysed and processed for immunoblot against pCREB and total CREB. Graphs show the densitometric analysis of pCREB from three independent experiments ($n = 3$). One-way ANOVA followed by Tukey's multiple comparison test was performed; significant p values are indicated. G, EP2 stimulation induces Rac activation in endothelial cells. Serum-starved PAE cells were stimulated with butaprost (500 nM) at the indicated times and lysed and processed for pulldown using GST-PAKN beads and analyzed by immunoblot with anti-Rac, pCREB, and total CREB antibodies. H, EP2 stimulation promotes P-REX1 activation in endothelial cells. Serum-starved PAE cells were stimulated with butaprost (1 μ M) at the indicated times and processed for pulldown assays to capture active P-REX1 using GST-RacG15A beads. Western blottings of anti-P-REX1, PKA-RI α , and C α were done in pulldowns, and total cell lysates, as well as pCREB and total CREB, were used as control. PKA-C α was not detected associated with active P-REX1 (not shown). Graph shows the densitometric analysis of active P-REX1 from three independent experiments ($n = 3$). One-way ANOVA followed by Tukey's multiple comparison test was performed, and significant p values are shown in the graph.

cAMP activates P-REX1 via type I PKA regulatory subunits

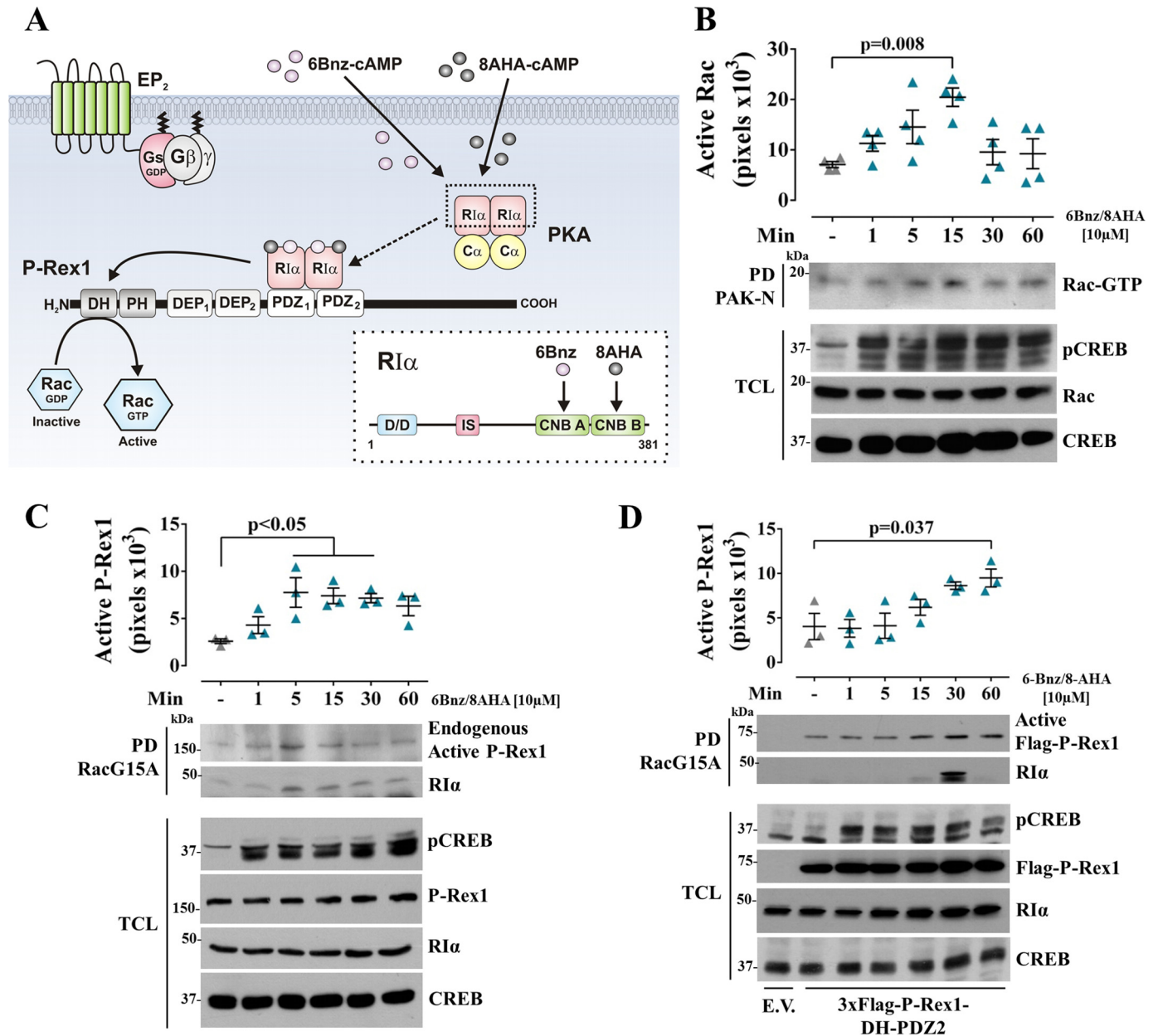


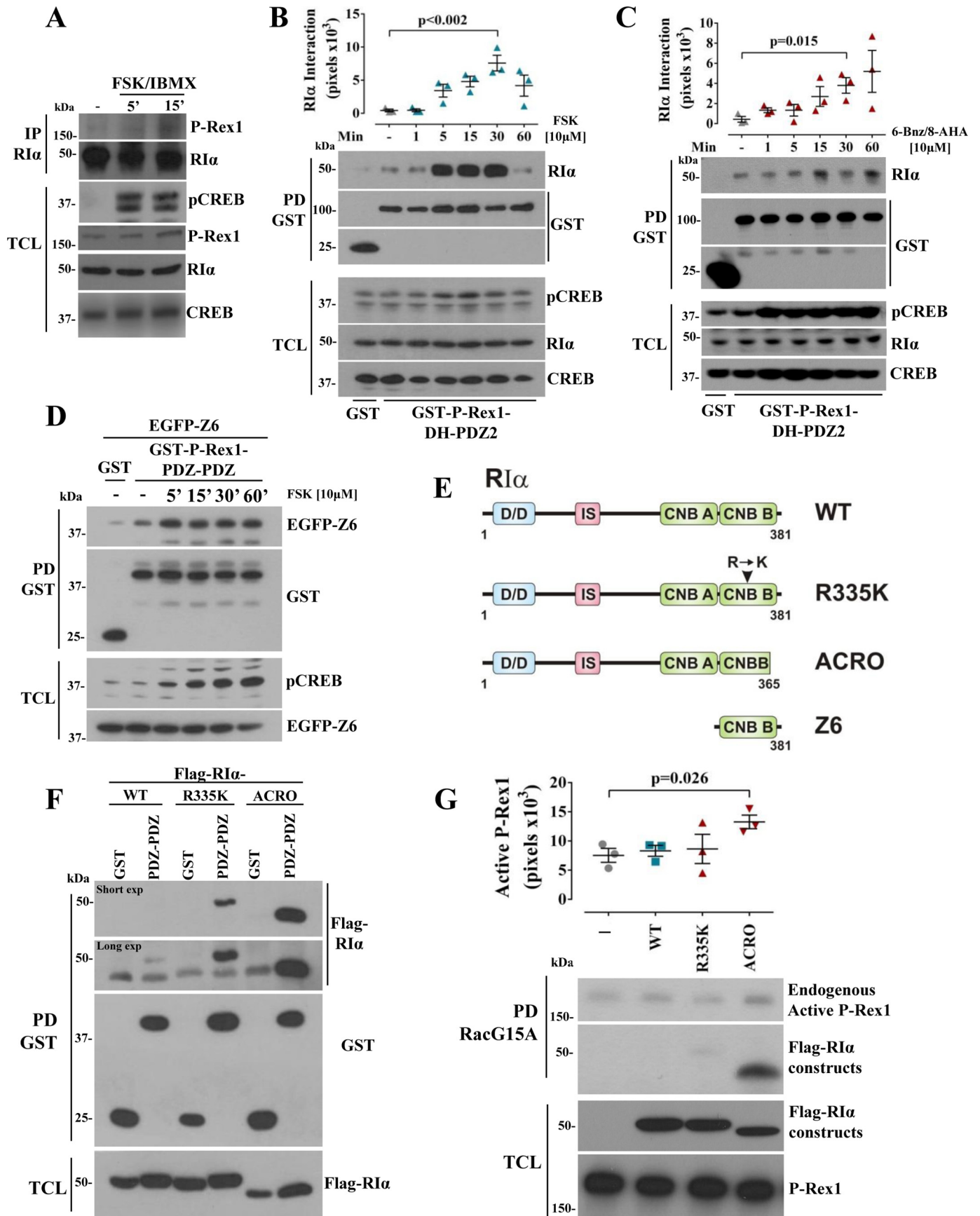
Figure 2. Direct stimulation of type I PKA promotes P-REX1 activation. *A*, hypothetical effect of direct stimulation of type I PKA on P-REX1. *B*, direct stimulation of type I PKA promotes Rac activation in endothelial cells. Serum-starved PAE cells were stimulated at the indicated times with 6-Bnz-cAMP (10 μ M) and 8-AHA-cAMP (10 μ M) cAMP analogs, and active Rac was isolated with GST-PAKN beads. Total and pCREB were detected by Western blotting in total cell lysates. Graph shows the densitometric analysis of four independent experiments of Rac activation ($n = 4$). One-way ANOVA followed by Tukey's multiple comparison test was performed, and significant p values are shown in the graph. *C*, direct stimulation of type I PKA promotes P-REX1 activation and association of RI α with the active fraction of P-REX1 in endothelial cells. PAE cells were stimulated as indicated in *B*, and active P-REX1 was isolated with GST-RacG15A and detected by immunoblot; RI α was revealed in the pull-down of active P-REX1 and in total cell lysates. pCREB and total CREB revealed in total cell lysates were used as controls. Graph shows the densitometric analysis of active P-REX1 from three independent experiments ($n = 3$). One-way ANOVA followed by Tukey's multiple comparison test was performed, and significant p values are shown in the graph. *D*, P-REX1 lacking the C-terminal region is activated in response to direct stimulation of type I PKA. HEK293T cells expressing FLAG-P-REX1-DH-PDZ2 construct were serum-starved and stimulated with cAMP analogs for type I PKA at the indicated times. Active P-REX1 was isolated with GST-RacG15A beads. Samples were processed for immunoblot against FLAG, RI α , pCREB, and total CREB. Graph shows the time course of FLAG-P-REX1 activation from three independent experiments ($n = 3$). One-way ANOVA followed by Tukey's multiple comparison test were performed, and the significant p value is shown in the graph.

type I PKA controls P-REX1 activity by independent intervention of regulatory and catalytic subunits.

Endogenous P-REX1 preferentially interacts with cAMP-bound RI α and, in vitro, they form an active RacGEF complex

Because pulldown experiments using lysates from cells stimulated with forskolin or RI α -specific cAMP analogs revealed a

positive effect of cAMP on the interaction between RI α and P-REX1, we wanted to directly assess the possible preferential interaction of P-REX1 with cAMP-bound RI α . To this end, we used specific cAMP affinity matrices to isolate either RI α or PKA-I holoenzyme from MCF7 cell lysates (12), and we compared whether P-REX1 preferentially remains bound with the fraction of RI α isolated with the cAMP affinity matrix (Fig. 5A).



