

Cell Migration and Signaling Specificity Is Determined by the Phosphatidylserine Recognition Motif of Rac1*[§]

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The Rho guanosine triphosphatases (GTPases) control cell shape and motility and are frequently overexpressed during malignant growth. These proteins act as molecular switches cycling between active GTP- and inactive GDP-bound forms. Despite being membrane anchored via their isoprenylated C termini, Rho GTPases rapidly translocate between membrane and cytosolic compartments. Here, we show that the Rho GTPase Rac1 preferentially interacts with phosphatidylserine (PS)-containing bilayers through its polybasic motif (PBM). Rac1 isoprenylation contributes to membrane avidity but is not critical for PS recognition. The similar protein Cdc42 (cell division cycle 42), however, only associates with PS when prenylated. Conversely, other Rho GTPases such as Rac2, Rac3, and RhoA do not bind to PS even when they are prenylated. Cell stimulation with PS induces translocation of Rac1 toward the plasma membrane and stimulates GTP loading, membrane ruffling, and filopodia formation. This stimulation also promotes Cdc42 activation and phosphorylation of mitogen-activated protein kinase through Rac1/PS signaling. Consequently, the PBM specifically directs Rac1 to effect cytoskeletal rearrangement and cell migration by selective membrane phospholipid targeting.

The Rho family of GTPases regulates a wide range of cellular processes including the actin cytoskeleton organization, gene transcription, and cell cycle progression (1). These Rho GTPases share high levels of sequence similarity despite their different biological functions (2). However, Rho proteins present a divergent C-terminal element, which is named the polybasic motif (PBM),² a stretch of basic residues contiguous to the CAAX isoprenylation-site motif (where “C” indicates a cysteine

residue, “A” is an aliphatic residue, and “X” any residue). The differential intracellular localization of various Rho GTPases has been characterized (3). Whereas overexpressed Cdc42 and Rac2 have been shown to be present in endocytic membranes in a Rho guanidine dissociation inhibitor-dependent fashion, Rac1 is localized to the plasma membrane. In contrast, RhoA has been observed predominantly in the cytosol. Thus, it is plausible that divergent features such as the PBM may help determine the localization of Rho GTPases, although the specific mechanisms are not clear.

Membrane association is critical for the function of Rho family proteins. Isoprenylation of their C termini plays a major role in membrane anchoring, although these proteins appear to shuttle rapidly between membrane and cytosolic compartments (4). Although the mechanism of specific targeting of these GTPases to plasma *versus* other internal membranes is still unsolved, their subcellular localization does appear to be influenced by electrostatic membrane interactions of the PBM (5). Both phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-triphosphate were initially proposed as Rac1 and RhoA ligands, respectively, binding preferentially to the nucleotide-free state (6). However, these phosphoinositide interactions were not detected by NMR spectroscopy or overlay assays (7), and their *in vivo* significance remains unclear.

To investigate the mechanism of Rac1 recruitment to the plasma membrane, we searched potential lipid ligands for Rho GTPases by liposome binding and NMR spectroscopy. We found that Rac1 preferentially interacts with phosphatidylserine (PS)-containing bilayers independently of the bound nucleotide. The PS binding site of Rac1 is located at the polybasic region, and prenylation of this motif increases the avidity for its ligand. Conversely, Cdc42 only binds PS when prenylated. Mutational analysis suggests that the PS-PBM interaction is required for Rac1' subcellular localization and GTP loading, and also triggers Cdc42-GTP formation, phosphorylation of the mitogen-activated protein kinase (MEK1) and rearrangement of the cytoskeleton. Thus, PS recognition by Rac1 through its PBM is centrally involved in cell motility and confers non-redundancy in signaling among GTPases proteins.

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1.

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² The abbreviations used are: PBM, polybasic motif; CHO-K1, Chinese hamster ovary K1; MEK1, mitogen-activated protein kinase 1; NMR, nuclear magnetic resonance; PAK1, p21-activated kinase 1; PC, phosphatidylcholine;

PS, phosphatidylserine; ERK, extracellular signal-regulated protein kinases; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GST, glutathione S-transferase; GTPγS, guanosine 5'-3-O-(thio)triphosphate; GEF, guanine nucleotide-exchange factor.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Rho Proteins—Full-length human Rac1, 2, 3, RhoA, and Cdc42 cDNA constructs (UMR cDNA Resource Center) were cloned into a pGEX2T vector (Amersham Biosciences). Site-directed mutagenesis of Rac1 was performed using the QuikChange exchange protocol (Stratagene). The C-terminal region of Rac1 comprising residues 185–192 was deleted in the truncated form (Δ C Rac1). Purification of all GST fusion proteins from *Escherichia coli* Rosetta cells (Novagen) on glutathione beads was performed according to the manufacturer's protocol (Amersham Biosciences). All Rho proteins were also cloned in pBAC-2cp (Novagen) and expressed using the eukaryotic baculovirus, *Spodoptera frugiperda* (Sf9) expression system. Prenylated Rho proteins were isolated from the membrane fraction of Sf9 cells by extraction with 0.5% CHAPS (8) and purified using Talon beads (Clontech). Purity of all proteins was over 95% as judged by SDS-PAGE gels. All purified proteins were stable in the presence of protease inhibitors as judged by mass spectrometric analysis (data not shown).

