Membrane Translocation of P-Rex1 Is Mediated by G Protein $\beta\gamma$ Subunits and Phosphoinositide 3-Kinase[*][S]

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P-Rex1 is a guanine-nucleotide exchange factor (GEF) for the small GTPase Rac that is directly activated by the $\beta\gamma$ subunits of heterotrimeric G proteins and by the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$), which is generated by phosphoinositide 3-kinase (PI3K). G$\beta\gamma$ subunits and PIP$_3$ are membrane-bound, whereas the intracellular localization of P-Rex1 in basal cells is cytosolic. Activation of PI3K alone is not sufficient to promote significant membrane translocation of P-Rex1. Here we investigated the subcellular localization of P-Rex1 by fractionation of Sf9 cells co-expressing P-Rex1 with G$\beta\gamma$ and/or PI3K. In basal, serum-starved cells, P-Rex1 was mainly cytosolic, but 7% of the total was present in the $117,000 \times g$ membrane fraction. Co-expression of P-Rex1 with either G$\beta\gamma$ or PI3K caused only an insignificant increase in P-Rex1 membrane localization, whereas G$\beta\gamma$ and PI3K together synergistically caused a robust increase in membrane-localized P-Rex1 to 23% of the total. PI3K-driven P-Rex1 membrane recruitment was wortmannin-sensitive. The use of P-Rex1 mutants showed that the isolated Dbl homology/pleckstrin homology domain tandem of P-Rex1 is sufficient for synergistic G$\beta\gamma$- and PI3K-driven membrane localization; that the enzymatic GEF activity of P-Rex1 is not required for membrane translocation; and that the other domains of P-Rex1 (DEP, PDZ, and IP4P) contribute to keeping the enzyme localized in the cytosol of basal cells. *In vitro* Rac2-GEF activity assays showed that membrane-derived purified P-Rex1 has a higher basal activity than cytosol-derived P-Rex1, but both can be further activated by PIP$_3$ and G$\beta\gamma$ subunits.

The small GTPase Rac (isoforms Rac1, Rac2, and Rac3) is a member of the Rho family of GTPases that regulates a range of important cellular functions, including gene expression, cytoskeletal structure, and reactive oxygen species formation (1). Like all small GTPases, Rac is directly activated by guanine-

nucleotide exchange factors (GEFs)[3] (2). The P-Rex family of Rac-GEFs is composed of P-Rex1, P-Rex2, and P-Rex2b (3–5). P-Rex1 is expressed in white blood cells and brain (3), P-Rex2 expression is more widespread but low or absent in leukocytes (4), and P-Rex2b, a splice variant of P-Rex2 that lacks the C-terminal half, is expressed mainly in the heart (5). Studies in P-Rex1-deficient mice have shown that P-Rex1 is involved in the regulation of G protein-coupled receptor (GPCR)-dependent Rac2 activation in neutrophils, production of reactive oxygen species, chemotaxis, and neutrophil recruitment to inflammatory sites (6, 7). In the neuronal PC12 cell line, RNA interference-mediated down-regulation of P-Rex1 leads to impaired neurotrophin-stimulated cell migration (8). In the endothelial HUVEC cell line, suppression of P-Rex2b levels by RNA interference results in reduced Rac1 activation and cell migration in response to sphingosine 1-phosphate (9).

P-Rex family GEFs are directly and synergistically activated *in vitro* and *in vivo* by the $\beta\gamma$ subunits of heterotrimeric G proteins (G$\beta\gamma$ subunits) and by the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$), which is generated by phosphoinositide 3-kinase (PI3K) (3). A range of different Gβ and Gγ subunit combinations, with the exception of G$\beta_5\gamma_2$, are capable of activating P-Rex1 (10). Phosphorylation by cyclic AMP-dependent kinase (PKA) inhibits the stimulation of P-Rex1 activity by PIP$_3$ and G$\beta\gamma$ subunits (11). Mutagenesis studies have shown that P-Rex1 is activated via its PH domain by PIP$_3$ and via its catalytic DH domain by G$\beta\gamma$ subunits and that the presence of its other domains (two DEP and two PDZ protein interaction domains and a region of homology over its C-terminal half to inositol polyphosphate 4-phosphatase) helps to keep the catalytic activity of the full-length enzyme low in the resting state (12).

P-Rex1 was initially identified on the basis of its enzymatic activity from the cytosolic fraction of pig neutrophils (3). P-Rex1 was highly abundant in the neutrophil cytosol, representing around 0.1% of total protein (3). When overexpressed in the pig aortic endothelial (PAE) cell line, P-Rex1 was also localized in the cytosol (3). Stimulation of P-Rex1-transfected PAE cells with PDGF resulted in PI3K-dependent (wortmannin-sensitive) and P-Rex1-dependent activation of Rac, as was clear

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[3] The abbreviations used are: GEF, guanine-nucleotide exchange factor; GPCR, G protein-coupled receptor; G$\beta\gamma$, $\beta\gamma$ subunits of heterotrimeric G proteins; PIP$_3$, phosphatidylinositol (3,4,5)-trisphosphate; PI3K, phosphoinositide 3-kinase; PKA, cyclic AMP-dependent kinase; PDGF, platelet-derived growth factor; GTPγS, guanosine 5′-3-O-(thio)triphosphate; PAE, pig aortic endothelial; GFP, green fluorescent protein; EGFP, enhanced GFP; DH, Dbl homology; PH, pleckstrin homology; iDH, isolated DH.

from the resulting cytoskeletal changes of lamellipodia formation and membrane ruffling. However, activation of PI3K did not result in any significant membrane translocation of P-Rex1 (3). Hence, although the PI3K-dependent activator of P-Rex1, PIP_3, is membrane-bound, its formation alone is not sufficient to induce significant P-Rex1 membrane localization.

The Bokoch laboratory has recently studied the membrane translocation of endogenous P-Rex1 in neutrophils (13). Using a mainly microscopy-based approach, they showed that endogenous neutrophil P-Rex1 translocates from the cytosol to the plasma membrane in response to stimulation of GPCRs and that translocation can be inhibited by several pharmacological inhibitors, including wortmannin, which inhibits PI3K, M119, which inhibits $G\beta$-subunit binding to effectors, and several protein-tyrosine kinase inhibitors as well as stimulators of PKA (13).

Here we investigated whether $G\beta\gamma$ subunits or the concomitant presence of both $G\beta\gamma$ and PIP_3 are sufficient signal to mediate P-Rex1 membrane translocation, using fractionation of Sf9 cells expressing P-Rex1 either alone or with $G\beta\gamma$ and/or PI3K. We provide information on the relative contributions of $G\beta\gamma$ subunits and PI3K to P-Rex1 membrane translocation, the roles of the various domains of P-Rex1 in membrane translocation, and the effects of the subcellular localization on the catalytic activity of P-Rex1. Our results reinforce the notion that P-Rex1 acts as a coincidence detector for the concomitant activation of PI3Ks and GPCRs.

EXPERIMENTAL PROCEDURES

P-Rex1, $G\beta\gamma$ Subunit, and PI3K Constructs—The construction of most of the panel of cDNAs, encoding human full-length and mutant N-terminally EE-tagged P-Rex1, in the baculovirus transfer vector pAcoG1 used here was described previously (12, 14). The isolated DH (iDH) domain construct, a C-terminal truncation consisting of P-Rex1 residues Met^1–Glu^{245}, was generated by standard PCR cloning and subcloning into pAcoG1, which gave it an N-terminal EE epitope tag for use in Sf9 cells. For microscopy experiments, full-length P-Rex1 was constructed with NH_2-terminal EGFP and GFP-myc tags. The heterotrimeric G protein subunits EE-$G\beta_1\gamma_2$ and the PI3K catalytic His_6-$p110\gamma$ and regulatory EE-p101 subunits were expressed as described (3, 15).

