Protein Kinase A (PKA) Type I Interacts with P-Rex1, a Rac Guanine Nucleotide Exchange Factor

EFFECT ON PKA LOCALIZATION AND P-Rex1 SIGNALING*

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Morphology of migrating cells is regulated by Rho GTPases and fine-tuned by protein interactions and phosphorylation. PKA affects cell migration potentially through spatiotemporal interactions with regulators of Rho GTPases. Here we show that the endogenous regulatory (R) subunit of type I PKA interacts with P-Rex1, a Rac guanine nucleotide exchange factor that integrates chemotactic signals. Type I PKA holoenzyme interacts with P-Rex1 PDZ domains via the CNB B domain of RIα, which when expressed by itself facilitates endothelial cell migration. P-Rex1 activation localizes PKA to the cell periphery, whereas stimulation of PKA phosphorylates P-Rex1 and prevents its activation in cells responding to SDF-1 (stromal cell-derived factor 1). The P-Rex1 DEP domain is phosphorylated at Ser-436, which inhibits the DH-PH catalytic cassette by direct interaction. In addition, the P-Rex1 C terminus is indirectly targeted by PKA, promoting inhibitory interactions independently of the DEP1-PDZ2 region. A P-Rex1 S436A mutant construct shows increased RacGEF activity and prevents the inhibitory effect of forskolin on sphingosine 1-phosphate-dependent endothelial cell migration. Altogether, these results support the idea that P-Rex1 contributes to the spatiotemporal localization of type I PKA, which tightly regulates this guanine exchange factor by a multistep mechanism, initiated by interaction with the PDZ domains of P-Rex1 followed by direct phosphorylation at the first DEP domain and putatively indirect regulation of the C terminus, thus promoting inhibitory intramolecular interactions. This reciprocal regulation between PKA and P-Rex1 might represent a key node of integration by which chemotactic signaling is fine-tuned by PKA.

Rho guanine exchange factors (RhoGEFs)3 are mechanistically linked to fundamental cellular processes, such as migration, adhesion, and morphogenesis. Based on their ability to integrate signaling inputs that result in the activation of Rho GTPases, RhoGEFs indirectly contribute to establish nucleation sites for actin polymerization, thus exerting a tight control on cytoskeleton dynamics (1, 2). RhoGEFs can also play a role as scaffolds of different signaling cascades, thus representing regulatory nodes for spatial and temporal control of signaling (3). These fundamental processes are fine-tuned by phosphorylation of RhoGEFs. However, the complex interplay between RhoGEFs and kinases is not completely understood; relevant examples include RhoGEFs known to be activated or inhibited by phosphorylation, some of them by PKA (4, 5).

The ability of PKA to modulate the activity of RhoGEFs might be further facilitated by direct interactions that would also influence the subcellular localization of PKA and its accessibility to specific substrates. The paradigmatic example of this situation is AKAP-Lbc, which is a RacGEF activated by G12α-coupled receptors that promotes polymerization of actin filaments downstream of Rho and also interacts with multiple kinases regulating mitogenic inputs (6, 7). AKAP-Lbc interacts with KSR-1, a scaffold of the ERK1/2 signaling cascade, allowing PKA bound to AKAP-Lbc to enhance control of this mitogenic pathway in response to cyclic AMP (8, 9). Although type II PKA translocates to the leading edge of migrating fibroblasts (10), the binding partners that restrict the kinase to this location are currently undefined. Moreover, whether type I PKA regulates RhoGEFs through direct interactions during a chemotactic response is currently unknown, although this possibility might help to explain the apparent paradox according to which PKA either activates (11–14) or inhibits (15–19) cell migration, depending on the chemoattractant and cell type.

P-Rex1 is a multidomain Rac guanine nucleotide exchange factor involved in chemotactic migration of normal and metastasizing cancer cells, thereby playing a critical role in tumor invasion and metastasis.

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4 The abbreviations used are: RhoGEF, Rho guanine exchange factor; GEF, guanine nucleotide exchange factor; P-Rex1, phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchange 1 protein; PRKAR1a, cAMP-dependent protein kinase type Iα regulatory subunit; PRKACa, cAMP-dependent protein kinase catalytic subunit α; AKAP, A-kinase anchoring protein; PKAS, PKA substrate; (p)CREB, (phospho)cAMP response element binding; S1P, sphingosine 1-phosphate; DEP, Dishevelled, Egl-10, and Pleckstrin; PDZ domains, mTOR, mammalian target of rapamycin; SD1-1, stromal cell-derived factor 1; HUVEC, human umbilical vein endothelial cell; PAE, porcine aortic endothelial; DH, homology domain; PH, pleckstrin homology; IBMX, isobutylmethylxanthine; EGFP, enhanced GFP; CNB B, cyclic nucleotide-binding domain B.
static cancer cells (20–27). This RacGEF is activated by Gβγ and phosphatidylinositol-3,4,5-trisphosphate (28–32), by growth factor receptors, and regulators of neuronal morphology (33–36) as well as by direct interaction with mTORC2, a fundamental multimeric kinase with affinity for P-Rex1 DEP domains (20, 37) and by protein phosphatase 1α, which dephosphorylates P-Rex1 at Ser-1165 (38). In endothelial cells, in which this guanine nucleotide exchange factor (GEF) is among the most highly expressed RhoGEFs (39), it influences changes in cell morphology in response to PDGF (28, 38) and participates in the chemotactic response to sphingosine 1-phosphate and SDF-1 (23, 27). P-Rex1 structure is constituted by a DH-PH cassette characteristic of the family of RhoGEFs with homology to Dbl (homology (DH) domain and a pleckstrin homology (PH) domain) followed by two DEP domains in tandem, two PDZ domains, and a long C terminus with homology to inositol polyphosphate-4-phosphatase (28). Phosphorylation of P-Rex1 exerts a positive or negative role on its activity, putatively depending on the kinase involved and the phosphorylation site (38, 40). PKA, in particular, phosphorylates and prevents P-Rex1 from being activated by Gβγ and phosphatidylinositol-3,4,5-trisphosphate (41, 42). Although the precise mechanisms of this regulation remain unknown, these findings open the interesting possibility that PKA might maintain interactions with P-Rex1 and also might be regulated by this RacGEF. Here we identified type I PKA as a novel P-Rex1 interactor and study the molecular aspects and functional consequences of this interaction on P-Rex1 regulation and PKA localization.

Experimental Procedures

Endothelial cDNA Library Generation and Yeast Two-hybrid Screening—An endothelial cDNA library from human microvascular endothelial cells (HMEC-1) was generated according to the MatchmakerT™ yeast two hybrid library construction and screening kit user manual (Clontech) and used to screen P-Rex1-PDZ-PDZ as bait according to protocols previously described in detail (43). In brief, total RNA extracted from human microvascular endothelial cells was retro-transcribed using reverse transcriptase and amplified using the Clontech SMART system containing Moloney human microvascular endothelial cells was retro-transcribed described in detail (43). In brief, total RNA extracted from HMEC-1 was digested with BglII and BamHI, enzymes with compatible cohesive ends, and then the new vector containing the first fragment of P-Rex1 was digested again with BamHI and XbaI to introduce the second fragment of P-Rex1 to finally obtain pEGFP-C1-P-Rex1 full-length. pCEFL-GST-P-Rex1-Nter (DH-PDZ, M1-1788) was prepared from pCEFL-EGFP-P-Rex1 by PCR using 5′-Nter-P-Rex1BamHI atgaGATCCatg-gagccgccacgccgacgac and 3′-Nter-P-Rex1EcoRI atgaATTCtgcagacgccgacgac primers. P-Rex1 DEP1 and DEP2 and P-Rex1 PDZ1 and PDZ2 domains were amplified by PCR and cloned as 5′-BamHI/3′-EcoRI into pCEFL-mammalian expression vector. P-Rex1 DEP1 primers were atgaGATCCAGAGAAGTTGAACCTGACCACAG and atgaGATTCctgcagacgccgacgac primers. P-Rex1 PDZ1 primers were atgaGATCCAGAGAAGTTGAACCTGACCACTCG and atgaGATTCctgcagacgccgacgac primers. P-Rex1 PDZ2 primers were atgaGATCCAGAGAAGTTGAACCTGACCACTCG and atgaGATTCctgcagacgccgacgac primers. P-Rex1-DEP2 primers were atgaGATTCCTCTACACCCCGGTGATCAAAGACC and atgaGATTCGACACATGGCGTGGCCACCAAGGAG and P-Rex1-PDZ2 primers were 5′-ataGGATCCGAC-ACACTGTGGCTTCAGATCCAGATCG and atgaGATTCtgcagacgccgacgac primers. P-Rex1 N-terminal S436A and S436D mutant constructs were prepared using the QuikChange site-directed mutagenesis kit (Stratagene #200518) and pCEFL-GST-P-Rex1-N terminus as template. The plasmid was amplified using the following primers: 5′-GGAGCAGCGGAGAAGAGTGCgCgCTGATCCCCCAAGTGCGTTTTC-3′ and 3′-GGAACGACATTGGGGAGACGACGACGCTTTCTCCGGCGGTC-5′ for the S436A mutant and 5′-GGA-CCGCGGAGAAGAGTGCgCgCTGATCCCCCAAGTGCGTTTTC-3′ and 3′-GGAACGACATTGGGGAGACGACGACGCTTTCTCCGGCGGTC-5′ for the S436D mutant. The point