Liposome Binding Assay—Phospholipids including dipalmitoyl phosphatidylcholine (PC), dipalmitoyl PS, phosphatidylinositol, phosphatidylglycerol, and dipalmitoyl PE (Avanti Polar Lipids) were prepared as a lipid film by lyophilization overnight. Lipid films were hydrated in 20 mM Tris-HCl (pH 6.8), 100 mM NaCl, 2 mM dithiothreitol at 1 mg/ml concentration and freeze-thawed 3 times. Large unilamellar vesicles were formed by extruding the lipid through a 0.1- μ m filter (Nucleopore track-etch membrane, Whatman) 20 times and used immediately. Recombinant proteins were centrifuged at 44,000 rpm (TLA-45 rotor) for 20 min at 25 °C prior to the assay to remove protein aggregates. Ten micrograms of each of the proteins was added to \sim 50 μ g of liposomes. The mixture was incubated at room temperature for 1 h and then centrifuged at 44,000 rpm (TLA-45 rotor) for 10 min at 25 °C. Pellets and supernatants were analyzed by SDS-PAGE.

Nuclear Magnetic Resonance Spectroscopy—NMR experiments were performed at 25 °C on Varian INOVA 600 and 500 MHz spectrometers equipped with triple resonance probes with z axis pulse field gradients. 15 N-labeled Rac1 was prepared by growing *E. coli* cells in M9 minimal media, with 15 NH₄Cl (1 g/liter) as the sole nitrogen source (9). Protein was purified by fast protein liquid chromatography over a Superdex S75 column (Amersham Biosciences). Titrations with PS/PC (30/70%) or PC (100%) large unilamellar vesicles were performed by recording a series of two-dimensional 1 H, 15 N-heteronuclear single quantum coherence spectra of Rac1 (100 μ M) at different liposome concentrations. All samples were prepared in 20 mM d₁₁-Tris-HCl buffer (pH 6.8), 100 mM NaCl, 1 mM sodium azide, and 1 mM dithiothreitol. Chemical shift assignments of Rac1 were taken from Thapar *et al.* (7).

Lipid Overlay Assay—Lipid strips were prepared by spotting 1 μ l of PS dissolved in chloroform:methanol:water (65:25:4) onto Hybond-C extra membranes (Amersham Biosciences). Membrane strips were incubated with 0.2 μ g/ml of full-length Rac1 or its mutants in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20, and 3% fatty acid-free bovine serum albumin

overnight at 4 °C. Following extensive washes with the same buffer, proteins bound to the membrane strips were probed with rabbit anti-GST antibody (Santa Cruz Biotechnology). Donkey anti-rabbit horseradish peroxidase antibody was obtained from Amersham Biosciences. Detection was carried out using ECL reagent (enhanced chemiluminescence, Pierce).

Preparation of Nucleotide Free and Bound Rac1—Proteins were first depleted of nucleotide for 15 min at room temperature in a buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM dithiothreitol, and 2 mM EDTA (6). Proteins were extensively washed with the same buffer without EDTA in 10-kDa concentrators (Millipore) and incubated in the presence of either GDP or GTP γ S (100 μ M) for 15 min at 37 °C under gentle agitation. Reactions were stopped with 1 mM MgCl₂. Binding to PS was carried out by the lipid-membrane overlay assay.

Surface Plasmon Resonance Spectroscopy—Surface plasmon resonance binding experiments were performed on a BIAcore 3000 instrument using L1 chips coated with \sim 0.5 mM mixed PS/PC (30/70%) size-calibrated liposomes. PC liposomes were used as a negative control. Prenylated and non-prenylated Rac1 or its mutants binding experiments were performed in a degassed solution containing 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 1 mM sodium azide. This buffer was used during equilibration, association, and dissociation phases. Proteins were added to this buffer at the indicated concentrations. Regeneration of the phospholipid bilayer after the dissociation phase was carried out using 20 mM NaOH. The data were globally fit using a two-state reaction model with mass transfer (BIAevaluation 3.0 software).