cAMP activates P-REX1 via type I PKA regulatory subunits

As shown in Fig. 5, B and C, P-REX1 was present in the pull-down of cAMP-bound RI α (isolated with cAMP-agarose) but not in the one used to isolate the PKA-I holoenzyme (with (R_p)-8-AHA-cAMP-agarose). These results indicated that, endogenously, P-REX1 preferentially interacts with the cAMP-bound active RI α rather than with RI α that is part of the type I holoenzyme (Fig. 5, B and C). Besides, we tested whether direct interaction of purified RI α with P-REX1 is sufficient to activate P-REX1. To confirm this possibility, we first purified bacterial recombinant RI α with cAMP-resin and P-REX1, from HEK293T cells, using the HaloTag system and analyzed their interaction. Both P-REX1 and RI α were detected as single bands by Coomassie staining (Fig. 5D). To further confirm that the interaction between cAMP-bound RI α and P-REX1 detected in cell lysates was in fact direct, we incubated purified RI α with purified P-REX1 and subjected them to pull-down assays with cAMP- and (R_p)-cAMP-agaroses. Again, we precipitated RI α with both resins, but P-REX1 was preferentially associated to RI α in the cAMP-resin (Fig. 5E). To assess whether *in vitro* RI α and P-REX1 formed an active RacGEF complex, we used recombinant nucleotide-free Rac fused to GST to isolate the putative active RacGEF complex formed by P-REX1 and RI α . We found that RI α did activate P-REX1 and remained associated to the isolated fraction of active P-REX1 (Fig. 5F), suggesting that RI α promoted P-REX1 activation by direct interaction.

P-REX1 interaction with PKA-RI α is necessary for EP2-dependent endothelial cell migration

Stimulation of the P-REX1/Rac pathway by RI α suggests the cAMP-dependent interaction of this PKA regulatory subunit with P-REX1 is especially relevant for cell migration elicited by chemotactic G_s-coupled receptors. To test this possibility, we decided to perturb the interaction of RI α with P-REX1 by expressing the P-REX1-PDZ1 domain, mapped as the minimal RI α -interacting domain, to compete the endogenous interaction (Fig. 6A). Consistent with previous results (Fig. 3, A–D), endogenous RI α interacted with P-REX1-PDZ domains in response to forskolin stimulation (Fig. 6B). As predicted, expression of EGFP-P-REX1-PDZ1 domain competed in the interaction between endogenous P-REX1 and RI α isolated by

pull-down with cAMP-resin (Fig. 6C, left panel). Moreover, P-REX1-PDZ1 domain prevented the activation of endogenous P-REX1 in response to direct stimulation of type I PKA, without significantly perturbing PKA catalytic activity measured by CREB phosphorylation (Fig. 6D). Notably, in PAE cells expression of the P-REX1-PDZ1 domain decreased the effect of EP2 agonists, but not serum (FBS), on cell migration (Fig. 6, E and F). These results suggest that EP2 promotes endothelial cell migration via a cAMP-RI α /P-REX1 pathway.

PKA regulatory and catalytic subunits target distinct P-REX1 molecules

The positive effect that RI α exerts on P-REX1 signaling might counteract the inhibitory effect of P-REX1 phosphorylation by PKA C α subunits. These opposite effects might fine-tune P-REX1 activity or target distinct P-REX1 molecules. To understand the apparent paradoxical effects of PKA on P-REX1, we stimulated EP2 receptors in COS7 cells and isolated active and inactive pools of FLAG-P-REX1 at different times of stimulation. Notably, active P-REX1 molecules, isolated by pull-down with GST-RacG15A, were not phosphorylated (Fig. 7A, PD-RacG15A), whereas the inactive fraction, isolated by subsequent immunoprecipitation, contained phosphorylated P-REX1, detected by Western blotting with anti-phospho-PKA substrate (PKAS) antibodies (Fig. 7A, IP FLAG). Our results suggest that upon PKA activation and dissociation, RI α activates nonphosphorylated P-REX1, whereas C α targets different P-REX1 molecules restricting them by phosphorylation. Eventually, because the fraction of active P-REX1 decreases and the inactive phosphorylated fraction increases (Fig. 7A), the dual signaling by PKA leans toward the desensitizing effect on P-REX1 by direct phosphorylation (Fig. 7B).