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X-α-Gal (α-galactosidase reporter, indicative of positive interactions). Plasmids containing putative interacting prey clones were isolated with Zymoprep™ yeast plasmid miniprep kit (Zymo research) and used to transform Escherichia coli DH5α strain. To confirm specific interactions, yeast were cotransformed with P-Rex1-PDZ-PDZ and the different prey plasmids and plated on DOBA/−/AHLT (selecting for interactions) or DOBA/−/LT (selecting only for the plasmids). PTD1/p53 plasmids were used as controls as indicated by the Matchmaker III system. Specific P-Rex1-PDZ-PDZ-interacting clones were sequenced and identified by BLAST at the NCBI web page.

Constructs and Plasmids—Z6 prey, coding for the C-terminal region of type 1α PKA regulatory subunit (including CNB B, the second cAMP binding domain), identified as a P-Rex1-PDZ-PDZ-interacting clone, was subcloned into the mammalian expression vector pCEFL-EGFP-3XFLAG. pEGFP-C1-PRKAR1a and pCDNA3.1-HA-PRKAR1a plasmids were kindly donated by Dr. Manos Mavrakis from the NICHD, National Institutes of Health, Bethesda, MD. PRKAR1a from pEGFP-C1-PRKAR1a was subcloned into pmCherry-C1 vector using BglII/NheI restriction sites. P-Rex1 from pCEFL-EGFP-P-Rex1 was cloned into pEGFP-C1-P-Rex1 in two parts, and pCEFL-EGFP-P-Rex1 was digested with BamHI and EcoRI enzymes releasing two fragments of P-Rex1, one comprising the first 3262 bp of P-Rex1 (fragment 1, BamHI/BamHI) and the second fragment of 1377 bp corresponding to the last part of P-Rex1 (BamHI/XbaI). Fragment 1 was introduced into pEGFP-C1 vector linearized with BglII and BamHI, enzymes with compatible cohesive ends, and then the new vector containing the first fragment of P-Rex1 was digested again with BamHI and XbaI to introduce the second fragment of P-Rex1 to finally obtain pEGFP-C1-P-Rex1 full-length. pCEFL-GST-P-Rex1-Nter (DH-PDZ, M1-1788) was prepared from pCEFL-EGFP-P-Rex1 by PCR using 5′-Nter-P-Rex1BamHI atgaGATCCatg-gagccgccacgccgacgac and 3′-Nter-P-Rex1EcoRI atgaATTCtgcagacgccgacgac primers. P-Rex1 DEP1 and DEP2 and P-Rex1 PDZ1 and PDZ2 domains were amplified by PCR and cloned as 5′-BamHI/3′-EcoRI into pCEFL-mammalian expression vector. P-Rex1 DEP1 primers were atgaGATCCAGAGAAGTTGAACCTGACCACAG and atgaGATTCctgcagacgccgacgac primers. P-Rex1 PDZ1 primers were atgaGATCCAGAGAAGTTGAACCTGACCACTCG and atgaGATTCtgcagacgccgacgac primers. P-Rex1 PDZ2 primers were atgaGATCCAGAGAAGTTGAACCTGACCACTCG and atgaGATTCtgcagacgccgacgac primers. P-Rex1 N-terminal S436A and S436D mutant constructs were prepared using the QuikChange site-directed mutagenesis kit (Stratagene #200518) and pCEFL-GST-P-Rex1-N terminus as template. The plasmid was amplified using the following primers: 5′-GGACCGGCGGAGAAGAGTGCgCgCTGATCCCCCAAGTGCGTTTTC-3′ and 3′-GGAACGACATTGGGGAGACGACGACGCTTTCTCCGGCGGTC-5′ for the S436A mutant and 5′-GGA-CCGCGGAGAAGAGTGCgCgCTGATCCCCCAAGTGCGTTTTC-3′ and 3′-GGAACGACATTGGGGAGACGACGACGCTTTCTCCGGCGGTC-5′ for the S436D mutant. The point
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mutations were confirmed by sequencing using BigDye Terminator v3.1 Cycle Sequencing kit. Other constructs have been previously described (20). The EGFP-P-Rex1-CAAX constructs were generated by amplifying the P-Rex1 regions of interest, omitting a stop codon in the reverse primers, and cloning the fragments into pCEFL-EGFP-CAAX using 5'-Bam-HI/3'-EcoRI restriction sites (located between the EGFP and CAAX coding sequences). DH-Ph primers were ataGGATCCATGGAGGCAGCCAGGCACG and ataGAATTCGGAATCTGCTGGGCTCCGCCTCGGAGAT, DH-DEP2 primers were ataGGATCCATGGAGGCAGCCAGGCACG and ataGAATTCGCGCTGCTGAC and ataGAATTCCAGTGCATGGTATACAGGCCCCAG and ataGAATTCCAGTGCATGGTATACAGGCCCCAG and ataGAATTCCAGTGCATGGTATACAGGCCCCAG and ataGAATTCCAGTGCATGGTATACAGGCCCCAG.

Cell Culture, Transfection, and Stimulation—HEK-293T, COS-7, 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) (HEK-293T and COS-7) or PolyFECT (Qiagen) PAE, according to the manufacturer’s protocol. Experiments were done 48 h after transfection. When indicated, cells were starved for 16 h with serum-free DMEM before stimulation. HUVEC cells were used before passage 8 and maintained in HuMedia-EG2 medium (Kurabo). Transfection was performed using Lipofectamine 2000 (Invitrogen) and Plus reagent (Invitrogen) according to the manufacturer’s protocol, removing complexes 40 min after transfection. Transfection efficiency of PAE cells used for chemotaxis experiments was between 29 and 35%. Stimulation of cells was done with SDF-1/CXCL12 (Peprotech, catalog #300-28A) or sphingosine 1-phosphate (S1P, Sigma, catalog #S9666) as indicated in figure legends (Figs. 3 and 5). The effect of PKA on S1P-dependent PAE cell migration was assessed with 10 μM forskolin (Sigma, catalog #F6886) and 100 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma) catalog #I5879 in the absence of presence of 10 μM H89 (Sigma, catalog #B1427) as indicated.

Pulldown and Immunoprecipitation—Cells grown in 10-cm dishes were washed with 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 μg/ml leupeptin, and 10 μg/ml aprotinin), and phosphatase inhibitors (1 mM NaF, 1 mM sodium orthovanadate, and 1 mM β-glycerophosphate), and incubated for 10 min on ice. Cell lysates were centrifuged at 13,000 rpm for 10 min at 4 °C. Supernatants were incubated with the indicated antibodies (1–10 μl, as required) for immunoprecipitation and incubated overnight at 4 °C on a rocking platform, then 35 μl of protein G-agarose beads (Mililipore) were added and incubated for 3 h. Samples were centrifuged at 13,000 rpm for 2 min at 4 °C, the supernatant was discarded, and beads were washed 3 times with lysis buffer. Finally, beads were resuspended in 1× Laemmli sample buffer containing mercaptoethanol and boiled for 5 min. For pull-down assays of GST-tagged proteins, cell lysates were incubated with 25 μl of glutathione-Sepharose beads (catalog #17-5279-01, GE Healthcare) for 30–45 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 blot using antibodies with the following specificities: HA (Covance), FLAG (Sigma), P-Rex1 (catalog #HPA001927, Sigma), PKA (R10a) (catalog #610165, BD Transduction Laboratories), Rac1 (BD Transduction Laboratories), GST (B-14, Santa Cruz Biotechnology), GFP (B-2, Santa Cruz Biotechnology), ERK-2 (C-14, Santa Cruz Biotechnology), phospho-p42/44 MAP kinase Thr-202/Tyr-204 (Cell Signaling), phospho-Akt1/2/3 Ser-473 (Santa Cruz, sc-7985), Akt1 (Sigma catalog #P2482), phospho-CREB Ser-133 (Cell Signaling #9191), and phospho-(Ser/Thr) PKA substrate (catalog #9621, Cell Signaling). Secondary antibodies were goat anti mouse monoclonal (Zymed Laboratories Inc., Invitrogen or KPL) or goat anti-rabbit (Rockland Immunocchemicals or KPL).