Loading PS into Cell Membranes and Immunofluorescence Staining—Lipid vesicles containing a mixture of 70% PC and 30% PS was prepared by extrusion as described above. Growing and transfection of Chinese hamster ovary-K1 (CHO-K1) cells were performed as described (10). Briefly, $4-8 \times 10^4$ CHO-K1 cells/well were grown in 6-well plates in 2 ml of F-12 media with serum. Cells were incubated at 37 °C in a CO₂ incubator until they are 50–80% confluent. For each well transfection, 0.5 μ g of DNA (pCS2+ (*myc*)₆-tagged Rac1 constructs) and 40 μ g of Lipofectamine reagent (Invitrogen) were combined and incubated for 5 h with F-12 media without gentamicin/serum. Following incubation, medium containing twice the normal concentration of serum was added without removing the transfection mixture. Cells were kept in this condition until Rac1 constructs were expressed (about 11 h). Fresh PC or PC/PS liposomes (100 μ M) in phosphate-buffered saline were added to *myc*-tagged Rac1 (or its mutants)-transfected CHO-K1 cells in a culture medium deprived of serum as reported (11). Briefly, following 1 h of incubation at 37 °C, free lipid vesicles were removed by washing the cells with serum-containing medium. Cells were then incubated in normal medium at 37 °C for 2 h to allow translocation of loaded PS from the outer leaflet to the inner leaflet (11). To detect intracellular localization of either Rac1 or its mutants, cell were fixed directly with 3.7% formaldehyde and stained with Cy3-conjugated anti-*myc* antibody as described (10). Nuclei and actin stress fibers were detected using 4',6'-diamino-2-phenylindol and Alexa 488-phalloidin (Molecular Probes), respectively. Flu-

orescent images were visualized by using a Nikon Eclipse TE300 microscope and captured with a charge-coupled device camera.

PAK1 CRIB Pulldown Assay—The activity of both Rac1 and Cdc42 were assessed using the p21-binding domain (CRIB) of p21-activated kinase 1 (PAK1) (12). Adherent Rac1-transfected CHO-K1 cells ($1-2 \times 10^7$) were stimulated either with PC or PC/PS liposomes as described above and lysed with cold lysis buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 200 mM NaCl, 1% Nonidet P-40, 5% glycerol, and a mixture of EDTA-free protein inhibitors) in the presence of 8 μg of GST-PAK1 CRIB for 30 min at 4 °C. The cell lysates were clarified by centrifugation at $13,600 \times g$ for 10 min at 4 °C. Equivalent amounts of protein in each supernatant were incubated with glutathione beads for 1 h at 4 °C. Bead pellets was then centrifuged for 2 min at $1,000 \times g$ at 4 °C and washed 3 times with 25 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, 30 mM MgCl₂, 40 mM NaCl, 1% Nonidet P-40, and protein inhibitors and twice with the same buffer without Nonidet P-40. Proteins were separated by SDS-PAGE, blotted, and detected using mouse anti-*myc* (for Rac1) or rabbit anti-Cdc42 (for Cdc42) antibodies.

Immunoblotting Analysis—After transfection with the Rac1 constructs, non-stimulated and PS-stimulated CHO-K1 cells were lysed in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% CHAPS, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaVO₄, and 1 μg/ml leupeptin. Extracts were sonicated and samples subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% Tween 20, incubated with primary antibody (anti-MEK1, anti-phospho-MEK1 (Ser²⁹⁸) from Cell Signaling; anti-*c-myc* and anti-actin from Santa Cruz) overnight at 4 °C, washed, and incubated with the appropriate secondary antibody for 2 h at room temperature. All horseradish peroxidase-coupled antibodies were from Amersham Biosciences. Detection was performed using ECL reagent.

Wound Healing Assay—Untransfected or transfected CHO-K1 cells with the constructs described above were seeded at high density (1.5×10^5 cells on coverslips of 13 mm diameter) and incubated for 24 h until a confluent monolayer was formed. Mytomycin C (10 μg/ml) (Sigma) was then added to inhibit cell proliferation. Wounding was performed by scraping through the cell monolayer with a pipette tip. Afterward, cells were washed with medium. Cells were induced by addition of either PC or PS/PC liposomes as described above, or lipophosphatidic acid (Avanti Lipids) as a positive control. Individual fields of the coverslip were photographed at 20 h after wounding in a Zeiss Axiovert 200M microscope.

RESULTS AND DISCUSSION

We first investigated whether Rac1, the most representative member of Rho GTPases in eukaryotes, binds phospholipids that are predominantly present at the plasma membrane. We employed a liposome binding assay to identify specific lipid ligands. We found that Rac1 preferentially interacted with PS-containing bilayers as opposed to other anionic phospholipids (Fig. 1A), but no Rac1 association with other phospholipids or