Sf9 Cell Infection with High Titer Baculovirus—Aliquots of 6×10^6 Sf9 cells in triplicate 6-cm tissue culture dishes were infected with combinations of baculovirus encoding full-length or mutant EE-P-Rex1, the heterotrimeric G protein subunits $G\beta_1\gamma_2$, and/or the PI3K catalytic and regulatory subunits His_6-$p110\gamma$ and EE-p101 and cultured for 42 h in growth medium at 27 °C. Baculovirus titers and expression times were optimized for concomitant expression of P-Rex1 with $G\beta\gamma$ subunits and PI3K, as assayed by immunoprecipitation with EE tag antibody and Western blotting. Infected cells were serum-starved in Grace's insect medium (11605-045; Invitrogen) for 6 h at 27 °C prior to cell fractionation. In some experiments, Sf9 cell culture dishes were pretreated with wortmannin at a final concentra-

tion of 100 nM for 20 min at room temperature before cell fractionation.

Membrane Translocation Assay—Infected serum-starved Sf9 cells were washed in ice-cold lysis buffer (160 mM NaCl, 38 mM HEPES, pH 7.4, at 4 °C, 1 mM $MgCl_2$, 1 mM EGTA) before being resuspended in lysis buffer containing anti-proteases (10 μg/ml each of antipain, aprotinin, leupeptin, pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride) and 1 mM dithiothreitol. Wortmannin-pretreated cells were washed and collected into buffer containing 100 nM wortmannin. Cells were lysed by probe sonication using the microprobe of a Misonix sonicator on setting 5 (20 watts) by three pulses of 15 s with 15-s pauses on ice. A 10% aliquot of the total lysate was removed for Western blotting control; the rest was submitted to low speed centrifugation at $400 \times g$, 10 min at 4 °C, to remove unbroken cells, debris, and nuclei. A 10% aliquot of the postnuclear supernatant was removed for Western blotting control, and the remainder was separated by ultracentrifugation at $117,000 \times g$ for 30 min at 4 °C into cytosol and membrane fractions. Membrane pellets were resuspended in boiling $1.2\times$ SDS-PAGE sample buffer and boiled for 5 min. Boiling $4\times$ SDS-PAGE sample buffer was added to the total lysate, postnuclear supernatant, and cytosol fractions to a final concentration of $1\times$, and samples were boiled for 5 min as soon as each fraction became available during the course of fractionation. All fractions were snap-frozen and stored at -80 °C. Samples from all stages of cell fractionation were subjected to SDS-PAGE and Western blotting with EE tag antibody, followed by densitometric analysis. We chose the Sf9 cell system specifically because it supports concomitant expression of several exogenous proteins. However, expression levels of P-Rex1 did vary considerably between Sf9 cells that expressed P-Rex1 alone, P-Rex1 plus $G\beta\gamma$ or PI3K, or P-Rex1 plus $G\beta\gamma$ and PI3K. To control for variations in expression level, the amount of P-Rex1 in all subcellular fractions was always normalized to the average total lysate control for each fraction.

Isolation of Membrane-bound and Cytosolic P-Rex1 for in Vitro GEF Assays—Sf9 cells were infected to express full-length EE-P-Rex1, serum-starved, lysed, and fractionated as described for the membrane translocation assay except that production was scaled up from 6 cm to eight 175-cm^2 flasks per condition. Uninfected control cells were mock-treated through all stages. EE-P-Rex1 was extracted from the $150,000 \times g$ membrane fraction by resuspending the membrane pellet in the same lysis buffer used during sonication of the cells except that it additionally contained 1% Triton X-100 and phosphatase inhibitors 20 mM NaF and 17 mM β-glycerophosphate. Concentrated Triton X-100, NaF, and β-glycerophosphate stocks were added to the cytosol fraction ($150,000 \times g$ supernatant) to give the same final concentrations. Membrane and cytosol fractions were incubated on ice with periodic mixing and vortexing for 20 min. Insoluble material was removed by centrifugation at $117,000 \times g$ for 30 min at 4 °C. EE-P-Rex1 was immunoprecipitated from the lysates using EE antibody covalently coupled to Sepharose beads. Samples were washed extensively, and purified membrane-derived and cytosol-derived P-Rex1 were eluted from the beads by the addition of excess free antigenic EE peptide. Fatty acid-free bovine serum albumin (A6003; Sigma) was

added to a final concentration of 2 mg/ml, and ice-cold glycerol was added to 50%, immediately before samples were snap-frozen and stored at −80 °C. Aliquots of the purified membrane- and cytosol-derived P-Rex1 were subjected to anti-EE Western blot analysis, densitometric scanning, and ImageJ analysis for quantification, using recombinant EE-P-Rex1 as a standard.

Production of Recombinant Rac2 for in Vitro GEF Assays—Recombinant GDP-loaded post-translationally modified C-terminally EE epitope-tagged Rac2 for use in GEF activity assays *in vitro* was produced in Sf9 cells, purified by Triton X-114 phase separation and immunoprecipitation, and stored at −80 °C in 40 mM Hepes/NaOH, pH 7.4 (4 °C), 0.15 M NaCl, 1% (w/v) cholate, 5 μM GDP, 5 mM MgCl$_2$, 1 mM EGTA, 1 mM dithiothreitol, essentially as previously described (14).

Rac-GEF Activity Assay with Membrane- Versus Cytosol-derived P-Rex1—To assay the GEF activity of membrane-derived and cytosol-derived purified EE-P-Rex1, we adapted the *in vitro* Rac-GEF activity assay we routinely use with recombinant P-Rex1 (3). EE-P-Rex1 purified from membrane and cytosol fractions of Sf9 cells as described above was diluted in 20 mM HEPES, pH 7.0 (4 °C), 10% ethylene glycol, 1% betaine, 0.01% sodium azide, 0.5 mM EGTA, 0.2 M KCl, prior to the addition to the assay. GDP-loaded purified post-translationally modified recombinant Sf9 cell-derived EE-tagged Rac2 was incubated for 10 min on ice with liposomes made of phosphatidylcholine, phosphatidylserine, and phosphatidylinositol. Membrane-derived P-Rex1, cytosol-derived P-Rex1, or negative controls prepared in parallel from uninfected mock-treated Sf9 cells were added, together with GTPγS (including [^{35}S]GTPγS; NEG 030H; PerkinElmer Life Sciences), and samples were incubated at 30 °C for 10 min. Final concentrations were 30 nM Rac2, 10 nM membrane- or cytosol-derived P-Rex1, 5 μM GTPγS, and a 200 mM concentration of each lipid. Rac2 was isolated using its EE tag, and its [^{35}S]GTPγS loading was measured by β-counting. EDTA (2 mM) was used to artificially GTPγS-load Rac2 as a positive control. In some assays, synthetic D-stearoyl/arachidonoyl-PIP$_3$ (16) and purified post-translationally modified Sf9 cell-derived G$\beta_1\gamma_2$ subunits (3, 14) were added for stimulation of P-Rex1 GEF activity. In these assays, PIP$_3$ was incorporated into the liposomes to a final concentration of 0.2 μM, and G$\beta\gamma$ was added before the 10-min incubation on ice to a final concentration of 0.3 μM. To control for the presence of cholate in the G$\beta\gamma$ stock, cholate was added to controls to give the same concentration (0.005% final) in all samples.

Live Video Microscopy of P-Rex1 in PAE Cells—PAE cells were cultured and transiently transfected with EGFP-DAPP1 or EGFP-P-Rex1 as described (17). Transfected cells were grown on glass coverslips for 14 h and then serum-starved for 11 h before being mounted in the 37 °C chamber of an Ultra-View confocal microscope. After 1–2 min, cells were stimulated with 10 ng/ml PDGF for at least 10 min. Images were taken every 2–3 s throughout.