Activation of P-Rex1—Activation of P-Rex1 was assessed by detecting its interaction with nucleotide-free G15-Rac using a pulldown strategy with nucleotide-free G15-Rac fused to GST essentially as described for Net1 by García-Mata and co-workers (44).

Rac1 Activation Assay—Activation of Rac1 was assessed by detecting its interaction with the CRIB domain of PKA fused to GST using a pulldown strategy. HEK293T cells grown in 10-cm Petri dishes and transfected with EGFP-P-Rex1-CAAX constructs and other constructs (as indicated in the figure legends (Figs. 6 and 7)) were washed with PBS containing 10 mM MgCl₂ and lysed with 1 ml of ice-cold lysis buffer containing 10 mM MgCl₂. Cell lysates were incubated with 30 μl of GST-PK CRIB beads on ice for 30 min on a shaker. Beads were then centrifuged at 5000 rpm for 1 min and washed 3 times with lysis buffer. Beads were then resuspended in 50 μl of 1× Laemmli buffer, boiled for 5 min, centrifuged at 13,000 rpm for 1 min. Beads were then resolved in a 12% acrylamide, transferred to PVDF membranes, and immunoblotted using anti-Rac1 monoclonal antibodies. As controls, total cell lysates were analyzed in parallel.

Fluorescence Microscopy—Cells were seeded at low density on gelatin-coated glass-bottom dishes and transfected with pEGFP-C1-P-Rex1 or pmCherry-PKRAR1a or both plasmids. Experiments were carried out 24–48 h later. COS-7 cells were starved for 16 h and HUVEC cells for 2 h with M199 medium (Gibco) plus 0.5% BSA. Then cells were stimulated with CXCL12/SDF-1 for 5 min. Subsequently, cells were fixed in 4% paraformaldehyde in PBS for 15 min and then washed twice with PBS. 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 DS-Qi1Mc Nikon camera. Images for PRKAR1a and P-Rex1 were obtained with Texas Red and GFP filters, respectively, and analyzed with NIS-Elements software from Nikon.

Chemotaxis Assays—The effect of sphingosine 1-phosphate on PAE endothelial cells chemotaxis was assessed in Boyden chambers essentially as previously described (45) with minor modifications. 48-well chambers were used with gelatin-coated filters having 8-μm pores. Cells were transiently transfected with EGFP-CAAX, EGFP-Z6, EGFP-P-Rex1-DH-PDZ2-CAAX, or EGFP-P-Rex1-DH-PDZ2-S436A-CAAX, as described in the
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PKA Interacts with P-Rex1—To identify proteins with affinity for P-Rex1, a Gβγ- and phosphatidylinositol-3,4,5-trisphosphate-dependent RacGEF involved in cell migration (28), we used P-Rex1-PDZ-PDZ domains as bait in a yeast two-hybrid screen with a human endothelial cDNA library. We found that in yeast P-Rex1-PDZ-PDZ interacted with the C-terminal region of type I-α PKA regulatory subunit (PRKAR1α), including CNB B, the second cAMP binding domain. We designated this yeast two-hybrid prey as the Z6 clone (Fig. 1A). This clone was obtained only once in the screening. The interaction was specific, as confirmed by the growth in high stringency media lacking adenine, histidine, leucine, and tryptophan (−AHLT) of yeast transformed with the combination of P-Rex1-PDZ-PDZ and Z6 or with the positive control of the system composed of p53 and PTD1 (Fig. 1B, right panel). As control of transformation, the growth of yeast was confirmed in medium lacking leucine and tryptophan (−LT), indicating that each plasmid was effectively transformed (Fig. 1B, left panel). These results demonstrated a direct interaction in yeast between the C-terminal region of type I PKA regulatory subunit (including CNB B), represented by Z6, and the tandem PDZ domains of P-Rex1. Different complementary approaches were then used to assess the interaction between type I PKA and P-Rex1 in mammalian cells. Initially, we tested the interaction between PKA R1α prey and P-Rex1-PDZ-PDZ bait, both subcloned into mammalian expression vectors. HEK-293T cells were transfected with Z6, as a FLAG-EGFP-tagged protein, and P-Rex1-PDZ-PDZ fused to GST. Two days after transfection, cell lysates were subjected to pulldown assays with glutathione-Sepharose beads. As shown in Fig. 1C, left panel, Z6 prey interacted with P-Rex1-PDZ-PDZ domains (PD: GST), and this interaction was specific as Z6 did not interact with GST, used as negative control. The expression of the constructs was confirmed in total cell lysates (Fig. 1C, right panel, TCL). Similarly, full-length PRKAR1α (tagged with EGFP) interacted with P-Rex1-PDZ-PDZ domains (Fig. 1D). Next, we tested the interaction between both full-length proteins in HEK-293T cells transfected with EGFP-PRKAR1α and HA-P-Rex1. In this case P-Rex1 was immunoprecipitated with HA antibodies, and interacting PRKAR1α was revealed by Western blot using antibodies against-GFP (Fig. 1E, left panel), showing that, indeed, full-length PRKAR1α interacted with full-length P-Rex1. Furthermore, we also wanted to know whether the catalytic subunit of type I PKA could interact with P-Rex1. To test this possibility, EGFP-tagged catalytic PKA α subunit (PRKACA) and GST-P-Rex1-PDZ-PDZ were co-transfected into HEK-293T cells. As shown in Fig. 1F, left panel, we detected a specific interaction between type I PKA catalytic subunit and P-Rex1-PDZ-PDZ domains as revealed by the abundant presence of EGFP-PRKACA, detected with GFP antibodies, in the pulldown of GST-P-Rex1-PDZ-PDZ compared with the GST pulldown used as negative control. Next, to define whether other domains of P-Rex1 were able to participate in the interaction with PKA, different constructs of P-Rex1 (DH-Ph, DEP-DEP, PDZ-PDZ, and C terminus, all of them fused to GST) were obtained. These constructs were transfected into HEK-293T cells together with either Z6 (Fig. 1G), full-length PRKAR1α (Fig. 1H), or both type I PKA catalytic and regulatory subunits (Fig. 1I). The interacting PKA constructs were revealed by Western blot in the P-Rex1 pulldown assays; our results showed that all type I PKA constructs, either the C-terminal region containing the second cAMP binding domain, represented by Z6 (3XFLAG-EGFP-Z6), the full-length PRKAR1α (EGFP-PRKAR1α), or the full type I PKA holoenzyme (EGFP-PRKACA+HA-PRKAR1α) mainly interacted with the PDZ-PDZ domains of P-Rex1 (Fig. 1, G, H, and I, respectively). All together these results demonstrate that P-Rex1 specifically interacts with PKA throughout its PDZ-PDZ domains. Likewise, we analyzed the endogenous interaction between these proteins. The interaction of endogenous type I PKA regulatory subunit (PRKAR1α, R1α) and endogenous P-Rex1 was assessed by immunoprecipitating PRKAR1α and detecting P-Rex1. As shown in Fig. 1I, endogenous P-Rex1 co-immunoprecipitated with endogenous PKA R1 α subunit but not with a control negative antibody. Anti-P-Rex1 antibody detected two main bands in the total cell lysate. The upper band (above the 150-kDa marker) corresponds to full-length P-Rex1, and the lower molecular mass band (above 100 kDa) might correspond to a splice variant or might represent a degradation product of full-length P-Rex1. According to...
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Nexprot beta database, P-Rex1 isoform 2 starts at the second PDZ domain and has a predicted molecular mass of 106 kDa (nextprot). The P-Rex1 antibody used for these Western blots was generated using a peptide corresponding to P-Rex1 C570-P700, which in P-Rex1 isoform 2 corresponds to Cys-43–Pro-173.