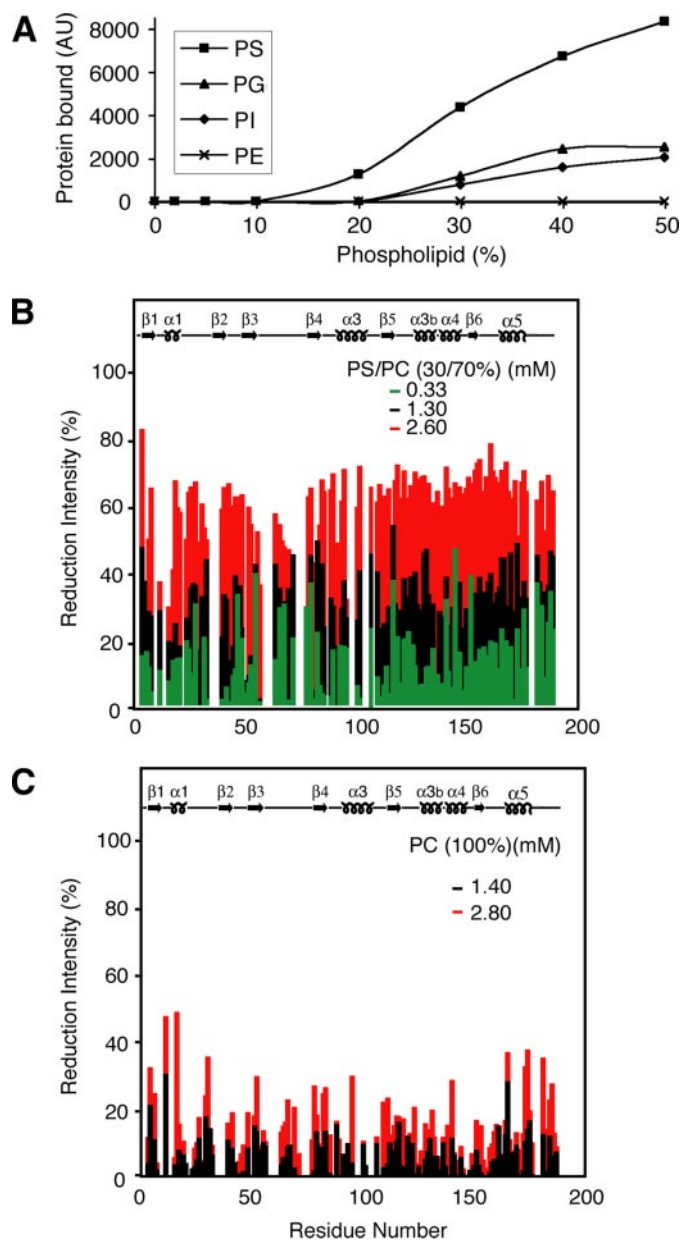


FIGURE 1. Rac1 binds to PS through its PBM. A, analysis of the phospholipid binding properties of Rac1 was investigated by a liposome-binding assay. Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol. B, heteronuclear single quantum coherence experiments of ¹⁵N-labeled Rac1 were performed after addition of 0.33, 1.30, and 2.6 mM PS/PC (30/70%) liposomes and subsequent signal intensity reductions of backbone NH resonance are shown for each residue. The secondary structure of the protein is indicated above the histogram (42). C, heteronuclear single quantum coherence spectra of ¹⁵N-labeled Rac1 were collected after 1.4 and 2.8 mM PC (100%) liposomes were added, and the resulting line broadening of each NH signal residue upon binding is shown in the histogram.

sphingolipids was detected (Fig. 1A and data not shown). The binding of Rac1 to PS was independently verified by assaying for vesicle binding using NMR spectroscopy. Stepwise addition of large unilamellar vesicles composed of 30% dipalmitoyl PS and 70% dipalmitoyl PC to the ¹⁵N-labeled Rac1 sample caused broadening of the amide NMR signals, indicating the formation of a slower tumbling complex (Fig. 1B). On the other hand, addition of PC alone did not induce comparable line broadening (Fig. 1C), confirming the requirement of Rac1 to bind to PS.

Phosphatidylserine Recognition by Rac1

Interestingly, addition of equimolar concentrations of the soluble dicaproyl PS (diC₆PS) to the Rac1 sample did not induce significant changes in its ¹⁵N-¹H NMR signals. However, when diC₆PS binding was tested above its critical micellar concentration, significant line broadening of the amide signals was evident (data not shown). These observations indicate an essential role for presenting PS within the context of a micellar or bilayer environment for Rac1 interaction. This may be provided *in vivo* by the plasma membrane, which contains 67% of the total available PS being enriched in the inner leaflet (13). Because Rac1 signals from the plasma membrane, our results suggest that this protein directly interacts with PS-enriched regions where it may direct PS-dependent cellular processes.

The preference of Rac1 for PS suggested that other members of the Rho family GTPases might also bind to this phospholipid. However, we found that bacterially expressed forms of Cdc42, RhoA, Rac2, and -3 did not associate with PS under the same experimental conditions (Fig. 2A). Plasma membrane recruitment of Rac1 is driven by both the lipid modification at the Cys¹⁸⁹ and the PBM (14). To study whether isoprenylation influences PS binding, we purified native Rho proteins from Sf9 membranes (15). Prenylated Rac1 and to a lesser extent Cdc42, but not other Rho proteins, interacted with PS-containing liposomes (Fig. 2A). In addition, substitution of a Ser residue for Cys¹⁸⁹ reduced but did not eliminate Rac1 binding to PS liposomes (Fig. 2A). Thus, the interaction of Rac1 with bilayers containing PS benefits from but does not require prenylation.