Immunofluorescence Microscopy of P-Rex1 in THP-1 Cells—Monocytic THP-1 cells were grown in RPMI 1640 with 10% heat-inactivated fetal bovine serum (HighClone), Glutamax and penicillin/streptomycin (Invitrogen). Cells were transfected with GFP-myc-P-Rex1 using the Amaxa Nucleofection kit, and positive clones were selected with 0.7 mg/ml G418

(Invitrogen). Stable clones were maintained in the absence of G418. Before stimulation, cells were starved for 4 h in fetal bovine serum-free medium with 25 mM HEPES (pH 7.3), washed in phosphate-buffered saline, resuspended at 2×10^6 cells/ml in starvation medium, and then stimulated with or without 100 nM MCP-1 for 90 s. Cells were fixed in an equal volume of 8% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature, sedimented, washed three times in phosphate-buffered saline, transferred into 96-well plates, and permeabilized with 0.1% saponin in phosphate-buffered saline for 10 min at room temperature. Cells were stained with Alexa-488 rabbit anti-GFP (Molecular Probes) to enhance the GFP signal, with Alexa-594-phalloidin (Molecular Probes) and 0.1 μg/ml 4′,6-diamidino-2-phenylindole (Sigma), washed, and transferred onto glass slides in ProLong Antifade embedding medium (Molecular Probes) under coverslips. Wide field microscopy was performed with a Nikon T-800, and images were deconvoluted with Openlab software (Improvision).

Calculations and Statistics—To quantify P-Rex1 membrane translocation, densitometric scanning and ImageJ analysis were performed on triplicate Western blot bands of EE-P-Rex1 from total lysate, postnuclear supernatant, cytosol, and membrane fractions. The different fractions were compared on the same autoradiography film, using unsaturated exposures, and normalized for cell equivalents loaded. Levels of EE-P-Rex1 in the membrane fraction were expressed as a percentage of EE-P-Rex1 in the total lysate. Unless otherwise indicated, data are means ± S.E. of all experiments. Statistics were calculated using Student's *t* test.

RESULTS

Activation of PI3K Is Not Sufficient to Induce P-Rex1 Membrane Recruitment—In our earlier work, we found that intracellular localization of P-Rex1 in unstimulated cells was cytosolic, both for endogenous protein from pig neutrophils and for overexpressed P-Rex1 in the endothelial PAE cell line (3). Activation of PI3K through PDGF stimulation of PAE cells was not sufficient to promote a convincing membrane translocation of P-Rex1 (3). In order to investigate whether we might have missed a minor or transient translocation of P-Rex1 in PDGF-stimulated PAE cells, we performed here similar experiments using live video microscopy with NH$_2$-terminally EGFP-tagged P-Rex1. We compared the localization of P-Rex1 during PDGF stimulation of PAE cells with that of DAPP1, which is well known to translocate to the plasma membrane (17) (Fig. 1*A* and supplemental Videos 1–3). Under conditions where PDGF stimulation led to activation of Rac, as was obvious from cell spreading, lamellipodia formation, and membrane ruffling, and where DAPP1 translocation was very clear, we did not find membrane translocation of EGFP-P-Rex1. Hence, the live video microscopy experiments confirmed our earlier findings in fixed cells that PI3K activation alone is not sufficient to stimulate membrane translocation of P-Rex1.
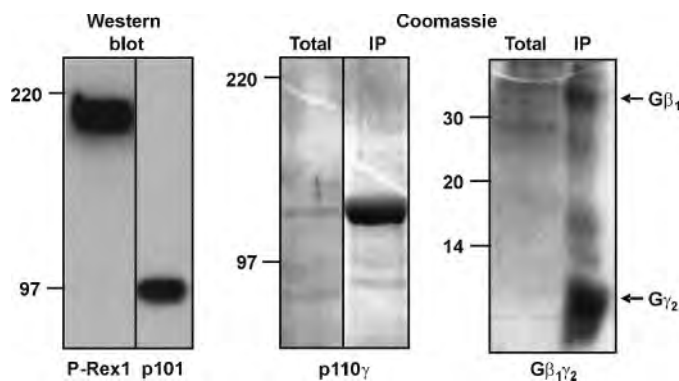
Stimulation of Myeloid Cells through GPCR Induces P-Rex1 Membrane Translocation—Stimulation of myeloid cells through GPCRs leads to concomitant production of G$\beta\gamma$ subunits and PIP$_3$ due to the presence of G$\beta\gamma$-dependent PI3K in these cells. To determine whether we could observe P-Rex1
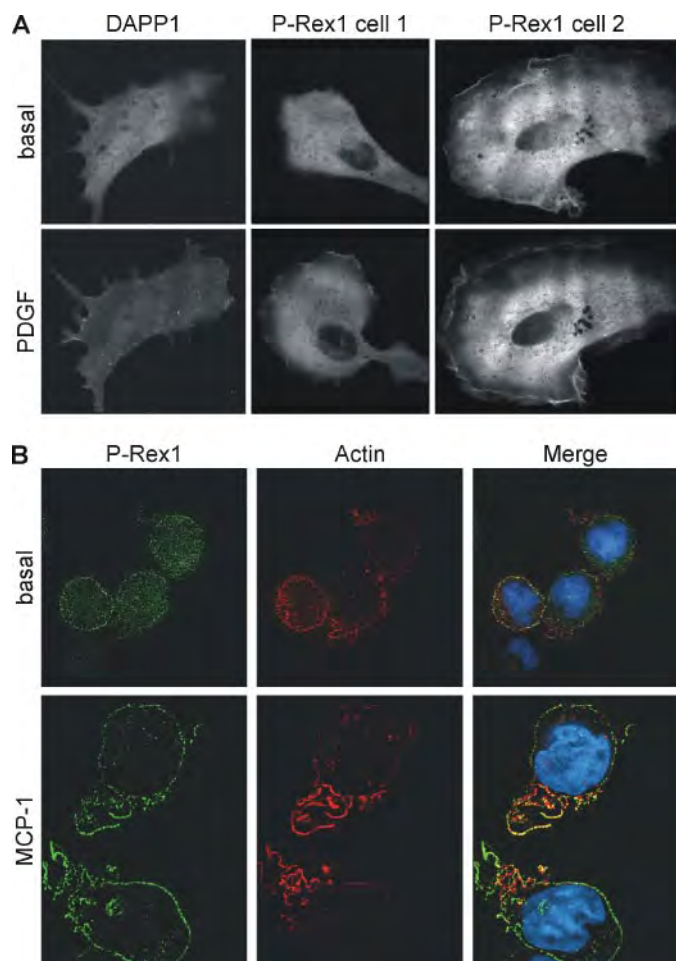
FIGURE 1. **PI3K activation is not sufficient to cause P-Rex1 translocation to the plasma membrane, but stimulation of GPCRs in myeloid cells is.** *A,* PAE cells were transfected with EGFP-DAPP1 or EGFP-P-Rex1, grown on coverslips, serum-starved, and observed by live confocal microscopy. After 1–2 min, cells were stimulated with PDGF. Photographs are stills from videos (see supplemental materials) taken from the same cells before and after stimulation with PDGF and are representative of four videos for P-Rex1 and three for DAPP1. P-Rex1 cell 1 is representative of low level P-Rex1 expression, and cell 2 is representative of high expression. *B,* monocytic THP-1 cells were stably transfected with GFP-myc-P-Rex1, serum-starved, and stimulated or not with 100 nM MCP-1 for 90 s as detailed under "Experimental Procedures." Cells were fixed and stained with Alexa-488-labeled rabbit anti-GFP for detection of P-Rex1 (*green*), Alexa-594-phalloidin for detection of F-actin (*red*), and 4′,6-diamidino-2-phenylindole (*blue*).