Discussion

P-REX1, a multidomain RacGEF centrally involved in chemotactic G protein-coupled receptor signaling, is synergistically activated by G $\beta\gamma$ and PIP₃ (13–16). Previously, we reported a reciprocal communication between P-REX1 and PKA. Accordingly, P-REX1 localizes PKA RI α to the plasma membrane and PKA inhibits P-REX1 by phosphorylating it (7). In the current study, we extend our understanding of this recip-

Figure 3. P-REX1 activation by type I PKA is linked to its interaction with RI α . A, stimulation of the cAMP pathway promotes interaction between endogenous P-REX1 and RI α in endothelial cells. Serum-starved PAE cells were stimulated with forskolin (10 μ M) at the indicated times. Then endogenous RI α was immunoprecipitated, and bound P-REX1 was detected by Western blotting. Total P-REX1, RI α , pCREB (stimulation control), and total CREB were revealed in total cell lysates. B, stimulation of the cAMP pathway promotes interaction between endogenous RI α and the DH-PDZ2 region of P-REX1. HEK293T cells expressing GST-P-REX1-DH-PDZ2 construct were serum-starved and stimulated with forskolin (10 μ M) as indicated. P-REX1-DH-PDZ2 was isolated using GSH-Sepharose beads. Immunoblot was performed against RI α , GST, pCREB (stimulation control), and total CREB. Graph shows the time course of forskolin-induced RI α interaction with P-REX1 from three independent experiments ($n = 3$). One-way ANOVA followed by Tukey's multiple comparison test was performed, and significant p value is shown in the graph. C, direct stimulation of type I PKA promotes interaction of RI α with P-REX1. HEK293T cells expressing GST-P-REX1-DH-PDZ2 construct were serum-starved and then stimulated with 6Bnz-cAMP (10 μ M) and 8AHA-cAMP (10 μ M) as indicated. P-REX1 was isolated by GST pull-down, and samples were analyzed by Western blotting against RI α , GST, pCREB (stimulation control), and total CREB. Graph shows the time course of directly activated RI α interacting with P-REX1, from three independent experiments ($n = 3$). One-way ANOVA followed by Kruskal-Wallis test was performed, and significant p value is indicated. D, stimulation of the cAMP pathway promotes interaction between Z6 construct (RI α CNB-B domain) and the PDZ1-PDZ2 region of P-REX1. HEK293T cells co-expressing EGFP-Z6 and GST-P-REX1-PDZ1-PDZ2 constructs were serum-starved and stimulated with forskolin (10 μ M) as indicated. GST-P-REX1-PDZ1-PDZ2 was isolated with GSH-Sepharose beads. Immunoblot was performed against GFP, GST, pCREB (stimulation control), and total CREB. Three independent experiments were performed ($n = 3$). E, schematic representation of WT and mutant RI α constructs R335K and acrodysoyostosis(1–365) and Z6 (CNB-B). F, analysis of interaction between WT RI α and mutants at CNB-B domain and P-REX1 PDZ1-PDZ2 module. HEK293T cells co-expressing GST-P-REX1-PDZ1-PDZ2 domains and FLAG-RI α WT or mutants R335K or ACRO were serum-starved, lysed, and prepared for GST pull-down interaction assays as described previously. Immunoblots were performed against FLAG and GST. Three independent experiments were performed ($n = 3$). G, RI α ACRO mutant increases P-REX1 activity. MCF7 cells expressing empty vector or RI WT or R335K or ACRO mutants were serum-starved and lysed, and active P-REX1 was isolated with GST-RacG15A beads. Immunoblots were performed for P-REX1 and FLAG. Graph shows the densitometric analysis of three independent experiments ($n = 3$). The p value obtained by one-tail Student's t test comparing control versus ACRO is indicated.

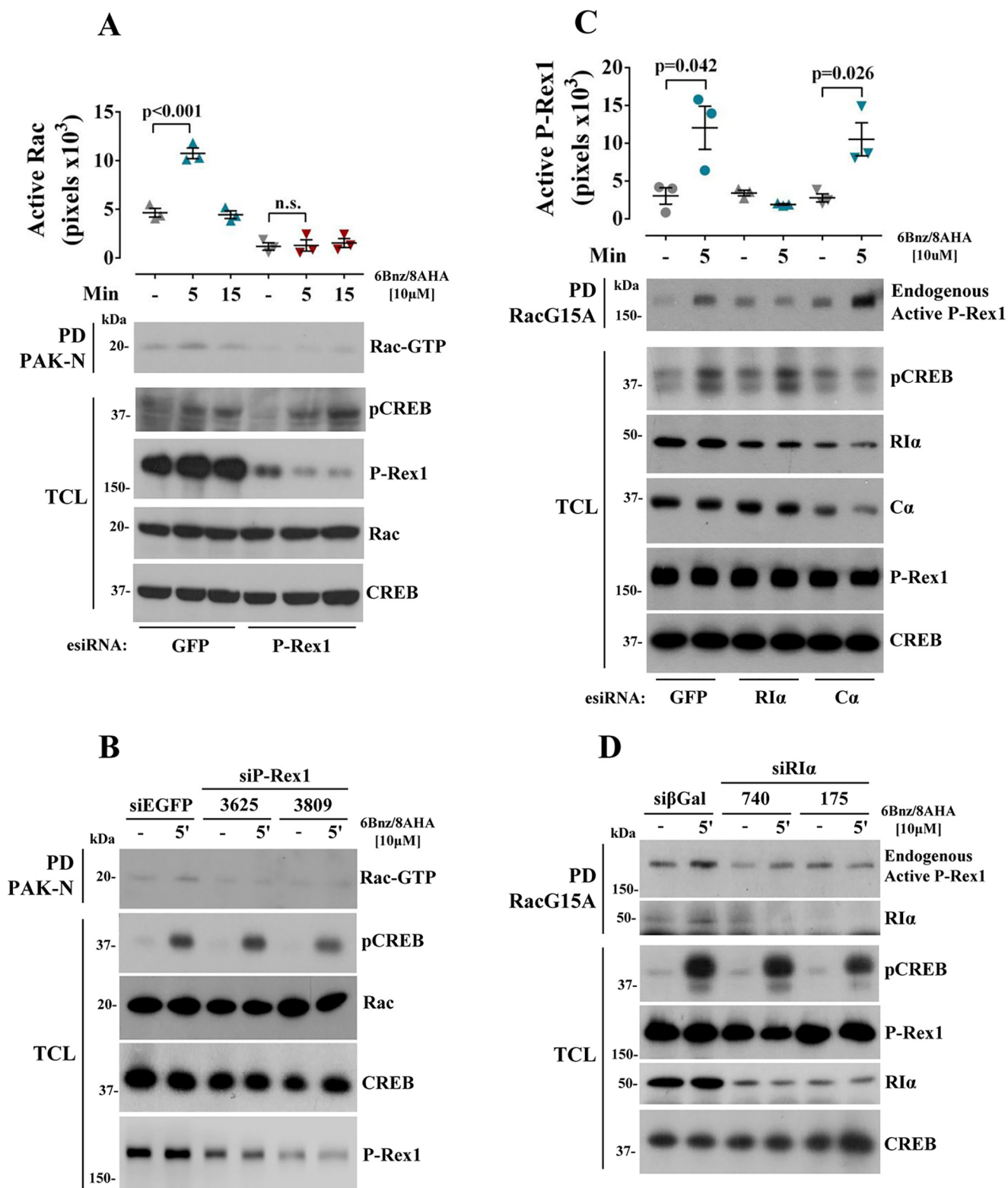


Figure 4. P-REX1 activation by type I PKA depends on regulatory, but not catalytic, subunit expression. *A* and *B*, Rac activation in response to direct stimulation of type I PKA requires P-REX1. MCF7 cells transfected with esiRNAs (*A*) or individual siRNAs (*B*) for P-REX1 or GFP (as negative control) were serum-starved and stimulated at the indicated times with type I PKA-specific cAMP analogs. Active Rac was isolated by pull-down with GST-PAKN and detected by immunoblot. Western blottings in total cell lysates were done for Rac, P-REX1, pCREB, and total CREB. *Graph* in *A* represents the densitometric analysis of three independent experiments ($n = 3$). Two-way ANOVA followed by Tukey test was performed, and significant p value is indicated. *n.s.*, no significant $p > 0.05$. *C* and *D*, P-REX1 activation in response to direct stimulation of type I PKA requires the R1 α subunit but not catalytic C α subunit. MCF7 cells transfected with esiRNAs for PKA-R1 α or -C α subunits (*C*), or siRNAs for PKA-R1 α or β Gal (as negative control) (*D*), were serum-starved and stimulated with type I PKA-specific cAMP analogs at the indicated times. Active P-REX1 was isolated with GST-RacG15A and detected by immunoblot. Phosphorylation of CREB as well as expression of P-REX1, R1 α , C α , and total CREB were assessed by Western blotting in total cell lysates. *Graph* in *C* represents the densitometric analysis of active P-REX1 from three independent experiments ($n = 3$). One-tail Student's t test was performed for the comparisons, and significant p values are indicated.