Rho GTPases share a high level of homology in their amino acid sequence, despite their different effects on cell morphology and signaling. The C-terminal PBM is the most divergent region, with Rac1 presenting the most basic PBM sequence among Rho GTPases (Fig. 2B). Because PS is negatively charged, a likely candidate for direct interaction is the PBM near the CAAX box. Mutation of C-terminal residues Lys¹⁸⁶ or Arg¹⁸⁷ to Ala of Rac1 in the non-isoprenylated protein significantly reduced PS binding (Fig. 2C). Both mutations combined (K186A/R187A) abolished the interaction, producing the same effect as the deletion of the C-terminal region of the protein (Fig. 2C), and confirming the essential role of these residues in PS binding. Liposome binding assays confirmed the requirement of an intact PBM (Fig. 2C). Mutations at the PBM did not perturb GTP loading as tested by the PAK1 CRIB pull-down assay (Fig. 2D), and did not affect the overall structure (data not shown). Because Lys¹⁸⁶ and Arg¹⁸⁷ residues are conserved in Rac2, the results suggest that although they are essential, other residues are also involved in PS recognition. By analogy, plasma membrane recruitment of the Ras protein KRas4B is dependent on the net charge of its highly basic PBM (5). Thus, it is plausible that a general mechanism occurs, in which basic PBM residues, such as Lys¹⁸³, Lys¹⁸⁴, Arg¹⁸⁵, Lys¹⁸⁶, Arg¹⁸⁷, and Lys¹⁸⁸ of Rac1, form electrostatic interactions with cluster of anionic phospholipids in the plasma membrane.

To explore the regulatory role of GTP, the PS interactions of the nucleotide-bound and depleted Rac1 protein were compared. As shown in Fig. 3A, nucleotide-free Rac1 recognized PS to the similar extent as the nucleotide-bound form. This result was supported by demonstration that Rac1 mutants that are either constitutively activated (Q61L) or dominant negative