FIGURE 2. **Expression of P-Rex1, PI3K, and G$\beta\gamma$ subunits in Sf9 cells.** Full-length EE-P-Rex1, PI3K (His$_6$-p110γ catalytic and EE-p101 regulatory subunits), and EE-G$\beta_1\gamma_2$ subunits were expressed in Sf9 cells using baculovirus titers and expression times and conditions that favor the concomitant presence of all components. Expression of the proteins was measured by anti-EE Western blotting for P-Rex1 and p101 or by immunoprecipitations (*IP*) for p110γ and G$\beta\gamma$ subunits, using the respective epitope tags followed by Coomassie staining of SDS-gels, as indicated.

comitantly express several exogenous proteins without compromising expression levels of each component too drastically.

First, we optimized conditions that favor the expression of all three components, EE-tagged P-Rex1, PI3K (consisting of the His-tagged catalytic p110γ and EE-tagged regulatory p101 subunits of class IB PI3K), and the heterotrimeric G protein subunits EE-Gβ_1/Gγ_2 (Fig. 2). Then we set up a protocol of cell fractionation by differential centrifugation to quantify membrane localization of P-Rex1. Sf9 cells were infected to express either P-Rex1 alone or P-Rex1 together with both G$\beta\gamma$ subunits and PI3K. After 42 h, the cells were serum-starved, washed, and lysed by sonication. The total lysate was cleared of nuclei and debris by low speed centrifugation, and the postnuclear supernatant was further fractionated by ultracentrifugation into cytosol and membrane fractions. Aliquots were collected at each step of the fractionation procedure. Subcellular localization of P-Rex1 was assessed by anti-EE Western blotting of each fraction (Fig. 3*A*). We quantified P-Rex levels in each fraction by densitometric scanning of the Western blots and NIH ImageJ analysis. The expression of P-Rex1 was significantly reduced by coexpression with both G$\beta\gamma$ and PI3K. To test whether a change in expression level would alter the degree of membrane localization of P-Rex1, we titrated P-Rex1 expression over 3 orders of magnitude by altering the baculovirus titer of Sf9 cell infection and compared the level of P-Rex1 in the membrane fraction with those in the postnuclear supernatant (Fig. 3*B*). Over the range of P-Rex1 expression levels we obtained in any of our experiments, the relative amount of P-Rex1 at the membrane in basal cells remained constant.

Next, we compared the membrane localization of P-Rex1 in Sf9 cells that expressed either P-Rex1 alone, P-Rex1 together with G$\beta\gamma$ subunits, P-Rex1 together with PI3K, or P-Rex1 with both G$\beta\gamma$ and PI3K. When expressed alone, 7% of total cellular P-Rex1 was expressed at the membrane (Fig. 3*C*). Around 80% of the total was in the cytosol, and the remainder was in the low speed pellet (not shown). When coexpressed with either G$\beta\gamma$ subunits or PI3K alone, we found a small but not statistically significant increase of P-Rex1 in the membrane fraction to 10 or 11% of the total, respectively. In contrast, when expressed in the

membrane translocation under these conditions, we stably transfected monocytic THP-1 with NH$_2$-terminally GFP-myc-tagged P-Rex1 and stimulated them with MCP-1 (CCL2), the ligand of the CCR2. The subcellular localization of P-Rex1 was analyzed by immunofluorescence microscopy. Although P-Rex1 localization was mainly cytosolic in basal, serum-starved cells, MCP-1 stimulation induced a robust translocation of P-Rex1 to the plasma membrane, where it co-localized with filamentous actin (Fig. 1*B*).

PI3K Activity and G$\beta\gamma$ Subunits Together Synergistically Induce P-Rex1 Membrane Recruitment—To investigate whether G$\beta\gamma$ subunits alone are a sufficient signal to drive membrane recruitment of P-Rex1 or whether G$\beta\gamma$ subunits and PIP$_3$ together are, we expressed P-Rex1 with G$\beta\gamma$ subunits and/or PI3K in Sf9 insect cells. We chose this cell line, because Sf9 cells, in contrast to mammalian cell lines, are able to con-
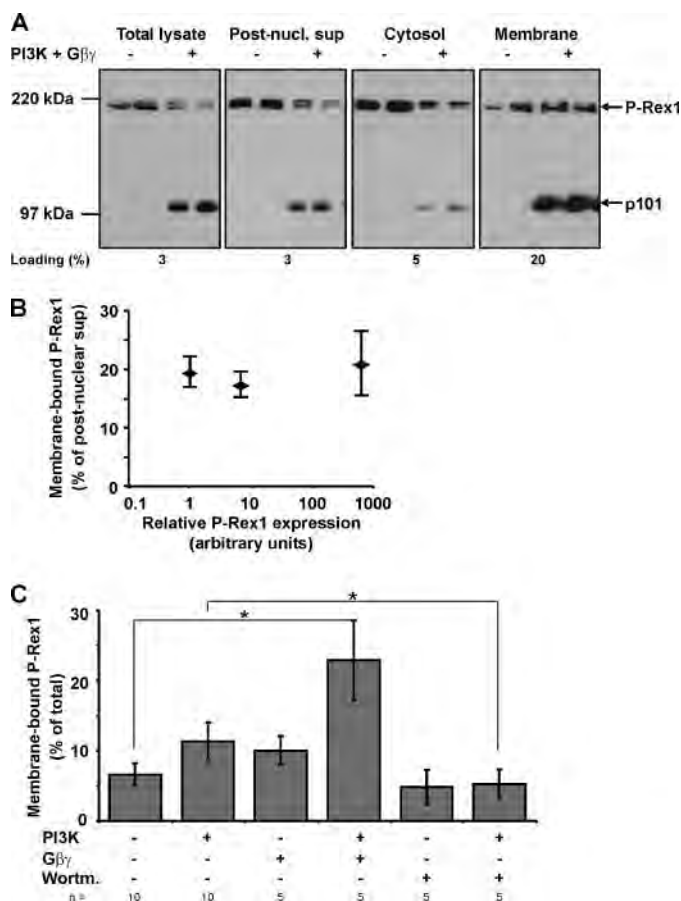
FIGURE 3. **PI3K activity and Gβγ subunits together synergistically induce P-Rex1 membrane recruitment in Sf9 cells.** *A*, anti-EE Western blots of subcellular fractions total lysate, postnuclear supernatant, cytosol, and membrane obtained from serum-starved Sf9 cells expressing EE-P-Rex1 or co-expressing EE-P-Rex1 with PI3K (His$_6$-p110γ, EE-p101) and EE-Gβ$_1$γ$_2$ subunits by differential centrifugation. The blots are from one experiment representative of five. *B*, basal membrane localization of EE-P-Rex1 as a function of its expression level in Sf9 cells. P-Rex1 expression levels were titrated over 3 orders of magnitude by altering the baculovirus titers used to infect Sf9 cells. The percentage of EE-P-Rex1 localized in the membrane fraction of Sf9 cells was quantified by densitometric scanning and ImageJ analysis of anti-EE Western blots as detailed under "Experimental Procedures" and is plotted as a function of its expression level in the postnuclear supernatant. Data are the average from one experiment performed in triplicates ± S.D. *C*, membrane localization of P-Rex1 in fractions of serum-starved Sf9 cells when expressed alone, co-expressed with either Gβγ subunits or PI3K, or co-expressed with Gβγ subunits and PI3K together and with or without a 20-min pretreatment with 100 nM wortmannin was measured by anti-EE Western blotting of fractions and quantified by densitometric scanning and ImageJ analysis and is plotted as the percentage of P-Rex1 in the corresponding total lysate fraction. Data are mean ± S.E. of five or 10 experiments, respectively, as indicated. Statistics were calculated using *t* test.