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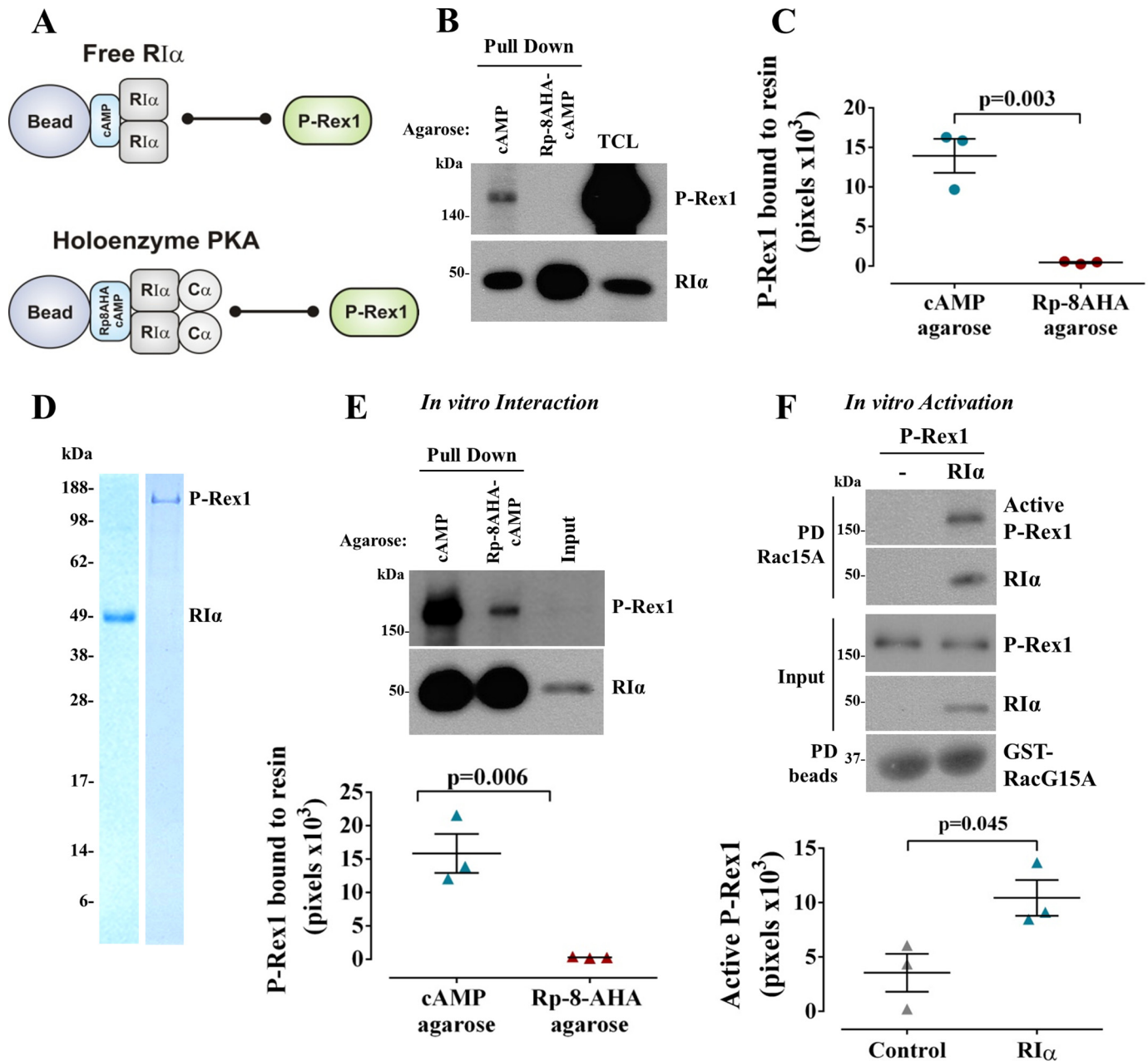