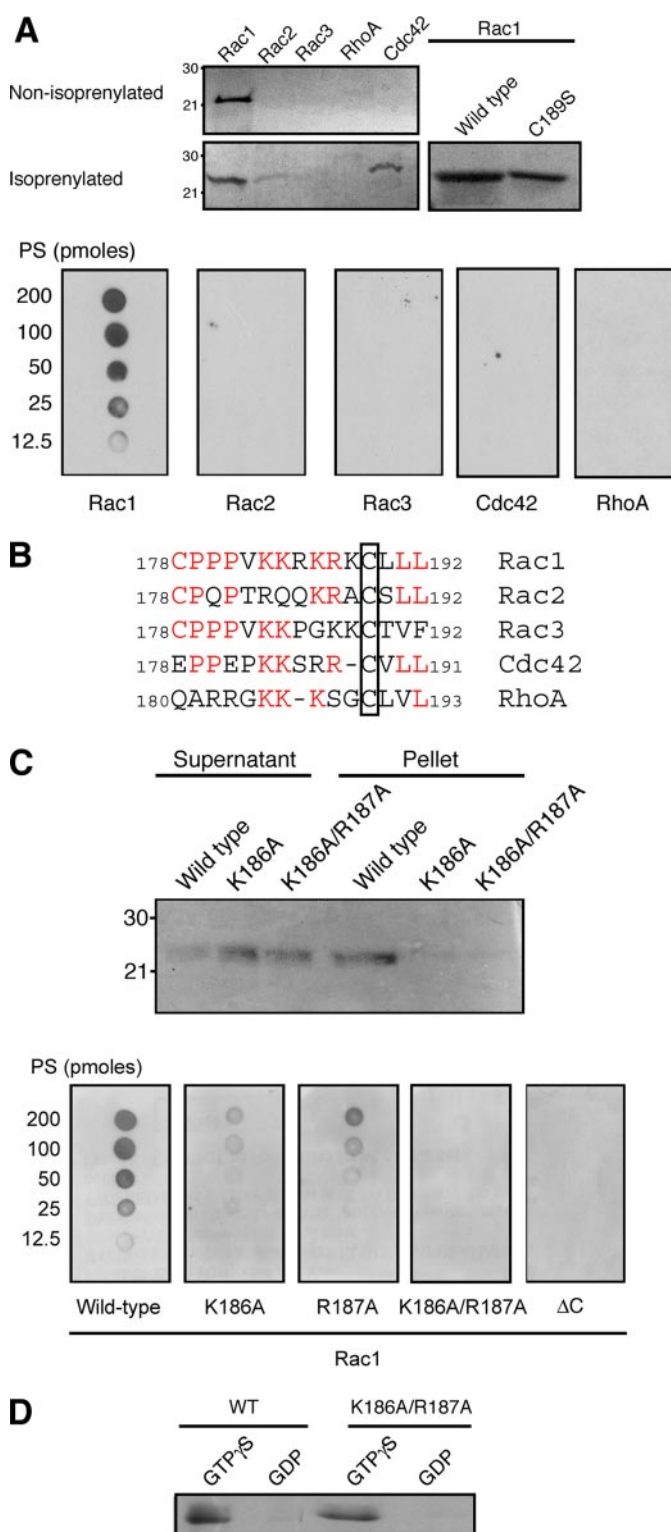


FIGURE 2. PS recognition by Rho GTPases. A, Rho proteins from bacteria (upper panel) and Sf9 membranes (lower panel) were incubated with PS/PC liposomes (top). The liposome-bound (as shown) and soluble protein were separated by ultracentrifugation. A similar approach was carried out by the lipid overlay assay (bottom). Strips with different amounts of PS were spotted on Hybond-C extra and different bacterial expressed GST-Rho proteins were tested for binding. B, alignment of the C termini of the human Rho family sequences. The most conserved residues are colored red and the isoprenylated cysteine is boxed. C, binding of the wild type and mutants of Rac1 to PS was monitored by the liposome binding assay (top). Mutants of Rac1 were also tested by the lipid overlay assay (bottom). D, GTP loading of the wild type and PS-binding mutant forms of Rac1 was tested by the PAK1 CRIB pull-down assay.

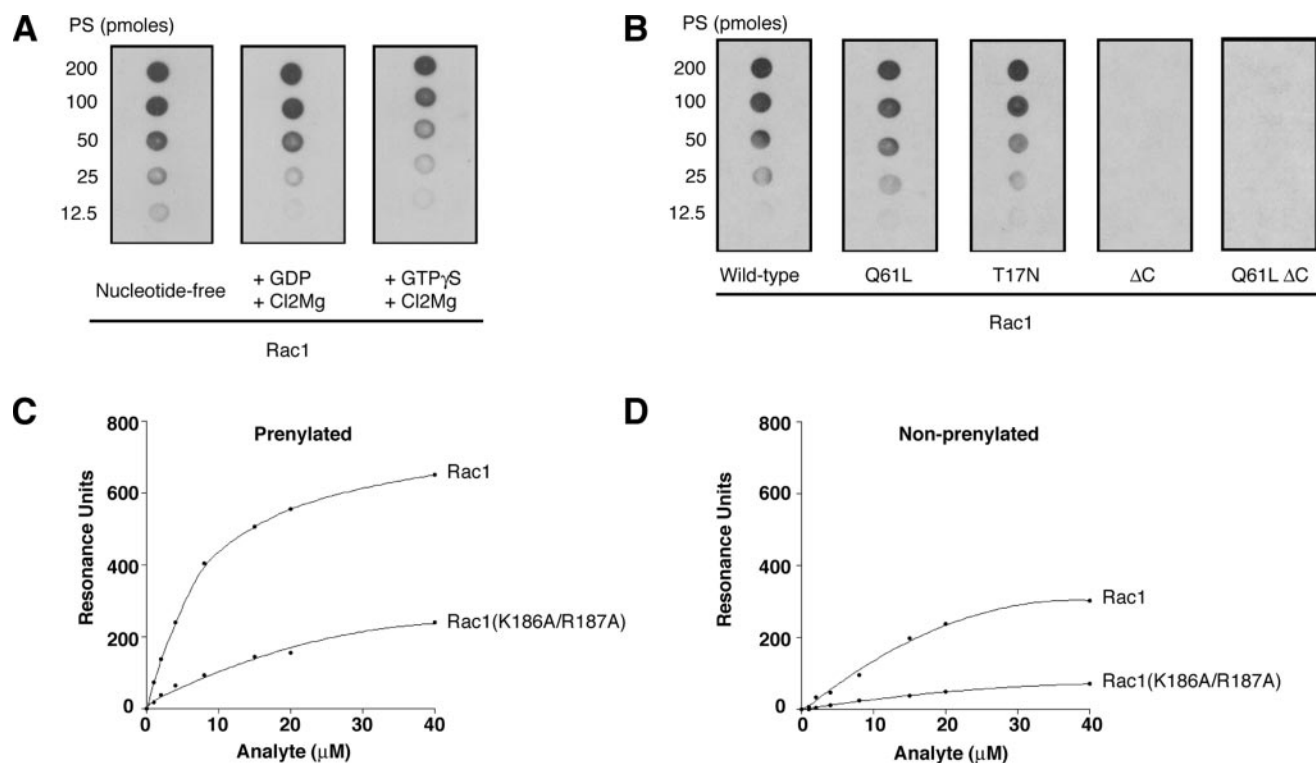


FIGURE 3. **PS binding properties of Rac1.** A, nucleotide-free, GDP- and GTP-bound GST-Rac1 proteins were tested for PS binding using the lipid overlay assay. B, Q61L, T17N, Δ C, and Q61L Δ C Rac1 mutants were also tested by the PS-bound lipid overlay assay. C and D, the interactions of isoprenylated wild type and K186A/R187A (C) and non-isoprenylated wild type and K186A/R187A Rac1 proteins (D) with immobilized PS/PC liposomes were analyzed by surface plasmon resonance detection with the resonance units (RU) indicating the bound protein fraction at increasing Rac1 concentrations.