PRKAR1a CNB B Preferentially Interacts with P-Rex1–PDZ1—We explored a possible preferential interaction between endogenous PRKAR1a and either one of the two PDZ domains of P-Rex1. First, individual P-Rex1 PDZ domains fused to GST were isolated from transfected COS7 cells, and endogenous PRKAR1a bound to them was revealed by Western blot. As shown in Fig. 2A, PRKAR1a interacted with P-Rex1 PDZ1 at comparable levels as it did with the P-Rex1-PDZ1–PDZ2 tandem cassette. In comparison, the interaction of PRKAR1a with P-Rex1-PDZ2 was significantly lower. Additionally, to know the minimal structural requirements on PRKAR1a to interact with P-Rex1-PDZ1, to address the question of whether the C-terminal region of this PKA regulatory subunit is required, and to confirm that the interaction between these regions was direct, we used a peptide array strategy using overlapping peptides (15-mers) representing different regions of PRKAR1a schematically shown in Fig. 2B. Interestingly, a series of seven overlapping peptides whose sequence corresponds to a region of human PRKAR1a, extending from Gly-346 to Ser-378, interacted directly with the P-Rex1-PDZ1 recombinant domain. The interacting peptides did not include the last three amino acids of PRKAR1a, suggesting a non-canonical mechanism of interaction between the CNB B cAMP binding domain of PRKAR1a and P-Rex1 PDZ1.

SDF-1 Promotes P-Rex1 Activation and Mobilization to the Cell Membrane Together with PRKAR1a—To determine the effect of P-Rex1 activation on its interaction with PRKAR1a and the localization of PRKAR1a–P-Rex1 complex, we first analyzed the kinetics of P-Rex1 activation detected by its interaction with nucleotide-free Rac (GST–RacG15A) (44, 47) by pulldown. As shown in Fig. 3A, in COS-7 cells, the maximum activation of P-Rex1 was detected at 30 min of SDF-1 stimulation. Time course of interaction between endogenous type I PKA and P-Rex1 in response to SDF-1 was assessed in cells transfected with GST–P-Rex1-DH–PDZ2 (including DH–PH–DEP1–DEP2–PDZ1–PDZ2 domains, as represented in Fig. 3B). We detected that the interaction between endogenous PRKAR1a and the indicated GST–P-Rex1 construct increased with time in cells stimulated with SDF-1 (Fig. 3B, graph and upper panel).

The mobilization of P-Rex1 to the cell membrane and its potential effect on PKA R1α localization was studied in COS-7 and HUVEC cells stimulated with SDF-1. As shown in Fig. 3C, EGFP–P–Rex1 (panels a and e) and mCherry–PRKAR1a (panels b and f) showed a cytosolic distribution in serum-starved control cells. As expected, P-Rex1 translocated to the membrane upon SDF-1 stimulation (panels c, g, and k), whereas PRKAR1a depended on P-Rex1 co-expression to translocate to the membrane, remaining cytosolic in cells not transfected with P-Rex1 but localized to membranes together with P-Rex1 in cells transfected with both PRKAR1a and P–Rex1 (Fig. 3C, compare panel d with panels h and l). In this case mCherry–PRKAR1a co-localized with P–Rex1 at the cell membrane of SDF-1-stimulated cells (panels i and j and panels m and n for overlap and amplification in Cos-7 and HUVEC cells, respectively). Thus, PRKAR1a mobilization to the cell membrane upon SDF-1 stimulation likely depends on its interaction with P-Rex1. Interestingly, endothelial cells expressing Z6 (the domain of R1α initially identified as a P-Rex1 PDZ–PDZ interactor in the yeast two hybrid assay) showed a higher chemotactic response to lower doses of sphingosine 1-phosphate (Fig. 3D), suggesting that PKA exerts a tonic inhibitory control over chemotactic migration in which P-Rex1 is involved.

PKA Phosphorylates P-Rex1–DEP1 Domain at Ser-436 and Modulates P-Rex1 Activity—The characterization of type 1 PKA as a novel P-Rex1 interacting protein together with previous reports showing that P-Rex1 is desensitized by PKA (41) led us to characterize the potential phosphorylation of P-Rex1 by type I PKA and whether this potential phosphorylation correlates with the kinetics of activation of this GEF in response to SDF-1 and the potential effect of the interaction between PKA and P-Rex1 on the regulation of this GEF. First, we searched for potential PKA-dependent phosphorylation sites of P-Rex1 using ScanSite. This algorithm predicted the existence of four possible sites on P-Rex1 (Fig. 4A): one located at the PH domain

FIGURE 1. PKA interacts with P-Rex1. A, schematic representation of the domains of P-Rex1 depicting the bait used in the yeast two-hybrid screen and the domains of PRKAR1a depicting the prey that was obtained, B, the specificity of the interaction between Z6 prey and PDZ–PDZ domains of P-Rex1 was tested in yeast using p53 as negative control. All yeast grew in media lacking leucine and tryptophan (∼LT) which select for the presence of Z6 prey, P-Rex1 bait, p53 and PTD1 plasmids (right panel). Only those displaying interaction were able to grow under high stringency conditions (media lacking adenine, histidine, leucine, and tryptophan, ∼AHLT). The interaction between p53 and PTD1 was used as a positive control. C–I, characterization of PKA and P-Rex1 interaction in transfected HEK-293T cells. Pulldown assays and immunoprecipitation experiments were resolved on SDS-polyacrylamide gels and analyzed by immunoblotting using the indicated antibodies. C, P-Rex1–PDZ–PDZ domains interact with Z6 prey. Cell lysates containing Z6 prey (tagged with 3XFLAG and EGFP) and P-Rex1–PDZ–PDZ as GST–fusion protein (GST–P-Rex1–PDZ–PDZ) were incubated with glutathione beads, and both total cell lysates (TCL; right panel) and pulldown assays (PD; GST, left panel) were analyzed using the indicated antibodies. D, P-Rex1–PDZ–PDZ domains interact with full-length PRKAR1a. Immunoblots show GST–P-Rex1–PDZ–PDZ and EGFP–PRKAR1 in total cell lysates (right panel) and pulldown assays (PD; GST, left panel). E, full-length P-Rex1 interacts with full-length PRKAR1a. HA–P-Rex1 was immunoprecipitated using HA antibody (IP; HA), and PRKAR1a association was detected with GST antibody in immunoprecipitated fractions (IP; HA, left panel) and total cell lysates (right panel). F, GST–P-Rex1–PDZ–PDZ interacts with PRKAc subunit. Pulldown assays from cells expressing EGFP–PRKAc and either GST or GST–P-Rex1–PDZ–PDZ were immunoblotted using anti-GFP and GST antibodies. G, Z6 prey mainly interacts with P-Rex1–PDZ–PDZ domains. Pulldown assays from cells transfected with 3XFLAG–EGFP–Z6 prey and GST–fusion protein were analyzed using the indicated antibodies. H, full-length PRKAR1a mainly interacts with P-Rex1–PDZ–PDZ domains. Pulldown assays from cells transfected with EGFP–PRKAR1a and GST–fusion protein were analyzed using the indicated antibodies. I, endogenous P-Rex1 interacts with endogenous PRKAR1a. The interaction between endogenous P-Rex1 and PRKAR1a was assessed by immunoprecipitation, IP: R1α (PRKAR1a), followed by Western blotting (P-Rex1) using total cell lysates from Cos7 cells. The specificity of the assay was determined by using a mouse antibody as negative control (Ctrl). Results shown are representative of at least three independent experiments.
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(Ser-305), one located at the DEP1 domain (Ser-436), and two located at the C terminus (Ser-1001 and Ser-1272). To map the precise domain of P-Rex1 to be phosphorylated by PKA, we transfected HEK-293T cells with different constructs of this GEF together with type I PKA. We first compared the potential phosphorylation sites of P-Rex1 in the N terminus versus the P-Rex1 C terminus. The phosphorylation, detected with antibodies recognizing PKA substrates (PKAS), was associated with the P-Rex1 N terminus, which includes the DH, PH, two DEP, and two PDZ domains (Fig. 4B, left panel). We then compared the potential phosphorylation site in P-Rex1 constructs containing the DH and PH domains and constructs containing
both DEP and both PDZ domains. In this case the phosphorylation of P-Rex1 was detected in the construct comprising from the first DEP domain until the second PDZ domain (Fig. 4B, right panel). Finally, we assessed the potential phosphorylation of PKA at the DEP1, DEP2, PDZ1, and PDZ2 domains of P-Rex1, all expressed as individual constructs. With this approach, we detected the phosphorylation of P-Rex1 by PKA at the first DEP domain, GST-DEP1 (Fig. 4C), where a predicted consensus PKA phosphorylation site at Ser-436 was identified (Fig. 4A). With the aim of directly assessing whether P-Rex1 is phosphorylated by PKA at Ser-436, we engineered a GST-DEP1 S436A mutant and tested its potential phosphorylation by PKA. As shown in Fig. 4D, this mutant is not phosphorylated by PKA, as indicated by the lack of signal in the Western blot using antibodies recognizing PKA substrates that, in parallel, gave a positive signal with the P-Rex1-DEP1 domain. Altogether our results demonstrate that P-Rex1 is a novel PKA interactor that contributes to the mobilization of PKA R1α to the plasma membrane, indicating a potential reciprocal regulation, as P-Rex1 is also phosphorylated by PKA at Ser-436 at its DEP1 domain.