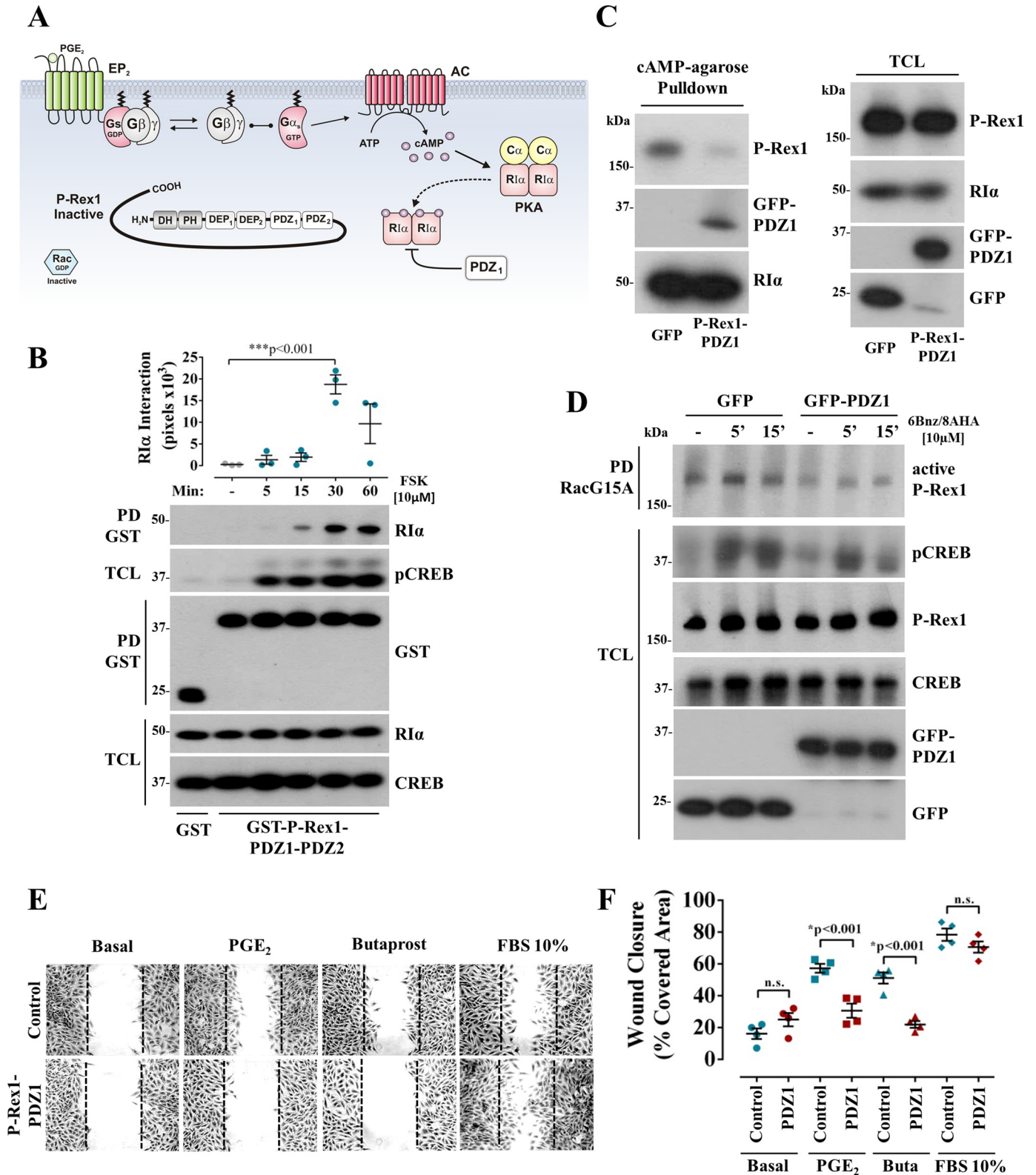
Figure 5. Endogenous P-REX1 preferentially interacts with cAMP-bound RI α , and *in vitro* they form an active RacGEF complex. *A*, drawing shows the experimental strategy to assess whether P-REX1 preferentially interacts with cAMP-bound RI α with respect to type I PKA holoenzyme, isolated by pull-down with affinity matrices containing cAMP or the antagonist analog (R_p)-8AHA-cAMP. *B*, P-REX1 preferentially interacts with free cAMP-RI α but not with inactive PKA holoenzyme. Lysates of serum-starved MCF7 cells were used for pull-down assays with cAMP or (R_p)-cAMP-agaroses. P-REX1 and RI α in the pull-down, and total cell lysate, were detected by Western blotting. *C*, graph represents the densitometric analysis of three independent experiments ($n = 3$) like the one shown in *B*. Results were analyzed by one-tail Student's *t* test, and the *p* value is indicated in the graph. *D*, Coomassie staining of isolated full-length RI α and P-REX1 used for *in vitro* assays. RI α was expressed in *E. coli* BL21 strain and purified using cAMP-agarose followed by gel filtration. HA-HaloTag-P-REX1, expressed in HEK293T cells, was isolated by pull-down with HaloLink resin. P-REX1 was released from the resin using HaloTag-tobacco etch virus protease, removing the HA-HaloTag. *E*, isolated P-REX1 and RI α were incubated *in vitro* and subjected to pull down analysis with cAMP or (R_p)-agaroses. Samples were immunoblotted against P-REX1 and RI α . The graph below represents the densitometric analysis of three independent experiments ($n = 3$). Results were analyzed by one-tail Student's *t* test, and *p* value is indicated. *F*, P-REX1 is directly activated by RI α . Purified P-REX1 was incubated in the presence or absence of RI α for 15 min, and the active fraction of P-REX1 was isolated with GST-RacG15A beads. P-REX1 and RI α were detected by immunoblot. Recombinant GST-RacG15A was stained with Ponceau red. The graph below represents the densitometric analysis of three independent experiments ($n = 3$). Results were analyzed by one tail Student's *t* test, and *p* value is indicated.

rocal regulation and demonstrate the potential of type I PKA regulatory subunits to directly activate P-REX1. We show that endothelial EP2, a G_s -coupled receptor, promotes P-REX1 activation and interaction of RI α subunit of PKA with the active fraction of P-REX1. This finding is compatible with a previous report showing P-REX1-dependent activation of Rac by β -ad-

renergic receptors in MCF7 cells (17). EP2 was previously described as a G_s -coupled angiogenic receptor (8); however, the molecular mechanisms by which EP2 stimulates Rho GTPases involved in typical angiogenic responses such as endothelial cell migration, and the identity of RhoGEFs putatively activated downstream of EP2, had remained unknown. Here, we demon-

stated that P-REX1, known to be involved in angiogenic signaling by chemotactic G_i -coupled receptors such as CXCR4 (18, 19), and its effector Rac were activated upon EP2 stimulation, which simultaneously promoted the phosphorylation of CREB, a paradigmatic PKA substrate. Intriguingly, these findings would involve a complex, and apparently paradoxical,

mechanism of P-REX1 regulation by PKA. We previously demonstrated that PKA inhibits P-REX1 by promoting phosphorylation-dependent intramolecular inhibitory interactions, and we are now revealing a positive effect of PKA on P-REX1, which is mediated by the $RI\alpha$ subunit. Our current results point to a mechanism depicted in Fig. 7B by which PKA regulatory and



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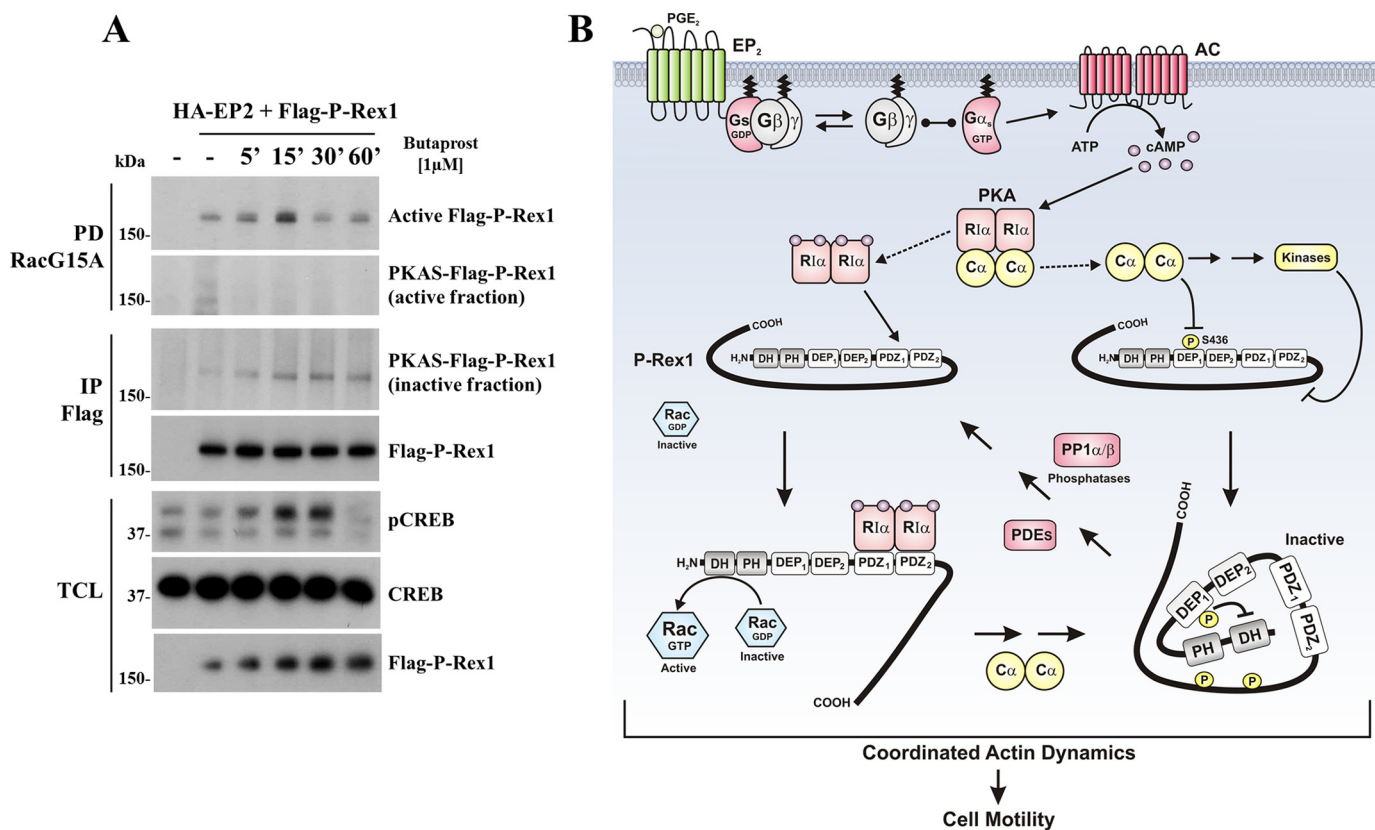