TABLE 1

Kinetic analysis of PS recognition by Rac1

Binding constants for the association of Rac1 to PS-containing bilayers as estimated by surface plasmon resonance detection. χ^2 is a measure in resonance units of the average standard deviation between the fitted curve and experimental data. Analysis of the data was obtained using the BIAevaluation 3.0 software (BIAcore) using the two-state reaction model with mass transfer. This analysis is an average of two independent experiments.

Rac1	Isoprenylation	k_{a1} $M^{-1} s^{-1}$	k_{d1} s^{-1}	k_{a2} $M^{-1} s^{-1}$	k_{d2} s^{-1}	K_D M	Fit χ^2	Change
Wild type	+	$1.1 \pm 0.6 \times 10^3$	$1.2 \pm 0.01 \times 10^{-2}$	$2.2 \pm 0.04 \times 10^{-3}$	$2.3 \pm 0.8 \times 10^{-6}$	1.1×10^{-8}	5.8	1
K186A/R187A	+	$4.1 \pm 2.2 \times 10^2$	$7.6 \pm 0.48 \times 10^{-2}$	$4.7 \pm 0.10 \times 10^{-2}$	$1.7 \pm 0.04 \times 10^{-3}$	6.7×10^{-6}	2.6	613
Wild type	-	$9.1 \pm 1.0 \times 10^2$	$1.8 \pm 0.01 \times 10^{-2}$	$1.2 \pm 0.01 \times 10^{-2}$	$1.2 \pm 0.03 \times 10^{-3}$	1.9×10^{-6}	6.1	177
K186A/R187A	-	$5.6 \pm 1.2 \times 10^2$	$6.7 \pm 0.31 \times 10^{-2}$	$3.5 \pm 0.20 \times 10^{-3}$	$6.1 \pm 0.42 \times 10^{-3}$	2.1×10^{-4}	10.7	19160

(T17N) bound PS similarly to the wild type protein (Fig. 3B). This is in agreement with the observation that the GTPase activity of Rac1 is independent of the presence of PS (16). Moreover, deletion of the last eight residues in Q61L Rac1 (Q61L Δ C) abrogated PS binding (Fig. 3B), indicating that Rac1 activation is not sufficient for PS association. Likewise, GTP loading appears to be dispensable for the phospholipid binding by the Cdc11 GTPase (17). Thus, recruitment to PS-containing membranes may control the activities of Rac1 through co-localization with its effectors rather than through GTP-dependent regulation of its lipid interactions.

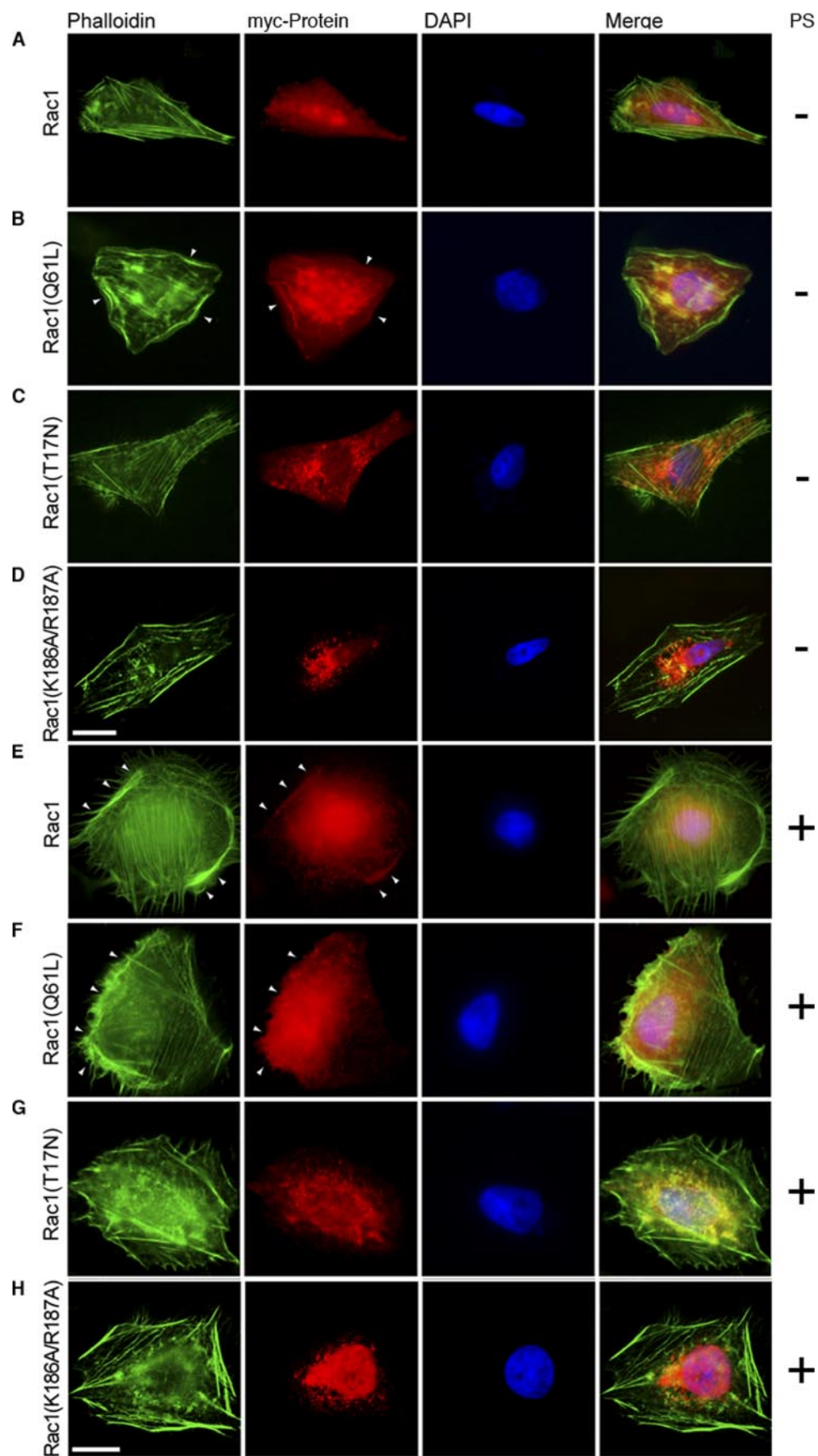
The kinetics of the interaction between Rac1 and PS were investigated by surface plasmon resonance detection. All Rac1 proteins displayed reversible binding to immobilized PS/PC liposomes (data not shown). Prenylated Rac1 showed the strongest binding, with an estimated dissociation constant (K_D) of 1.1×10^{-8} M (Fig. 3C and Table 1). This affinity is similar to that reported for the PS binding of the Src protein, in which both myristoylation and polybasic regions