To examine the functional effect of P-Rex1 phosphorylation at Ser-436 by PKA, we assessed the effect of forskolin on SDF-1-dependent P-Rex1 activation and phosphorylation. As shown in Fig. 5A, SDF-1-dependent P-Rex1 activation was inhibited by forskolin, coincident with an increase of P-Rex1 phosphorylation detected with antibodies recognizing PKAS (Fig. 5A, second panel). As positive controls of the effect of SDF-1 signaling...
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and forskolin-dependent increase of cAMP, we demonstrated the phosphorylation of Akt and CREB, respectively (Fig. 5A, pCREB and pAkt; TCL). To address whether this inhibition of P-Rex1 by PKA correlated with a potential inhibitory effect of PKA on sphingosine 1-phosphate-elicted endothelial chemotaxis, we initially explored the effect of forskolin/IBMX and H89 on this process. As shown in Fig. 5B, sphingosine 1-phosphate promoted endothelial cell chemotaxis in a dose-response manner that was partially inhibited by PKA (as depicted by the inhibitory effect of forskolin/IBMX, reversed by H89, a widely used PKA inhibitor). To address the hypothesis that desensitization of P-Rex1 and inhibition of chemotactic migration by PKA in endothelial cells responding to sphingosine 1-phosphate were linked to phosphorylation of P-Rex1 at Ser-436, we analyzed the activity of non-phosphorylatable P-Rex1-N-term-S436A mutant compared with wild-type P-Rex1-N-term and the effect of these constructs on endothelial chemotaxis elicited by sphingosine 1-phosphate either in the absence or presence of forskolin/IBMX. Consistent with a regulatory role of PKA-dependent phosphorylation of P-Rex1 at Ser-436, the non-phosphorylatable P-Rex1 DH-PDZ2-S436A mutant showed increased activity, detected by higher interaction with nucleotide-free Rac in the pulldown assay (Fig. 5C) and prevented the inhibitory effect of FSK/IBMX in chemotactic cells stimulated with 100 nM sphingosine 1-phosphate (Fig. 5D). Altogether, our results indicate that P-Rex1 phosphorylation at Ser-436 by PKA inhibited its GEF function.

P-Rex1 DH-PH Catalytic Module Is Inhibited by Intramolecular Interactions within the N-terminal Region Facilitated by Phosphorylation of DEP1 Domain by PKA—Our experiments showing that PKA inhibited P-Rex1 by phosphorylating it at Ser-436 within the DEP1 domain and the lack of detectable phosphorylation within the catalytic DH-PH module (regardless of the existence of a predicted phosphorylation site at Thr-305, which might not have been detectable by the PKAS antibodies due to the absence of an arginine in the -3 position) raised questions about the mechanism of inhibition. Also, considering that other potential phosphorylation sites might not have been recognized by the PKAS antibodies, we followed an alternative, direct approach, to test the effect of PKA on P-Rex1 catalytic activity. We hypothesized that PKA-dependent phosphorylation of P-Rex1 at Ser-436 might promote inhibitory intramolecular interactions between the DEP1 domain and the DH-PH catalytic module that might also affect P-Rex1 interactions with its upstream activators; this was consistent with previous reports which postulated that intramolecular interactions modulate P-Rex1 activity (29, 38, 42). To assess these possibilities, we used transiently transfected HEK293T cells to test the activity of different P-Rex1 constructs that included the DH-PH catalytic module followed by a C-terminal CAAX to anchor them to the plasma membrane (as depicted in Fig. 6A). Furthermore, we assessed the effect of PKA on the membrane-anchored P-Rex1-DH-PH catalytic module either acting directly on it or through the DEP1 or C-terminal domains (as indicated in Figs. 6, D and G, and 7A). Our rational was that these constructs would directly activate Rac and might serve as readouts of the effect of direct modulators of the P-Rex1-DH-PH catalytic module. Thus, we cloned the DH-PH region fused to EGFP at the N terminus and a C-terminal isoprenylation box. To test potential intramolecular interactions within the N-terminal region of P-Rex1 (including DH-PH-DEP1-DEP2-PDZ1-PDZ2) affecting the DH-PH catalytic module, we tested the activity of constructs containing only these two domains or extended up to the second DEP or the second PDZ, as depicted in Fig. 6A. With pulldown experiments, we compared their affinity for RacG15A (considered as readout of RacGEF activity) and their effect on Rac activation. As expected, P-Rex1-DH-PH-CAAX protein showed constitutive activity, whereas the constructs that included the DEP1-DEP2 or DEP1-PD22 cassettes had marginal activity (Fig. 6, B and C), indicating an inhibitory role for the DEP and PDZ domains on the P-Rex1 DH-PH catalytic cassette.

Although our phosphorylation mapping approach on P-Rex1 using PKAS antibodies did not reveal phosphorylation at the PH domain (regardless of the existence of a putative phosphorylation site for PKA at Ser-305), it was still possible that PKA might have a direct effect on the catalytic module that was not