Figure 7. PKA regulatory and catalytic subunits target distinct P-REX1 molecules. A, COS7 cells co-expressing 3 × HA-EP2 receptors and 3 × FLAG-P-REX1 were serum-starved and stimulated with butaprost (1 μM) at the indicated times. Active P-REX1 was isolated with GST-RacG15A beads, and inactive P-REX1 was isolated from supernatants of RacG15A pulldowns by immunoprecipitation with anti-FLAG antibodies. Phosphorylation status of active and inactive P-REX1 was assessed by PKAS. Total cell lysates (TCL) were used to reveal pCREB (stimulation control), CREB, and FLAG-P-REX1. Three independent experiments were performed (n = 3). B, model explaining the effect of PKA regulatory and catalytic subunits on different P-REX1 molecules. G_s-coupled EP2 receptors stimulate adenylate cyclase generating cAMP that binds the RI α subunits of PKA holoenzyme promoting dissociation of cAMP-bound regulatory subunits and active kinase subunits. Based on our results, cAMP-Ri α directly activates a fraction of P-REX1 molecules, stimulating GTP loading to Rac (left, P-REX1 molecule, stimulatory input). In contrast, PKA C α subunits phosphorylate and inhibit a distinct pool of P-REX1 (right, P-REX1 molecule, inhibitory input). We previously demonstrated that inhibitory phosphorylation of P-REX1 by PKA occurs at Ser-436 on the DEP1 domain and PKA-regulated kinases that phosphorylate the P-REX1 C-terminal region, promoting intramolecular inhibitory interactions (7). Gradually, the fraction of active P-REX1 decreases, and the inactive, phosphorylated form increases, indicating that eventually the PKA C α inhibitory activity predominates. Later, P-REX1 is taken to its basal state by the intervention of protein phosphatases and cAMP-phosphodiesterases. Overall, our results suggest that P-REX1 activity is fine-tuned by the combined effects of PKA subunits leading to organized cytoskeleton dynamics and effective cell migration.

catalytic subunits would target distinct P-REX1 molecules once they are dissociated in response to signaling pathways stimulating cAMP production.

We demonstrated that cAMP analogs, used as type I-specific PKA agonists, lead to P-REX1 activation coincident with its interaction with the RI α subunit, suggesting that the RI α -P-

REX1 interaction, and consequently P-REX1 activation, depend on cAMP binding to RI α . Furthermore, we found that silencing RI α in MCF7 cells prevents P-REX1 activation by RI α -specific cAMP analogs. In contrast, C α knockdown has no effect on P-REX1 activation but prevents CREB phosphorylation. There are only few documented examples of a positive effect of cAMP

Figure 6. P-REX1 interaction with PKA-Ri α is necessary for EP2-dependent endothelial cell migration. A, drawing shows the experimental strategy to assess whether P-REX1-PDZ1 domain interferes on the interaction of PKA RI α subunits with P-REX1, preventing RI α -dependent P-REX1 activation and cell migration stimulated by EP2 receptors. B, endogenous RI α interacts with P-REX1-PDZ1-PDZ2 domains in a cAMP-dependent manner. HEK293T cells expressing GST-P-REX1-PDZ1-PDZ2 were serum-starved, stimulated with forskolin (10 μM) at the indicated times, and lysed. GST-P-REX1-PDZ1-PDZ2 was isolated with glutathione-Sepharose beads. Immunoblot was performed against RI α , GST, pCREB (stimulation control), and total CREB in pulldowns (PD GST) or total cell lysates (TCL) as indicated. Graph shows the time course of forskolin-induced RI α interaction with P-REX1-PDZ1-PDZ2 from three independent experiments (n = 3). One-way ANOVA followed by Tukey's multiple comparison test was performed, and significant p value is shown in the graph. C, P-REX1 PDZ1 domain competes with endogenous P-REX1 for the interaction with RI α pulled down with cAMP-agarose. MCF7 cells expressing EGFP or EGFP-P-REX1-PDZ1 were serum-starved and lysed. Endogenous RI α was isolated with cAMP-agarose beads. RI α , P-REX1, and GFP-PDZ1 in the pulldowns were revealed by immunoblot. Total cell lysates (TCL, right panel) were used to confirm the expression of the indicated proteins. Three independent experiments were performed (n = 3). D, expression of P-REX1-PDZ1 domain prevents P-REX1 activation in response to type I PKA-specific stimulation. MCF7 cells expressing EGFP or EGFP-P-REX1-PDZ1 were serum-starved, stimulated with type I PKA-specific cAMP analogs at the indicated times, and lysed. Active endogenous P-REX1 was isolated using GST-RacG15A beads and revealed by immunoblot. Total cell lysates (TCL) were used to detect P-REX1, pCREB (stimulation control), CREB, and GFP. Three independent experiments were performed (n = 3). E, expression of P-REX1-PDZ1 domain prevents EP2-dependent endothelial cell migration. Confluent PAE cells expressing EGFP or EGFP-P-REX1-PDZ1 were serum-starved and subjected to wound-healing assays. Cells were stimulated with PGE₂ (1 μM) or butaprost (1 μM). Pictures represent the wound closure after 16 h of stimulation; 10% fetal bovine serum (FBS) was used as control. F, graph represents the results of four independent experiments (n = 4). Statistical analysis was done by two-way ANOVA followed by Tukey; p values are indicated in the graph. n.s., no significant p > 0.05.

on the association between PKA regulatory subunits and putative signaling effectors. These include RII β interacting with G α_i in response to the coincident activation of G $_i$ - and G $_s$ -coupled receptors, enhancing MAPK signaling (20), and RI α interacting with Bim (B-cell lymphoma family protein), contributing to mitochondria-dependent apoptosis in S49 cells, also involving PKA catalytic activity (21). Consistent with a model in which RI α interacts with P-REX1 and acquires an active conformation that promotes P-REX1 activity, we found that RI α mutants with alterations at their CNB-B domain actually exhibited a better interaction with P-REX1 than WT RI α . However, only the ACRO mutant led to an increased P-REX1 activity, arguing that interaction and conformational adjustments are part of the mechanism by which RI α activates P-REX1. Interestingly, the RI α ACRO mutant is a cAMP-resistant mutant responsible for acrodysostosis, a genetic disease characterized by severe skeletal dysplasia, bone malformations, and hormone resistance (22). Cells expressing RI α ACRO have lower PKA kinase activity. Although the effect on P-REX1 of the cAMP-resistant RI α ACRO contrasts with the observed cAMP-dependent effect of WT RI α , the structure of the monomeric RI α ACRO1 (Lys-92–Ile-365) mutant presents a highly dynamic and disordered C-tail (Arg-357–Ile-365) (23). Because P-REX1 interacts with the CNB-B domain of RI α (Lys-346–Ser-378), the increased flexibility of the RI α ACRO1 (Lys-92–Ile-365) mutant C-tail might contribute to enhance its interaction with P-REX1, having a conformation suitable to activate it. Using peptide arrays, we previously mapped to the C-terminal region (Gly-343–Val-381) of RI α the sequences that interact with P-REX1 (7). Consistent with its increased effect on P-REX1, this region seems to be exposed in the RI α ACRO mutant.