FIGURE 4. **P-Rex1 mutants.** *A*, panel of the NH$_2$-terminally EE-tagged P-Rex1 mutants used in the membrane translocation assays. *B* and *C*, characterization of the *in vitro* GEF activity of the isolated DH domain of P-Rex1. The purified Sf9 cell-derived iDH domain of P-Rex1 was subjected to liposome-based *in vitro* Rac2-GEF activity assays in the presence of 0.3 μM EE-Gβ$_1$γ$_2$ subunits (*B*) or 10 μM D-stearoyl/arachidonoyl-PIP$_3$ (*C*). *C*, iDH is compared with full-length P-Rex1. Data in *B* are the mean ± S.E. of four experiments, and data in *C* are the mean ± S.D. from one experiment.

nin before cell fractionation. Wortmannin completely inhibited the PI3K-dependent arm of P-Rex1 membrane recruitment, confirming that PI3K activity (*i.e.* PIP$_3$ production) is required (Fig. 3*C*).

The DH/PH Domain Tandem Is Sufficient for Membrane Recruitment of P-Rex1 by PI3K and Gβγ Subunits—We asked which parts of the P-Rex1 protein confer membrane localization. From our previous work, we know that P-Rex1 GEF activity is stimulated via its PH domain by PIP$_3$ and via its catalytic DH domain by Gβγ subunits (12). We tested some of the P-Rex1 mutants used in that study here to compare their membrane localization with that of full-length P-Rex1 (Fig. 4*A*). Additionally, we made a new mutant consisting of only the isolated NH$_2$-terminal catalytic DH domain of P-Rex1 (iDH). To characterize the functionality of the new iDH mutant, we performed *in vitro* Rac2-GEF assays. As expected, Gβγ subunits could stimulate the GEF activity of the isolated DH domain (Fig. 4*B*), but PIP$_3$ could not (Fig. 4*C*).

Next, we investigated the membrane localization of the isolated DH domain of P-Rex1. Gβγ subunits or PI3K were not sufficient to stimulate membrane recruitment of the isolated DH domain; nor were Gβγ and PI3K together (Fig. 5*A*). Simi-

presence of both Gβγ and PI3K, we found a robust increase of P-Rex1 localization at the membrane to 23% of total, meaning the effect of Gβγ and PI3K together was 2.3-fold bigger than their additive effects. Hence, although neither PI3K alone nor Gβγ alone is a sufficient signal to recruit significant amounts of P-Rex1 to the membrane, PI3K and Gβγ together synergistically induce membrane recruitment of P-Rex1 (Fig. 3*C*).

We verified that the enzymatic activity of PI3K rather than the mere presence of the PI3K protein in the cell confers the PI3K-dependent arm of P-Rex1 membrane recruitment by treating serum-starved Sf9 cells expressing P-Rex1 alone or expressing P-Rex1 and PI3K with the PI3K inhibitor wortman-
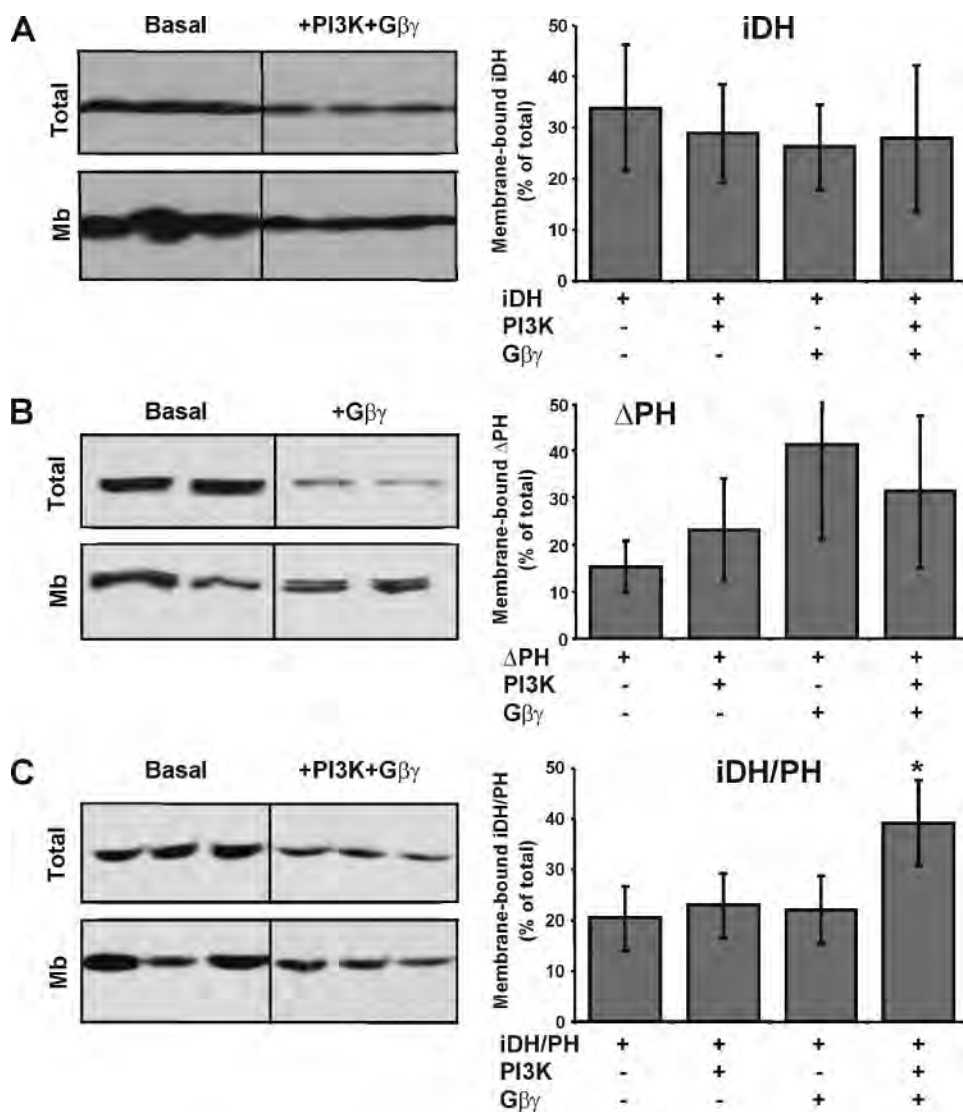
FIGURE 5. **The DH/PH domain tandem is sufficient for synergistic PI3K and Gβγ subunit-dependent membrane recruitment of P-Rex1.** Sf9 cells were infected to express the P-Rex1 mutants iDH (*A*), ΔPH (*B*), or iDH/PH (*C*), either alone, or with PI3K or Gβγ subunits, or with PI3K and Gβγ subunits together. Serum-starved cells were fractionated as in Fig. 3 into total lysate, postnuclear supernatant, cytosol, and membrane fractions. Three percent of total lysate and 20% of membrane fractions were subjected to EE-Western blotting, and membrane-localized P-Rex1 was quantified by densitometric scanning and ImageJ analysis and is plotted on the *right* as percentage of P-Rex1 in the corresponding total lysate fraction. Data in *A* are mean ± S.E. of three experiments performed in triplicates; data in *B* are mean ± S.E. of five experiments performed in duplicates; data in *C* are mean ± S.E. of three experiments performed in triplicates. Anti-EE Western blots of the membrane and total lysate fractions from representative experiments are shown on the *left*.

With each P-Rex1 deletion mutant, their basal level of P-Rex1 membrane attachment was much higher compared with the full-length protein (Figs. 3*C* and 5, *A–C*). This shows that the presence of the C-terminal domains of P-Rex1 (DEP, PDZ, and inositol polyphosphate 4-phosphatase) helps to keep the full-length enzyme localized to the cytosol in unstimulated cells. Similarly, the presence of these domains keeps the basal GEF activity of P-Rex1 low (12). However, none of these C-terminal domains are required for P-Rex1 membrane localization.

*GEF Activity Is Not Required for P-Rex1 Membrane Recruitment—*Next, we asked whether the GEF activity of P-Rex1 is required for membrane translocation. We measured membrane recruitment of a GEF-dead P-Rex1 mutant, which carries two point mutations in its DH domain that completely abolish GEF activity (12). Gβγ and PI3K could still synergistically cause membrane attachment of the GEF-dead P-Rex1 (Fig. 6). Hence, the GEF activity of P-Rex1 is not required for membrane translocation.