**FIGURE 3.** SDF-1 promotes P-Rex1 activation, interaction with PKA, and mobilization of them to the plasma membrane. A, SDF-1 promotes P-Rex1 activation. Cos7 cells were transfected with FLAG-tagged P-Rex1; 2 days post-transfection, cells were starved and stimulated with SDF-1 (50 ng/ml) for the indicated times. The fraction of active P-Rex1 was isolated by pulldown (PD) with nucleotide-free Rac-G15A fused to GST. The expression of transfected P-Rex1 (FLAG) and endogenous PRKAR1a (R1α) in total cell lysates (TCL) is shown as the control. The graph represents the mean densitometric values of active P-Rex1 obtained in four independent experiments. *, p < 0.05. B, effect of SDF-1 on P-Rex1 interaction with endogenous R1α. Serum-starved Cos7 cells expressing GST-P-Rex1-DH-PDZ2 (comprising the indicated domains, or GST, as control) were stimulated with SDF-1 (50 ng/ml) for 1–60 min. Endogenous PRKAR1a (R1α) interacting with P-Rex1 was revealed by Western blot in GST pulldown assays, and its expression was confirmed in total cell lysates (TCL). Expression and pulldown of P-Rex1 constructs and control GST were revealed by Western blot using anti-GST antibodies. The graph represents the mean densitometric values of endogenous R1α bound to P-Rex1 obtained in three independent experiments. *, p < 0.05. C, cellular distribution of P-Rex1 and PRKAR1a in Cos7 and HUVEC cells stimulated, as indicated, with SDF-1. Cells were transfected with eGFP-C-P-Rex1 either in the absence or presence of pmCherry-PRKAR1a. Cos7 cells were starved for 16 h and HUVEC cells for 2 h with M199 medium (Gibco) plus 0.5% BSA. Then cells were stimulated with SDF-1 for 5 min, fixed, and analyzed with Nikon Eclipse Ti inverted fluorescence microscope. GFP-P-Rex1 in the absence (a and c) and in the presence (e and g) of pmCherry-PRKAR1a in control (basal, a and e conditions) and upon SDF-1 stimulation (c, g, and k panels). P-Rex1 was relocated to cell membrane upon SDF-1 in 15 of 46 cells (transfected only with EGFP-P-Rex1). b, pmCherry-PRKAR1a transfected alone (b and d) or with P-Rex1 (f, h, and l) was visualized under control (basal, b and f conditions and upon SDF-1 stimulation (d, h, and l). In cells transfected only with PRKAR1a, this type I PKA regulatory subunit remained cytosolic in SDF-1-stimulated cells, whereas in cells co-transfected with PRKAR1a and P-Rex1, both proteins colocalized at the cell membrane in SDF-1-stimulated cells (16 from 36 cells). Representative images are shown in panels g and h and panels k and l. Merged images (i and m) and magnified images (j and n) showing GFP-P-Rex1 and pmCherry-PRKAR1a colocalization. D, Z6 (corresponding to the P-Rex1-interacting region in R1α, comprising the CNB B domain) had a positive effect on S1P-dependent cell chemotaxis. PAE cells were transfected either with Z6 or EGFP-CAAX (control) and subjected to chemotaxis assays in Boyden chambers as described under “Experimental Procedures.” The **right panel** shows representative areas where the nuclei of cells that migrated for 6 h under basal or stimulated conditions were visualized. Objective. Cell migration was determined by counting the nuclei of migrating cells using ImageJ software. The graph at the left shows the average and S.E. values for the effect of increasing doses of S1P of three independent experiments normalized to the number of cells that migrated under non-stimulated conditions, considered as 100%. Statistical significance was analyzed by two way analysis of variance followed by Tukey’s test. *, p < 0.05; **, p < 0.01.
revealed with the antibodies. Thus, we tested this possibility using the constitutively active P-Rex1-DH-PH-CAX construct co-transfected with PRKACA, the catalytic subunit of PKA (Fig. 6D). We found that PKA did not have a significant effect on the activation of Rac by the constitutively active P-Rex1-DH-PH-CAX construct (Fig. 6, E and F). These results are consistent with the idea that the inhibitory effect of PKA on P-Rex1 does not involve a direct effect on the DH-PH catalytic module.

Because the P-Rex1-DH-PH-DEP1-DEP2-CAAX construct was less active than P-Rex1-DH-PH-CAAX and because P-Rex1 phosphorylation by PKA mapped at DEP1-Ser-436, resulting in inhibition of P-Rex1 activity, we postulated that the P-Rex1-DH-PH catalytic cassette might be inhibited by the phosphorylated DEP1 domain via direct interactions. We tested this possibility by assessing the effect of DEP1 on the activation of Rac by P-Rex1-DH-PH-CAAX in the absence or presence of the PKA catalytic subunit (Fig. 6G). We found that FLAG-DEP1

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**FIGURE 4.** PKA phosphorylates P-Rex1 at Ser-436 of DEP1 domain. A, motif scan site analysis showing putative PKA-dependent phosphorylation sites within P-Rex1 protein (under medium stringency). B, PKA phosphorylates P-Rex1 at the N-terminal region. HEK-293T cells were co-transfected with EGFP-PRKACa and HA-PRKAR1a and either the N-terminal (DH-PDZ2), C-terminal, DH-PH, or DEP1-PDZ2 regions of P-Rex1 fused to GST. GST pulldown (PD) assays were performed, and PKA-dependent phosphorylation was detected in pulldown assays (GST-PD) using PKAS antibody. P-Rex1 was phosphorylated by PKA at the N-terminal but not at C-terminal region (left panel). Phosphorylation was found within the DEP1 to PDZ2 region of P-Rex1, excluding DH-PH domains (right panel). TCL, total cell lysates. TCLC, PKA phosphorolys P-Rex1 at DEP1 domain. Cells were co-transfected with EGFP-PRKACa and HA-PRKAR1a and single domains of P-Rex1 as GST fusion proteins (DEP1, DEP2, PDZ1, or PDZ2). GST was used as the negative control. PKA-dependent phosphorylation at the DEP1 domain of P-Rex1 was detected using PKAS antibody. D, PKA phosphorylates P-Rex1 at DEP1 domain. Phosphorylation of P-Rex1-DEP1-Ser-436 was confirmed by using P-Rex1-DEP1-S436A mutant. Pulldown assays were performed with cells lysates from HEK-293T cells co-transfected with EGFP-PRKACa and HA-PRKAR1a and either wild type P-Rex1-DEP1 or P-Rex1-DEP1-S436A mutant. PKA-dependent phosphorylation at the DEP1 domain of P-Rex1 was detected using PKAS antibody. Expression of transfected proteins is shown in total cell lysates, and isolated GST fusion proteins were detected with GST antibodies in the pulldown assays as indicated. Results shown are representative of at least three independent experiments.
inhibited the activation of Rac induced by P-Rex1-DH-PH-CAAX only when it was co-expressed with PRKACA (Fig. 6, H and I). Therefore, we investigated whether P-Rex1 DEP1 interacted with the DH-PH module in a manner affected by its phosphorylation at Ser-436. We assessed this hypothesis by a GST pulldown assay measuring the binding of P-Rex1-DH-PH (fused to FLAG) with P-Rex1-DEP1, either WT or S436A (fused to GST), in the absence or presence of PKA catalytic subunit (Catal). We found that phosphorylation of DEP1 at Ser-436 (demonstrated by Western blot with PKAS antibodies; Fig. 6 J) promoted its interaction with the DH-PH construct even in presence of PKA (Fig. 6 J). As controls, the expression of PPKA, P-Rex1-DH-PH, and the effectiveness of the GST
pulldown was demonstrated by Western blot using anti-GFP, FLAG, and GST antibodies, respectively. These results are consistent with the hypothesis that PKA inhibits P-Rex1 by promoting interactions between the phosphorylated DEP1 domain and the DH-PH catalytic module, potentially interfering with the access of Rac1 to the nucleotide exchanger groove.

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P-Rex1 DH-PH Catalytic Module Is Also Inhibited by the C-terminal Region via Interactions Facilitated by a PKA-dependent Mechanism without the Participation of the DEP1-PDZ2 Region—P-Rex1 maintains intramolecular interactions involving its N- and C-terminal regions (42). Interestingly, phosphorylation of Ser-1169 is required for P-Rex1 activation in response to neuregulin and insulin-like growth factor (40, 48), whereas dephosphorylation of its neighbor Ser-1165, both located in the P-Rex1 C-terminal region, is required to activate P-Rex1 with the involvement of protein phosphatase 1α (38). However, none of these sites is within the consensus sequence recognized by PKA; thus it remains to be defined whether PKA exerts a regulatory role on the P-Rex1 C-terminal region. As shown in Fig. 4A, Ser-1001 and Ser-1272 within the P-Rex1 C-terminal region correspond to PKA-phosphorylation consensus sites; however, the PKAS antibodies did not detect their phosphorylation in conditions in which phosphorylation of Ser-436 at P-Rex1 DEP1 was revealed (Fig. 4B). However, it was still possible that PKA-exerted a regulatory role on this region by the phosphorylation of residues that were not detected by the PKAS antibodies, possibly through a PKA-dependent indirect mechanism. Accordingly, a change in the electrophoretic mobility referred to as an electrophoretic delay and revealed as a doublet, occurred in a P-Rex1 C-terminal region construct co-transfected with PKA (Fig. 4B, left panel, C-ter, TCI/GST blot). This was consistent with the initial report of P-Rex1 regulation by PKA by Mayeenuddin et al. (41) who observed that this kinase caused an electrophoretic delay on P-Rex1. Thus, to assess a potential direct role of P-Rex1 C-terminal region on the activity of the DH-PH catalytic module, we assayed by pulldown the activity of the DH-PH-CAAX construct and assessed its effect on Rac activation in the presence of P-Rex1 C-terminal region and the PKA catalytic subunit, as represented in Fig. 7A. Consistent with a direct regulatory role of P-Rex1 C-terminal region on the DH-PH catalytic module, the single co-expression of the P-Rex1 C-terminal region was enough to attenuate the activity of the DH-PH construct (Fig. 7B) and the consequent activation of Rac (Fig. 7C). This inhibitory effect was strongly reinforced by simultaneous expression of the catalytic subunit of PKA (Fig. 7, B and C), which also promoted the interaction between P-Rex1 N- and C-terminal regions, in particular when Ser-436 was substituted by Ala. These findings further support the idea that phosphorylation at Ser-436 regulates interdomain interactions within the N-terminal region and between this and the C-terminal region (Fig. 7E). To explore if this effect was related to intramolecular interactions that might affect the association between P-Rex1 and its activators (Gβγ and mTOR) (Fig. 7D), we assessed the association between the P-Rex1 N-terminal region (DH-PDZ2) either WT or S436A and the P-Rex1 C-terminal region either in the presence or absence of PRKACA. We found, consistent with previous reports, that the P-Rex1 N-terminal and C-terminal regions did interact (42). Interestingly, this interaction was increased by the presence of PKA and was more effective in cells in which Ser-436 was substituted by Ala. Intriguingly, PKA-dependent changes on the interaction between P-Rex1 amino and C-terminal regions did not affect the interaction between the P-Rex1 amino and Gβγ heterodimer (Fig. 7E). Because phosphorylated DEP1 or the C-terminal region of P-Rex1 was directly inhibitory of the catalytic DH-PH region, the fact that P-Rex1 and Gβγ interaction was not decreased by PKA was consistent with the recently proposed model in which Gβγ recognizes a region of the P-Rex1-DH-PH module located at the opposite side of the catalytic interface (49). Previously, we demonstrated that P-Rex1 DEP-DEP domains interact with the mTOR C-terminal region, resulting in activation of P-Rex1 (20); here, we tested whether this interaction was affected by modifications at Ser-436, the site at P-Rex1 DEP1 that is phosphorylated by PKA. We used constructs corresponding to the P-Rex1 N-terminal region (DH-PDZ2) in which Ser-436 was substituted by Ala or Asp (S436A or S436D). They were transfected into HEK293T cells to test their ability to interact with mTOR C-terminal region (mTOR prey). We found that both P-Rex1 constructs interacted similarly with mTOR prey (Fig. 7F), suggesting that phosphorylation of P-Rex1 at Ser-436 does not affect the interaction between P-Rex1 and mTOR.