We demonstrated that P-REX1 preferentially interacts with RI α subunits in the presence of cAMP than with the inactive holoenzyme. Moreover, *in vitro*, using purified proteins, RI α is able to directly activate P-REX1. Moreover, EP2-dependent cell migration involves the interaction between RI α and P-REX1, as indicated by the inhibitory effect of the P-REX1 PDZ1 domain, which competes with the interaction and decreases the migratory effect of EP2 receptors. Thus, RI α , as a direct activator of P-REX1, joins the list of upstream regulators of this RacGEF originally including G $\beta\gamma$ and PIP $_3$ (13). Therefore, P-REX1 could potentially integrate diverse positive combinatorial inputs such as G $\beta\gamma$ + PIP $_3$, G $\beta\gamma$ + RI α , and PIP $_3$ + RI α . Remarkably, the co-existence of paradoxical effects of PKA on P-REX1 is explained by independent actions of PKA regulatory and catalytic subunits on distinct P-REX1 protein pools. Considering the known role of RhoGEFs as scaffolds (24), and the increasing number of P-REX1–interacting partners (25–28), our results support the idea that P-REX1 integrates multiple inputs and establishes the basis for a prototypic activation model for this family of multidomain RacGEFs. Additional possibilities include the potential direct regulation between P-REX1–binding partners. For instance, the mechanistic/mammalian target of rapamycin, an upstream regulator of P-REX1 (28), has been reported to be regulated by RI α (29). Moreover, because P-REX1 is in fact a PKA substrate, it could potentially influence cAMP-dependent holoenzyme dissociation and reassembly in which, in the case of type I PKA, it has

been documented that substrates play a role in the regulatory circuit of PKA activity (30).

Altogether, our results extend our current view of how PKA and P-REX1 are reciprocally regulated. The emerging picture includes a cAMP-dependent positive role of RI α directly activating a fraction of P-REX1 molecules downstream of chemotactic G $_s$ -coupled receptors. In our model (Fig. 7B), PKA activation by G $_s$ signaling and cAMP formation stimulates the activity of a pool of P-REX1 molecules via direct interaction with cAMP–RI α subunits, and another P-REX1 pool is phosphorylated and inhibited by PKA C α subunits. Gradually, PKA C α catalytic activity prevails and desensitizes P-REX1 signaling by phosphorylation at Ser-436 and the action of PKA-regulated kinases targeting the C-terminal region (7). Eventually, P-REX1 basal state is restored by protein phosphatases, like PP1 α , and cAMP–phosphodiesterases (31). Our results also contribute to define a new paradigm in which PKA regulatory subunits emerge as signaling proteins able to stimulate their own specific effectors via cAMP-dependent direct protein–protein interactions.

Experimental procedures

Plasmids and cDNA constructs

Plasmids have been previously described: pCDNA3.1–FLAG–RI α WT, R335K, and acrodysostosis mutant (1–365) (23); pCEFL–GST-, FLAG-, and HA-tagged P-REX1 constructs and pmCherry–RI α (7); and pCDNA3–EP2 (32). pCEFL–EGFP–P-REX1–PDZ1 construct was prepared by subcloning P-REX1 PDZ1 cDNA from pCEFL–GST–P-REX1–PDZ1 to pCEFL–EGFP vector by restriction with BamHI/EcoRI enzymes. pCEFL–HA–HaloTag–P-REX1, full-length construct, was prepared amplifying HaloTag sequence from pHTN–HaloTag vector using the following primers: 5'cgttcctgattacgctagcATGGCAGAAATCGGTACTGGC3' and 3'GCTGGGCGCCTCCATgctagcGCTCTGAAGTACAGATCC5'. The HaloTag cDNA fragment was inserted by Gibson Assembly (New England Biotechnology) into pCEFL–HA–P-REX1, between the HA and P-REX1 sequences, by restriction with NheI enzyme. The construct was confirmed by sequencing.

Cell culture, transfection, and stimulation

HEK293T, COS7, MCF7 and porcine aortic endothelial (PAE) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% bovine fetal serum. Cells were either transfected using Lipofectamine Plus reagent (Invitrogen) for HEK293T and COS7 cells, PolyFECT (Qiagen) for PAE cells, and Turbofect (ThermoFisher Scientific) or Lipofectamine RNAiMAX (Invitrogen) for MCF7 cells, according to the manufacturer's protocol. Experiments were done 48 h after transfection with plasmids or 72 h after esiRNA or siRNA for knockdown experiments. When indicated, HEK293T, PAE, and COS7 cells were starved for 16 h with serum-free DMEM before stimulation; MCF7 cells were starved for 24 h. For EP2 receptor stimulation, we used prostaglandin E $_2$ (Sigma, P5640) or butaprost, an EP-specific agonist (Sigma, B6309). Type I PKA was directly stimulated with a combination of cAMP analogs (9): 6-Bnz-cAMP (Sigma, B4560) and

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8-AHA-cAMP (Sigma, A2104) that target the CNB-A and CNB-B domains of RI subunits, respectively. As positive controls, we used sphingosine 1-phosphate (Sigma, S9666), hepatocyte growth factor (R&D Systems, 294-HGN), and fetal bovine serum.

Knockdown of P-REX1 and type I PKA subunits

Reverse transfection of MCF7 cells was performed with Lipofectamine RNAiMAX and 30 pmol of esiRNA or individual siRNA. The esiRNAs used were esi-P-REX1 (Sigma, EHU136571), esiRNA-PKA-RI α (Sigma, EHU071341), esiRNA-PKA-C α (Sigma, EHU132541), or esiRNA-EGFP (Sigma, EHUEGFP), as control. This system includes a pool of multiple siRNAs (esiRNAs are endoribonuclease-prepared siRNAs, all directed toward a fraction of the target mRNA). Individual siRNAs were obtained from Sigma; their sequences were siP-REX1–3625 GAGAU-GAGCUGCCCUGUGA, siP-REX1–3809 GAAAGAAGAGU-GUACAAUC, si β Gal-2891 GGACGCGCGAAUUGAAUUA, siEGFP GCCACAACGUCUAUAUCAU, siRI α -175 CCAUG-GAGUCUGGCAGUAC, and siRI α -740 UGAAUUGGCAAC-CAGUGUU. Lipofectamine and esiRNA or siRNA mixes were prepared in OptiMEM, according to the manufacturer's instructions. Cells were trypsinized and suspended in DMEM containing 5% FBS without antibiotics, and then Lipofectamine/esRNA complexes were added, and cells were seeded on p60 dishes and incubated overnight at 37 °C in a 5% CO₂ atmosphere. Next day, cells were washed with PBS and cultured with DMEM containing 10% FBS and antibiotics. Experiments with knockdown cells were done 72 h after transfection. Cells were starved in serum-free media for 24 h before being stimulated for 5 min with cAMP analogs 6-Bnz and 8-AHA-cAMP (10 μ M).

Pulldown and immunoprecipitation

Cells grown in 35- or 60-mm dishes were washed with cold phosphate-buffered saline (PBS), lysed in 1 ml of ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, pH 7.5, containing 1% Triton X-100, 5 mM EDTA), protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin), and phosphatase inhibitors (1 mM NaF, 1 mM sodium orthovanadate, and 1 mM β -glycerol phosphate), and incubated for 10 min on ice. Cell lysates were centrifuged at 13,000 rpm for 10 min at 4 °C. For immunoprecipitation, supernatants were incubated with 3 μ l of RI α antibody or 4 μ l of FLAG antibody (for 3 \times FLAG-tagged P-REX1) and incubated overnight at 4 °C on a rocking platform, and then 35 μ l of protein G-agarose beads (Millipore) were added and incubated for 3 h. Samples were centrifuged at 5000 rpm for 2 min at 4 °C, and beads were washed three times with lysis buffer. Finally, beads were resuspended in 1 \times Laemmli sample buffer containing mercaptoethanol and boiled for 5 min. For pulldown assays of GST-tagged proteins, cell lysates were incubated with 25 μ l of GSH-Sepharose beads (GE Healthcare, catalog no. 17-5279-01) for 30–45 min and processed as described for immunoprecipitations. To isolate free RI α or PKA holoenzyme cyclic nucleotides, agaroses were used for pulldowns. MCF7 cell lysates were prepared with 50 μ M IBMX and then incubated with 20 μ l of cAMP-agarose (Sigma, catalog no. A0144) or (*R*_p)-8-AHA-cAMP-agarose (MyBioSource catalog no. MBS256231) for 12 h at 4 °C.