*Membrane-bound P-Rex1 Has Higher GEF Activity than Cytosolic P-Rex1—*During our subcellular fractionation experiments, we noticed that P-Rex1 from the total lysate of Sf9 cells migrates as two bands on SDS-PAGE and that these separate differentially into the cytosol and membrane fractions (Fig. 3*A*). The cytosol was enriched in the higher molecular weight band, and the membrane was enriched in the lower molecular weight band. In their study of P-Rex1 regulation by PKA, the Garrison laboratory also found two bands of P-Rex1 in Sf9 cells (11). Phosphorylation of the purified P-Rex1 doublet by PKA resulted in a mobility shift toward the higher band and dephosphorylation with λ-phosphatase in a shift to the lower band. The PKA-treated phosphorylated P-Rex1 had lost its ability to be activated by Gβγ subunits and PIP_3, whereas the dephosphorylated P-Rex1 was much more activated by Gβγ subunits and PIP_3 than native P-Rex1 (11). To test whether the different forms of P-Rex1 in our subcellular fractions have different intrinsic GEF activities, we purified P-Rex1 by immunoprecipitation from the cytosol and membrane fractions of Sf9 cells using EE antibody coupled to Sepharose beads, eluted the purified membrane-derived and

larly, a P-Rex1 mutant missing the PH domain (ΔPH) could not be stimulated by Gβγ and PI3K to translocate to the membrane (Fig. 5*B*). Interestingly, we did find a marked stimulation of the membrane localization of P-Rex1-ΔPH by Gβγ subunits in some experiments, although this was quite variable, suggesting that the presence of the PH domain might somehow repress the access of Gβγ subunits to full-length P-Rex1. The minimal P-Rex1 construct that allowed Gβγ subunits and PI3K to synergistically induce membrane localization was the isolated DH/PH domain tandem (iDH/PH) (Fig. 5*C*). Hence, just like with the synergistic regulation of P-Rex1 GEF activity by PI3K and Gβγ subunits, the DH/PH tandem is sufficient for PI3K- and Gβγ-mediated membrane recruitment.
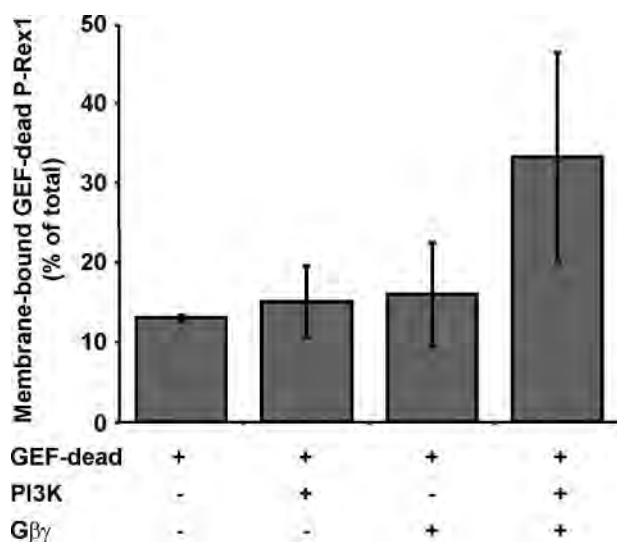
FIGURE 6. **The GEF activity of P-Rex1 is not required for membrane recruitment.** The GEF-dead E56A/N238A mutant of P-Rex1 was expressed in Sf9 cells either alone, with PI3K or $G\beta\gamma$ subunits, or with PI3K and $G\beta\gamma$ subunits together. Serum-starved cells were fractionated and analyzed for P-Rex1 membrane localization as in Fig. 5. Data are mean ± range from two experiments.

cytosol-derived P-Rex1 from the beads using free EE peptide, and assayed their Rac2-GEF activity *in vitro*. Membrane-derived P-Rex1 had a basal Rac2-GEF activity, comparable with the activity we generally see with recombinant P-Rex1 purified from whole Sf9 cells (see Figs. 4*C* and 7*A*). In contrast, we could not detect any basal Rac2-GEF activity of cytosol-derived P-Rex1 (Fig. 7*A*). This suggests that the pool of P-Rex1 at the membrane is preactivated, whereas the pool in the cytosol is inactive. However, the Rac2-GEF activities of both membrane-derived and cytosol-derived P-Rex1 could be stimulated by PIP$_3$ and $G\beta\gamma$ subunits, although the -fold activation of the membrane-derived P-Rex1 was lower than that of cytosol-derived P-Rex1 (Fig. 7*B*). This confirmed that the pool of P-Rex1 in the cytosol is not permanently inactive and can be mobilized for enzymatic function.

DISCUSSION

A conundrum over the past few years has been that both activators of P-Rex1, $G\beta\gamma$ subunits and PIP$_3$, are membrane-bound, but the intracellular localization of P-Rex1 in basal cells is cytosolic, both for endogenous and overexpressed protein. To activate Rac upon cell stimulation, P-Rex1 must translocate to the membrane. However, activation of PI3K alone is not sufficient to promote significant membrane translocation of P-Rex1. A recent study by the Bokoch laboratory has shown that endogenous P-Rex1 translocates to the plasma membrane of neutrophils in response to stimulation of GPCRs and that inhibition of PI3K activity, $G\beta\gamma$-effector interaction, and protein-tyrosine kinase activity and activation of PKA block membrane translocation. Here we confirm that P-Rex1 translocates to the plasma membrane in GPCR-stimulated myeloid cells by overexpressing P-Rex1 in monocytic THP-1 cells, where P-Rex1 translocates from the cytosol to the plasma membrane in response to stimulation with MCP-1. To investigate the molecular mechanisms through which P-Rex1 translocates to

the membrane, we assessed the subcellular localization of P-Rex1 through fractionation of Sf9 cells expressing full-length or mutant P-Rex1 with or without $G\beta\gamma$ subunits and/or PI3K. We show that $G\beta\gamma$ and PIP$_3$ together synergistically cause P-Rex1 membrane translocation, that the DH/PH domain tandem of P-Rex1 is sufficient for $G\beta\gamma$- and PI3K-dependent membrane recruitment, that translocation does not require P-Rex1 GEF activity, and that membrane-derived purified P-Rex1 has a higher intrinsic Rac-GEF activity than cytosol-derived P-Rex1.

As expected from previous work on P-Rex1 and other Dbl family GEFs, P-Rex1 was mainly localized in the cytosol of basal serum-starved cells, with only low levels in the $117,000 \times g$ membrane fraction. Co-expression of P-Rex1 with either $G\beta\gamma$ subunits or PI3K alone resulted in a modest increase of P-Rex1 membrane localization, showing that, like PIP$_3$ formation alone, the production of $G\beta\gamma$ subunits alone is not sufficient to induce P-Rex1 membrane translocation. Wortmannin treatment abolished the minor PI3K-driven membrane localization, confirming that PI3K activity (*i.e.* PIP$_3$ formation) rather than any potential activity-independent consequence of PI3K expression is responsible for the PI3K-dependent arm of P-Rex1 translocation. Co-expression of P-Rex1 with both $G\beta\gamma$ subunits and PI3K resulted in a synergistic (2.3-fold over additive) increase of membrane bound P-Rex1 to 23% of total, showing that $G\beta\gamma$ subunits and PI3K activity together are a required and sufficient signal for P-Rex1 membrane translocation. There is a possibility that stabilization of P-Rex1 at the membrane contributes to its increase in the membrane fraction of $G\beta\gamma$ subunit- and PI3K-expressing cells as well as net translocation of P-Rex1 from the cytosol to the membrane. However, we believe that net translocation is the predominant effect for two reasons: (i) the proportion of P-Rex1 recovered in the membrane fraction remains constant when we vary the expression levels of P-Rex1 over 3 orders of magnitude, which does not hint to changes in the stability of membrane association, and (ii) the immunofluorescence microscopy experiments in THP-1 cells, which clearly demonstrate net translocation of P-Rex1 from the cytosol to the membrane, would suggest that we also see net translocation in the fractionation experiments. Since neither $G\beta\gamma$ subunits nor PIP$_3$ can come out of the membrane to grab cytosolic P-Rex1, we imagine that cytosolic P-Rex1 that diffuses into the vicinity of the plasma membrane is captured by $G\beta\gamma$ subunits and PIP$_3$, when these are formed in the membrane. If only one of the translocation signals, either $G\beta\gamma$ subunits or PIP$_3$, is generated, their binding to P-Rex1 is not sufficient to retain P-Rex1 at the membrane, and P-Rex1 diffuses back into the cytosol.