FIGURE 6. P-Rex1 DH-PH catalytic module is inhibited by intramolecular interactions within the N-terminal region facilitated by phosphorylation of DEP1 domain by PKA. A, the left drawing shows the hypothetical constitutive activity of a P-Rex1 construct consisting of the DH-PH catalytic module anchored to the plasma membrane by isoprenylation and tagged with EGFP at the N-terminus. Drawings at the center and right depict potential inhibitory intramolecular interactions within P-Rex1 N-terminal region involving the DEP and PDZ domains. Functional assays comparing the activity of these constructs are shown in B and C. B, RacGEF activity of isoprenylated P-Rex1 constructs containing the indicated domains. HEK293T cells transfected with EGFP-CAAX empty vector or including P-Rex1-DH-PH, P-Rex1-DH-DEP2, or P-Rex1-DH-PDZ2 were starved of serum for 16 h and used for pulldown (PD) assays with GST-RacG15A beads. The amount of each RacGEF active construct was revealed by immunoblot against the EGFP tag. The graph represents the mean densitometry values of active EGFP-P-Rex1-CAAX constructs obtained in three independent experiments. Statistical analysis was done by t test, obtaining the value of p shown at the graph. A representative result is shown below the graph. TCI, total cell lysates. C, effect of isoprenylated P-Rex1 constructs as activators of Rac in serum-starved cells. Rac activation was assessed by GST-PAK-N pulldown assays done in parallel with GST-RacG15A using the same constructs and lysates described in B. The graph represents the mean and S.E. of densitometry values of active Rac obtained in three independent experiments. Statistical significance was analyzed by t test; the value of p is shown at the graph. A representative blot showing the fraction of active Rac and that in total cell lysates is shown below the graph. D, hypothetical model showing the potential effecting of PKA on the activity of the catalytic DH-PH module of P-Rex1, which was tested in E and F. E, PKA does not have a direct inhibitory effect on the activity of the catalytic DH-PH module of P-Rex1. A representative blot shows the activation of Rac in serum-starved HEK293T cells transfected with EGFP-CAAX or EGFP-DH-PH-CAAX together or not with EGFP-PRKACA. Rac activity was determined by GST-PAK pulldown and revealed by immunoblot against Rac as well as its presence in total cell lysates. F, the graph represents the results of experiments done to test the effect of PKA on the activation of Rac by isoprenylated P-Rex1-DH-PH construct. Bars represent the average and S.E. of three independent tests as the one shown in E. Statistical analysis was done by t test; ns, stands for nonsignificant difference. G, hypothetical model showing the potential effecting of PKA on the activity of the catalytic DH-PH module of P-Rex1, which was tested in H and I. H, phospho-DEP1 inhibits the DH-PH module of P-Rex1 as revealed by a lower level of active Rac detected in serum-starved HEK293T cells cotransfected with EGFP-DH-PH-CAAX and FLAG-DEP1 together or not with EGFP-PRKACA. I, the graph represents the mean densitometry values and S.E. of active Rac as detected in three independent experiments as the one shown in H. Statistical analysis was done by t test; the value of p is indicated. J, phospho-DEP1 interacts with the catalytic DH-PH module. Pulldown assays done with lysates from serum-starved HEK293T cells cotransfected with GST-P-Rex1-DEP1-WT or S436A and FLAG-DH-PH together or not with EGFP-PRKACA were analyzed by Western blot using antibodies against FLAG, PKAS, and GST as indicated. The expression of transfected constructs was confirmed in total cell lysates. The results are representative of three independent experiments.
**Discussion**

P-Rex1 is a multidomain GEF that couples G-protein-coupled receptor signaling to Rac activation through its binding to Gβγ and phosphatidylinositol-3,4,5-trisphosphate (28, 50). In the current study we demonstrate that the type I PKA regulatory subunit α (PRKAR1a) interacts with P-Rex1 and translocates with it to the cell membrane upon SDF-1 stimulation. Moreover, we demonstrate that PKA phosphorylates P-Rex1 at Ser-436 promoting intramolecular inhibitory interactions. Thus we reveal that PKA and P-Rex1 are reciprocally regulated. We previously showed that interaction of the oligomeric kinase mTORC2 with P-Rex1 DEP domains leads to Rac activation and cell migration (20). Indeed, mTOR was the first kinase identified as a direct interactor of P-Rex1 (20). Subsequent studies demonstrate that AKT1 interacts with P-Rex1 and phosphorylation of this kinase by mTORC2 is facilitated by its interaction with P-Rex1 (34). Thus, in addition to the role attributed to P-Rex1 as an integrator of G-protein-coupled receptor and growth factor receptor signaling leading to Rac activation and cell migration, we show here that this RacGEF is mechanistically regulated by PKA via a multistep mechanism involving direct interactions and phosphorylation-dependent intramolecular inhibitory interactions that interfere with the catalytic DH-PH cassette of P-Rex1, whereas activation of this RacGEF brings PKA to the...
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plasma membrane, revealing a novel scenario of reciprocal regulation between these important signaling proteins.

We also show here that PKAR1a is recruited to P-Rex1 by a non-canonical mechanism that involves interaction with the P-Rex1 PDZ domains with the CNB-B domain of PKAR1a. Although the C terminus of PRKAR1A contains a class II PDZ motif that we initially considered as the canonical motif for interaction with P-Rex1 PDZ domains, arrays using various peptides representing different regions of R1α revealed that CNB B, the second cAMP binding domain of R1α, preferentially interacts with P-Rex1-PDZ1. This interaction was effective even in the absence of the last three amino acids of R1α.

There is a clear contrast between this novel interaction described for P-Rex1 and PKA and the classical mechanism of interaction of PKA with AKAPs, which regularly occurs through the N terminus of types I and II regulatory subunits (51). Detailed biochemical, cellular, and structural studies have revealed a plethora of AKAPs that interact with the N terminus of PKA regulatory subunits and provide a mechanistic basis by which type II holoenzymes, in particular, are localized to different subcellular compartments (52–55). Less information is available for type I PKA, although some AKAPs are dual specific and bind to both RI and RII subunits, whereas a few such as sphingosine kinase-interacting protein (SKIP) and the recently discovered small myristoylated and palmitoylated AKAP are RI-specific (56–58). Sphingosine kinase interacting protein (SKIP) was also reported to be an R1-specific AKAP (56, 57).