Samples were centrifuged at 5000 rpm for 2 min at 4 °C, and beads were washed three times with lysis buffer. Finally, beads were resuspended in 1 \times Laemmli sample buffer containing mercaptoethanol, boiled for 5 min, and processed as described for immunoprecipitations.

Immunoblotting

Cell lysates, pulldown, and immunoprecipitation samples were resolved in SDS-PAGE and transferred to Immobilon membrane (Millipore). Proteins were detected by Western blotting using antibodies with the following specificity: FLAG (Sigma, catalog no. F3165); P-REX1 (catalog no. HPA001927 from Sigma and catalog no. 13168S from Cell Signaling Technology); PKA regulatory subunit (R1 α) (catalog no. 610165 from BD Transduction Laboratories and catalog no. 4782S from Cell Signaling Technology); PKA catalytic subunit (C α) (catalog no. 5675S Cell Signaling Technology); Rac1 (catalog no. 610651 BD Transduction Laboratories); GST (B-14, Santa Cruz Biotechnology); phospho-(S/T)-PKA substrates (Cell Signaling catalog no. 9621S); phospho-CREB Ser-133 (Cell Signaling catalog no. 9191); and CREB (Cell Signaling catalog no. 9197S). Secondary antibodies were goat anti-mouse monoclonal (Zymed Laboratories Inc., Invitrogen, or KPL) or goat anti-rabbit (Rockland Immunochemicals or KPL).

Rac1 activation assay

Activation of Rac1 was assessed by detecting its interaction with the CRIB domain of PAK fused to GST using a pulldown strategy. PAE and MCF7 cells grown in 60-mm Petri dishes were starved and stimulated (as indicated in figure legends), washed with PBS containing 10 mM MgCl₂, and lysed with 0.5 ml of ice-cold lysis buffer containing 10 mM MgCl₂. Cell lysates were incubated on ice with 30 μ l of GST-PAK CRIB beads for 30 min on a shaker. Beads were then centrifuged at 5000 rpm for 1 min and washed three times with lysis buffer. Beads were then resuspended in 20 μ l of 1 \times Laemmli buffer, boiled for 5 min, centrifuged at 13,000 rpm for 1 min. Beads were then resolved in 12% acrylamide, transferred to polyvinylidene difluoride membranes, and immunoblotted using anti-Rac1 monoclonal antibodies. As controls, total cell lysates were analyzed in parallel.

Activation of P-REX1

Serum-starved cells, stimulated as indicated in figure legends, were used to detect the activation of P-REX1 assessed by its interaction with nucleotide-free Rac using a pulldown strategy with nucleotide-free recombinant GST-RacG15A essentially as described for Net1 by García-Mata and co-workers (33).

Isolation of active and inactive fractions of FLAG-P-REX1

To address the phosphorylation status of active and inactive fractions of P-REX1, COS7 cells co-expressing EP2 receptors and P-REX1 (3 \times HA-EP2 and 3 \times FLAG-P-REX1) were stimulated with butaprost and subjected to pulldown assays with nucleotide-free Rac (GST-RacG15A) to isolate the active fraction of P-REX1. Then the supernatant, containing the inactive fraction of P-REX1, was subjected to immunoprecipitation

with anti-FLAG antibodies. Active and inactive P-REX1 fractions were then assayed by Western blotting with anti-phospho-PKA substrate antibodies.

Production and purification of recombinant P-REX1 and RI α proteins

RI α recombinant protein was produced in *Escherichia coli* BL21 strain and precipitated using cAMP-agarose. Resin was washed with saline buffer, and RI α was eluted with cGMP (40 mM). Then gel filtration was performed, and RI α fractions were collected, concentrated, and preserved at -20°C by dialysis with 40% glycerol. Recombinant P-REX1 protein was produced in mammalian cell cultures. HEK293T cells were grown in five 10-cm Petri dishes, transfected with HA-HaloTag-P-REX1 construct with Turbofect. 48 h after transfection, cell lysates from 2×10^8 cells were incubated with HaloLink resin following the HaloTag Quick purification protocol (Promega). Purified proteins were quantified by the Bradford method. SDS-PAGE, Coomassie staining and Western blotting were performed to confirm protein integrity.

P-REX1 *in vitro* activation and interaction assays

To assess the direct interaction, *in vitro*, of P-REX1 with cAMP-bound RI α or type I PKA holoenzyme cAMP-agarose or (R_p)-8-AHA-cAMP were used for pulldown experiments. A mix of purified P-REX1 and RI α (0.5 and 1 μg , considering the RI α as a constitutive dimer) was prepared in lysis buffer containing 50 μM IBMX. The mix was incubated with 20 μl of cAMP-agarose or (R_p)-8-AHA-cAMP-agarose for 12 h at 4°C . Samples were centrifuged at 5000 rpm for 2 min at 4°C , and beads were washed three times with lysis buffer containing 1% Triton. To assess *in vitro* activation of P-REX1 by direct interaction with RI α , a mix of P-REX1 and RI α was prepared in lysis buffer containing 1% Triton and 10 mM MgCl_2 . The mix was incubated with GST-RacG15A-Sepharose beads for 45 min in rocking agitator at 4°C . Samples were centrifuged at 5000 rpm for 2 min at 4°C , and beads were washed three times with lysis buffer. Finally (for both pulldowns), beads were resuspended in 1 \times Laemmli sample buffer containing mercaptoethanol, boiled for 5 min, and processed for SDS-PAGE and immunoblot.

Fluorescence microscopy

PAE cells were seeded at low density on gelatin-coated glass-bottom dishes. Cells were starved for 16 h with serum-free medium and stimulated with PGE_2 or butaprost (10 μM) for 15 min. Subsequently, cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed twice with PBS, and prepared for conventional phalloidin and DAPI staining. Cell images were visualized in a Nikon Eclipse Ti inverted fluorescence microscope using a Plan Apo VC 1.4 oil immersion objective and captured with a Digital sight iXon Ultra ANDOR EMCCD camera. Images for F-actin and nuclear staining were obtained with Texas Red and UV filters, respectively, and analyzed with NIS-Elements Advanced Nikon software.

Wound-closure assay

PAE cells were seeded on 0.02% gelatin in 12-well plates. After 24 h, cells were starved with serum-free DMEM for 12 h.

Mitomycin C was added (12 μM , Sigma, catalog no. M0440) after 10 h of starvation. Migration assays were initiated by wounding cell monolayers with a pipette tip. Cells were washed three times with PBS and stimulated with prostaglandin E_2 (1 μM), butaprost (10 μM), or 10% FBS in 2 ml of DMEM. After 24 h, cells were fixed with 4% paraformaldehyde, stained with crystal violet, washed with PBS, and photographed.

Chemotaxis assays

The effect of PGE_2 (1 μM), butaprost (10 μM), sphingosine 1-phosphate (1 μM) and hepatocyte growth factor (10 ng/ml) on PAE endothelial cells chemotaxis was assessed in Boyden chambers essentially as described previously (34). 48-Well chambers were used with gelatin-coated filters having 8- μm pores. Cells that were starved of serum for 14 h were washed with PBS, gently trypsinized, washed, and resuspended at 100,000 cells per 100 μl of serum free-DMEM. Chemotaxis assays were done with 50,000 cells per well leaving cells to migrate for 6 h at 37°C . Then, cells were fixed with methanol and stained with crystal violet. Cells on the top side of the filter were removed with a cotton swab, and those at the bottom of the filter were observed under a $\times 20$ objective to confirm the presence of stained cells. Densitometric analysis was performed with the ImageJ software.

Statistical analysis

Data are presented as means \pm S.E. of at least 3–5 independent experiments. Densitometric quantitation of Western blottings and chemotaxis assays were done with ImageJ software. Active proteins, phosphorylated proteins, and interactions in pulldowns were normalized with total proteins and pulldown efficiency. Statistical analysis was performed using Sigma Plot 11.0, and graphs using Prism software version 6.0. Statistical test are indicated at the figure legends.

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