Similar to membrane recruitment, the GEF activity of P-Rex1 is also synergistically stimulated by $G\beta\gamma$ subunits and PIP$_3$. Hence, through their dual effect on membrane recruitment and stimulation of GEF activity, $G\beta\gamma$ subunits and PIP$_3$ together seem to ensure that P-Rex1 is activated in the right location within the cell. In this way, P-Rex1 seems perfectly tuned to detect the coincident activation of GPCRs and PI3Ks upon cell stimulation and to activate Rac best under those conditions. In neutrophils, the presence of a GPCR-dependent PI3K makes it possible that translocation and full activation of P-Rex1 occurs
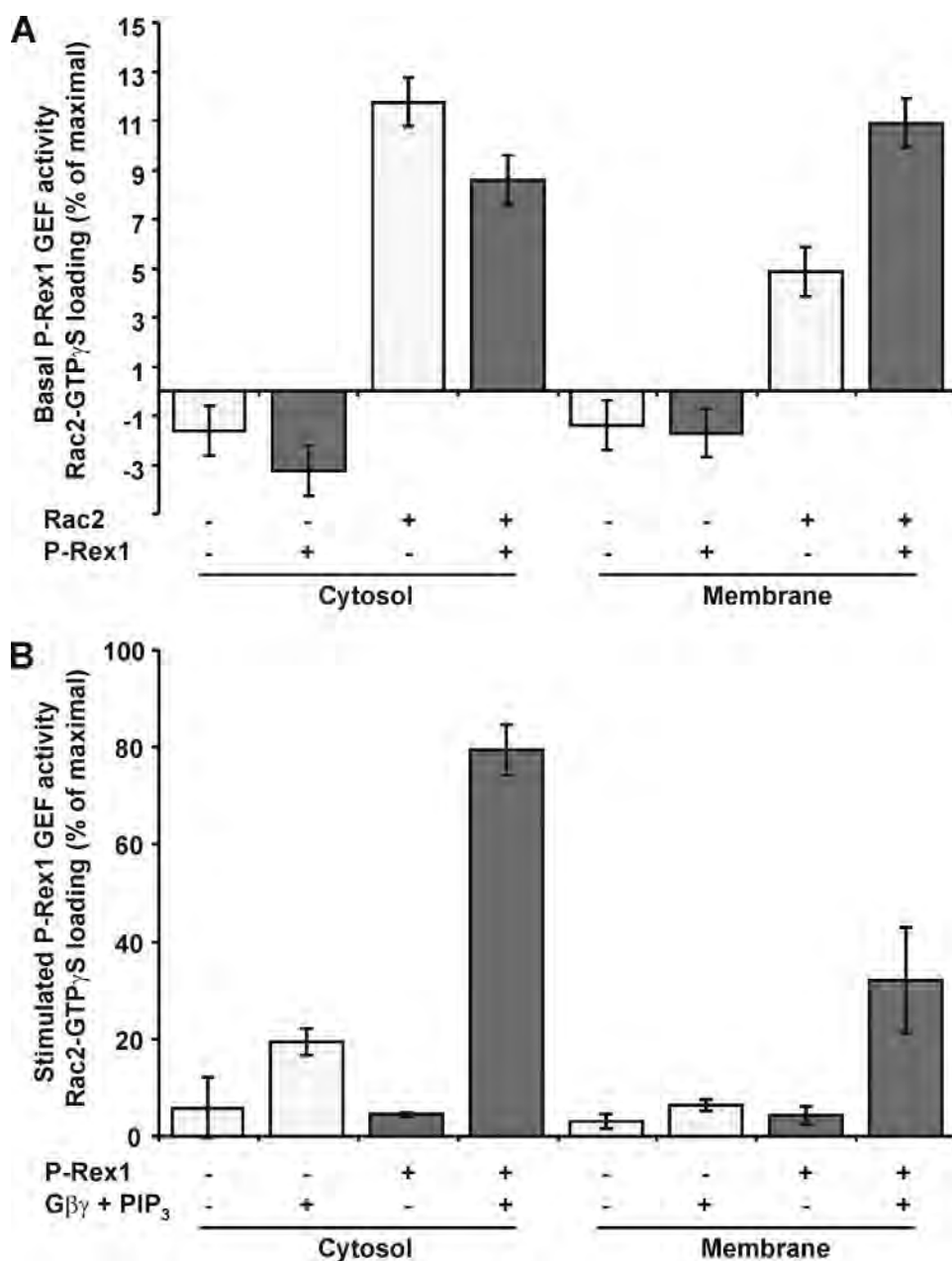
FIGURE 7. *In vitro* **Rac-GEF activities of membrane-derived *versus* cytosol-derived P-Rex1.** Serum-starved Sf9 cells expressing EE-P-Rex1 (*dark bars*) and uninfected control cells (*light bars*) were fractionated essentially as in Fig. 3, except on a larger scale. EE-P-Rex1 was purified by immunoprecipitation from the cytosol and membrane fractions, and controls were mock-treated as detailed under "Experimental Procedures." *A*, basal *in vitro* Rac2-GEF activity of purified membrane-derived and cytosol-derived P-Rex1. Data are mean ± S.E. of assays performed from three separate cell fractionation experiments. *B*, *in vitro* Rac2-GEF activity of purified membrane-derived and cytosol-derived P-Rex1 stimulated with 0.3 μM EE-G$\beta_1\gamma_2$ subunits and 0.2 μM D-stearoyl/arachidonoyl-PIP$_3$. Data are mean ± S.E. of three experiments, except for minus P-Rex1 controls, which are mean ± S.D. of triplicates from one of these experiments.

We have previously shown that G$\beta\gamma$ subunits activate P-Rex1 via the catalytic DH domain (12) (this report) and that PIP$_3$ activates P-Rex1 via the PH domain (12), although the PH domain lacks the consensus sequence for phosphoinositide binding. Use of P-Rex1 mutants here showed that the isolated DH/PH domain tandem of P-Rex1 is sufficient for synergistic G$\beta\gamma$ subunit- and PIP$_3$-dependent membrane translocation. The isolated DH domain of P-Rex1 and the P-Rex1 mutant lacking the PH domain were not able to translocate to the membrane in response to G$\beta\gamma$ subunits and PIP$_3$, showing that both the DH and the PH domain are required. It seems likely that, as for the stimulation of GEF activity, G$\beta\gamma$ act through the DH domain and PIP$_3$ through the PH domain when driving membrane translocation. Indeed, the ΔPH domain mutant translocated to the membrane in response to G$\beta\gamma$ subunits, although this was not statistically significant over all experiments. Together, the P-Rex1 mutants showed that there is no requirement for additional G$\beta\gamma$- and PIP$_3$-binding sites outside of the DH and PH domains for the stimulation of membrane localization. However, we have not yet pinpointed the precise sites for G$\beta\gamma$ and PIP$_3$ binding within the DH and PH domains, respectively, so we cannot preclude the possibility that there might be more than one such site within these domains.