Another small myristoylated and palmitoylated AKAP was also shown to be RI-specific and is recruited to the plasma membrane (58). R1α was also shown to be important for cell migration in response to α4 integrin, and recruitment of PKAR1a also did not involve a canonical AKAP mechanism (59–61). The non-canonical interaction that type I PKA establishes with P-Rex1 is the first in which CNB B, the second cAMP binding domain of this regulatory subunit, is recognized as the interacting interface with a protein other than the catalytic subunit of the kinase. Whether this represents the initial example of a common mechanism by which PDZ-containing proteins might control type I PKA subcellular dynamics and localization, equivalent to the role played by AKAPs for type II PKA, or might lead to the identification of additional AKAP-dependent R1α-regulated targets emerge as interesting possibilities that will be the focus of future studies. Interestingly, cAMP-bound R1I binds to Gαi and sensitizes δ-opioid Gβγ-coupled receptors signaling to ERK activation (62).

Understanding the particular intricacies by which PKA regulates Rho GTPases and cytoskeleton dynamics depends on the identification of specific targets and interacting partners that provide specificity for the effects of this serine/threonine kinase. These elements would help to explain the apparently conflicting results showing that PKA either promotes (11–14) or interferes (15–19, 28) with cell migration. Clearly such regulation is likely to be cell-specific. Although the PKA consensus phosphorylation sequence is present in thousands of potential protein substrates, its exquisite specificity is in part achieved through tight control of its subcellular distribution, and this is mediated in large part by its regulatory subunits that interact with a variety of targeting proteins. Here we identify a new class of PKA targeting protein that functions not only to target but also to bring a set of signaling domains to the membrane in response to G-protein-coupled receptor activation. Furthermore, we found that interaction of type I PKA with P-Rex1 PDZ-domains facilitates the phosphorylation of this GEF and its desensitization. Whether this interaction also facilitates the ability of type I PKA to recognize other substrates emerges as an interesting open question. Considering the mobilization of type I PKA to the cell membrane together with P-Rex1, upon stimulation of cells with SDF-1, it will be interesting to explore whether this interaction facilitates the potential regulation by PKA of plasma membrane proteins involved in chemotactic responses. Thus, it is likely that RI subunits play a far more important role at the plasma membrane than was previously appreciated.

Bachmann et al. (63) recently described the formation of a transient complex between Rac1 and PKA through the PKA RIIB regulatory subunit, and this interaction depends on the AKAP-like behavior of Rac1. Such an interaction would provide reciprocal regulation for signaling cascades of both components; particularly, Rac1-GTP stabilizes type II PKA holoenzyme (63). Here, we describe a new function for P-Rex1 interacting with PKA holoenzyme through the regulatory subunit Iα. This represents an alternative way of assembling a PKA signaling complex that is different from the traditional AKAP-mediated mechanism.

**FIGURE 7.** P-Rex1 DH-PH catalytic module is also inhibited by the C-terminal region via interactions facilitated by a PKA-dependent mechanism without the participation of the DEP1-PDZ2 region. A, hypothetical model showing the possible inhibitory effect of PKA contributing to the potential inhibitory action of P-Rex1 C-terminal region over the catalytic DH-PH module. Experiments assessing this possibility are shown in B and C. B, PKA enhances the inhibitory potential of P-Rex1 C-terminal region over the DH-PH module. Serum-starved HEK239T cells transfected with EGFP-DH-PH-CAAX and co-transfected together or not with EGFP-P-Rex1-Cter and EGFP-PRKACA were used for GST-RacG15A pulldown (PD) assays to isolate the active fraction of the DH-PH-CAAX construct revealed by immunoblot against the EGFP tag. The graph represents the average fraction of active P-Rex1-DH-PH-CAAX, confirms the expression of transfected constructs in total cell lysates (TCL) and shows the recombinant GST-RacG15A used in the pulldown assays. C, PKA enhances the inhibitory effect of P-Rex1 C-terminal region over the DH-PH module as revealed by the fraction of active Rac detected in serum-starved HEK239T cells cotransfected with EGFP-DH-PH-CAAX and EGFP-P-Rex1-Cter together or not with EGFP-PRKACA. Rac activation was assessed by GST-PAX-N pulldown assays done in parallel with GST-RacG15A using the same constructs and lysates described in B. The graph represents the mean values of active Rac obtained in three independent experiments. Statistical significance was analyzed by t test. *, p < 0.05. A representative blot below the graph shows the fraction of active Rac and that in total cell lysates is shown below the graph. D, hypothetical model showing the potential effect of P-Rex1 phosphorylation at Ser-436 by PKA on the interactions between the N- and C-terminal regions of P-Rex1 and with its upstream activators Gβγ and mTOR. Experiments addressing this model are shown in E and F. E, pulldown experiments assessing the effect of PKA on the interaction between P-Rex1 N- and C-terminal regions and with Gβγ, comparing the effect of wild type P-Rex1-DH-PDZ2 versus S436A non-phosphorylatable mutant. The presence of interacting proteins in the pulldown assays and their expression in total cell lysates of HEK293T cells transfected with the indicated constructs was detected by Western blot using anti-GFP, -GST, and -FLAG antibodies. F, pulldown experiments comparing the interaction between non-phosphorylatable and phosphomimetic P-Rex1-DH-PDZ2, mutated at Ser-436, with mTOR-prey expressed as a 3XFLAG-tagged protein (corresponds to the P-Rex1-interacting region within the C-terminal region of mTOR originally identified as a P-Rex1 interactor (20)). Results shown in E and F are representative of three independent experiments.
Our results confirm the previously reported ability of PKA to inhibit P-Rex1 signaling (41, 42). Furthermore, we extend these findings revealing a specific interaction of type I PKA CNB B preferentially with P-Rex1 PDZ1, the precise identification of the phosphorylation site located at Ser-436 at the first DEP domain of this GEF, and the mechanism of inhibition regulated by intramolecular interactions promoted by phosphorylation. Based on these findings and the influence of P-Rex1 on the localization of PKA, we propose a working model of P-Rex1 regulation by PKA (Fig. 8). Accordingly, P-Rex1 carries type I PKA as its own regulator. Thus, during a chemotactic response, the activity of P-Rex1 is fine-tuned by PKA, which accompanies the GEF when it goes to the membrane. Mechanistically, PKA attenuates P-Rex1 activity via two alternative inhibitory intramolecular interactions acting directly on the DH-PH module; one involving the phosphorylation of Ser-436 at DEP1 domain and the second the C-terminal region in which the action of PKA is likely indirect. According to our model, PKA switches the interactions between P-Rex1 N- and C-terminal regions, needed to keep the GEF sensitive to its activators (38, 42) toward inhibitory interactions within the N-terminal domains that depend on the phosphorylation of Ser-436. In summary, evidence shown here contributes to understanding how temporal regulation of P-Rex1 by PKA occurs. The fact that P-Rex1 is physically associated with PKA could partly explain how this GEF is tightly controlled during a chemotactic event, contributing to define a mechanism by which spatial organization of chemotactic signaling is achieved.

Author Contributions—L. C.-V. performed the two-hybrid screening and designed, performed, and analyzed most of the experiments in Figs. 1, 3C, 6G, and 7E. S. R. A.-G. designed, performed, and analyzed the experiments shown in Figs. 1, F and I, 3B (some repetitions), 4, 5C, and most of the experiments in Fig. 6 and Fig. 7, B, C, and F. R. D. C.-V. designed, performed, and analyzed the experiments shown in Figs. 1J, 2A, 3A, 3B (some repetitions), and 5A. S. C.-K. designed, performed, and analyzed the experiments shown in Figs. 3D, 5B, and 5D. J. G. H. B. designed, performed, and analyzed the experiments shown in Fig. 2B. S. F. and N. M. provided technical assistance and contributed with the overall design, execution, and analysis of the experiments shown in Fig. 3C and contributed to the analysis and interpretation of data. S. S. T. contributed with the overall design and analysis of the experiments shown in Fig. 2C and contributed to the analysis and interpretation of data and edited the final version of the manuscript. G. R.-C. provided technical assistance and contributed to design, analysis, and interpretation of data. J. V. P. conceived and coordinated the study and wrote the paper together with L. C.-V. and S. R. A.-G. All authors reviewed the results and approved the final version of the manuscript.

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