As is often found with GEFs homologous to Dbl (*i.e.* having a DH/PH domain tandem), the isolated DH/PH tandem of P-Rex1 has high constitutive GEF activity (12). It also shows high constitutive membrane binding in serum-starved cells. Hence, the presence of the DEP, PDZ, and inositol polyphosphate 4-phosphatase homology domains in full-length P-Rex1 contributes both to keeping the basal catalytic activity of P-Rex1 low and to keeping the enzyme in the cytosol in unstimulated cells, presumably through some undefined intramolecular interaction between the NH$_2$-terminal and C-terminal parts of the enzyme in its resting state.

Membrane-derived full-length P-Rex1 has a higher basal catalytic GEF activity *in vitro* than cytosol-derived P-Rex1, sug-

after stimulation of a single receptor type. In other cell types (*e.g.* in the brain, which express P-Rex1 but no GPCR-dependent PI3K), concomitant activation of GPCRs with another class of receptor that couples to protein-tyrosine kinase-regulated PI3K could provide the required P-Rex1 translocation and activation signals. Equally, it seems likely that similar coincidence detection applies to membrane recruitment and activation of the other members of the P-Rex family, P-Rex2 and P-Rex2b (4, 5), that are not expressed in hemopoietic cells.

gesting that only a somehow preactivated subpopulation of the enzyme can reach the membrane. We suppose that this preactivation is likely to be through the dephosphorylation of native P-Rex1, since the Garrison laboratory has shown that phosphorylation of purified Sf9 cell-derived P-Rex1 results in a gel shift and in an enzyme that cannot be activated by $G\beta\gamma$ subunits and PIP_3, whereas dephosphorylated P-Rex1 can (11). In our experiments, the higher migrating band of P-Rex1 was enriched in the cytosol, and the lower band was enriched in the membrane fraction. Hence, it is tempting to speculate that a phosphorylated form of P-Rex1 (presumably by PKA) is an inactive pool that resides mainly in the cytosol, whereas the dephosphorylated form can translocate to the membrane in response to $G\beta\gamma$ formation and PI3K activity. A further intriguing finding was that the iDH/PH and iDH P-Rex1 proteins did not migrate as doublets, suggesting that the phosphorylation sites giving rise to the mobility shift probably lie outside of the DH and PH domains. We also found that the *in vitro* GEF activities of both membrane-derived and cytosol-derived P-Rex1 can be stimulated by $G\beta\gamma$ subunits and PIP_3. Although these *in vitro* data by no means imply that $G\beta\gamma$ and PIP_3 can activate P-Rex1 when it is remote from the membrane within the cytosol, they do suggest that the cytosolic P-Rex1 is not permanently inactive and can be mobilized. These findings helped us to refine our working model of the mechanisms governing P-Rex1 membrane translocation; cytosolic P-Rex1 exists in the cell both in phosphorylated and nonphosphorylated form. Phosphorylated P-Rex1 remains cytosolic upon cell stimulation, but nonphosphorylated cytosolic P-Rex1 that diffuses into the vicinity of the plasma membrane can be captured by either $G\beta\gamma$ subunits or PIP_3 but is retained at the membrane only if both translocation signals are present concomitantly. In this refined model, dephosphorylation of cytosolic P-Rex1 regulates the size of the pool of P-Rex1 available to be mobilized for signaling at the membrane.

$G\beta\gamma$ subunits have been shown to be necessary and sufficient membrane targeting signals for a variety of proteins in organisms ranging from yeast to mammals. For example, membrane recruitment of the yeast scaffold protein Ste5 by $G\beta\gamma$ binding is important for the pheromone response pathway (18), CRAC recruitment by $G\beta\gamma$ is required for adenylyl cyclase activation through GPCRs in Dictyostelium (19), and recruitment of βARK by $G\beta\gamma$ regulates desensitization of the β-adrenergic receptor (20). The P-Rex family is so far the only Dbl family enzyme known to be directly activated and/or translocated by $G\beta\gamma$ subunits. Two other Dbl family GEFs are activated *in vivo* upon $G\beta\gamma$ formation, Ras-Grf1 and p114RhoGEF. $G\beta\gamma$-dependent stimulation of the Rac-GEF activity of Ras-Grf1, a dual Ras and Rac-GEF, is indirect via Src family protein-tyrosine kinases (21). The mechanism of $G\beta\gamma$-dependent stimulation of p114RhoGEF, which activates RhoA and Rac1, is unknown (22), and membrane translocation of Ras-Grf1 and p114RhoGEF upon $G\beta\gamma$ formation has not yet been studied. $G\alpha$ subunits (of the $G_{12/13}$ family) can also act as membrane targeting modules for Dbl family enzymes, but only for those GEFs that contain an RGS domain, such as p115RhoGEF, LARG, and PDZ-RhoGEF (23, 24).

PH domains were first recognized as phosphoinositide binding domains and are necessary and sufficient for membrane binding of many proteins, including DAPP1 and Grp1 (25, 26). Apart from P-Rex, several other Dbl family GEFs have been shown to bind phosphoinositides via the PH domains in their DH/PH tandem, including members of the Tiam, Vav, and Sos families (27). However, this is never sufficient to induce membrane translocation. Membrane localization mechanisms for Dbl family GEFs are varied and include phosphoinositide binding to various domains, phosphorylation by various protein kinases, and the formation of multiprotein complexes. The membrane translocation of Tiam1 has been most intensively studied. Tiam1, which has two PH domains, an NH_2-terminal one and the C-terminal one in the DH/PH tandem, translocates from the cytosol to the plasma membrane in response to cell stimulation with serum, lysophosphatidic acid, or PDGF (28, 29), and its membrane localization is necessary for Rac activation *in vivo* (28, 30). Both PH domains of Tiam1 bind phosphoinositides (31, 32), but membrane translocation is dependent on $PI(4,5)P_2$ binding to the NH_2-terminal PH domain (28, 31) and independent from PI3K activity (29) and from PI3P binding to the C-terminal PH domain in the DH/PH tandem (33). Vav recruitment to the plasma membrane upon FcϵRI engagement requires its SH2 domain and the activity of the protein-tyrosine kinase Syk (34). Sos has recently been shown to translocate to the plasma membrane in 293 cells in response to SLIT stimulation of the Robo receptor and by forming a complex with the receptor and the adaptor protein Dock (35). The Rac-GEF βPIX is involved in regulating contact inhibition by translocating from the cytosol to focal adhesion complexes in a way that depends on its interaction with and the kinase activity of PAK (36). A C-terminal coiled-coil domain of βPIX is also involved in its recruitment to the cell periphery and necessary for the formation of membrane ruffles and microvilli (37). Localization of Trio8, an isoform of the dual Rac1- and RhoA-GEF, to endosomal membranes of developing Purkinje neurons is mediated via a hydrophobic C-terminal domain and required for neurite elongation (38). Although phosphoinositide binding to the PH domain in the DH/PH tandem is never sufficient for membrane translocation, it has been shown to be required for some GEFs other than P-Rex1. For example, proto-Dbl and Dbs both require phosphoinositide binding to the PH domain for membrane recruitment and to maintain their transforming abilities (39, 40). The emerging consensus is that the PH domain in the DH/PH tandem often plays a dual role; it can participate in membrane recruitment, and it regulates GEF activity through allosteric interaction with the DH domain and/or the GTPase (41).

The most distinguishing feature of the P-Rex family is its dual regulation via $G\beta\gamma$ subunits and PIP_3. In the past, we have shown that the GEF activities of P-Rex1 and P-Rex2 are synergistically stimulated by $G\beta\gamma$ and PIP_3 *in vitro* and *in vivo* (3, 4). Here we have shown that $G\beta\gamma$ subunits and PI3K activity also synergistically induce P-Rex1 membrane localization. It will be especially interesting in the future to identify the functional roles of P-Rex family enzymes in the convergence of signaling pathways downstream of concomitant activation of two different classes of cell surface receptors in those cell types that do not express a $G\beta\gamma$-stimulated PI3K.

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