mediate phospholipid recognition (18). Simultaneous mutation of the two basic residues in Rac1 reduced the affinity over 600-fold, whereas unmodified wild type Rac1 had 177 times lower affinity than prenylated protein (Fig. 3, C and D, and Table 1). The role of the PBM in membrane binding is also seen in Src, because addition of PS to neutral membranes increases its affinity by 3 orders of magnitude (19). Thus, the hydrophobic and electrostatic interactions of Rac1 with PS-containing bilayers may be synergistic, with electrostatic contacts playing a dominant role.

Rac1 shares the G-domain fold common to all Rho GTPases, which consists of a six-stranded β sheet flanked by six α helices (20). The PBM forms an exposed and apparently unstructured extremity of the folded domain. Based on our CD and NMR data, which do not indicate major structural changes, we propose that the flexible C-terminal element of Rac1 is the primary mediator of the interaction with PS-containing bilayers.

Rho proteins regulate the actin cytoskeleton at different levels. Whereas RhoA regulates focal adhesion assembly and

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actin stress fiber formation, Cdc42 triggers the formation of filopodia and focal complexes in the cell periphery and the direction of the migration; and Rac induces membrane ruffling and lamellipodia formation at the plasma membrane upon growth factor stimulation (21). In CHO-K1 cells, Rac1 was diffusely distributed in the cytosol (Fig. 4A), in agreement with previous observations (22). The activated Q61L Rac1 induced robust localization to and ruffling of the plasma membrane, although some of the protein was also cytoplasmic (Fig. 4B). In contrast, T17N Rac1 was only cytoplasmic (Fig. 4C). Interestingly, mutation in the PS-binding site of Rac1 induced a punctuate cytoplasmic distribution of the protein, suggesting vesicular compartmentalization (Fig. 4D). This observation is consistent with the proposal that the CAAX motif targets proteins to endomembrane systems, whereas the additional presence of the PBM provides plasma membrane localization (23).

Given the PS recognition by Rac1, we examined the subcellular distribution of the Rac1 constructs upon PS stimulation. Loading of Rac1-transfected cells with PS induced partial translocation of Rac1 to the membrane and stimulated the formation of membrane ruffles, prominent filopodia structures, and reduction of actin stress fibers (Fig. 4E), reminiscent of the morphological changes in cells transfected with activated Q61L protein (Fig. 4F). Formation of the membrane ruffles is indicative of Rac1 activation (Fig. 4, B and F) (21). Moreover, appearance of filopodia after PS stimulation suggests a PS-dependent activation of Cdc42, in agreement with the observation that prenylated Cdc42 binds PS liposomes *in vitro* (Fig. 2A). Overexpressed *myc*-transfected cells showed no translocation of this protein to the plasma membrane after PS stimulation (supplemental Fig. S1). Membrane translocation of endogenous Rac1 was also observed after PS stimulation of CHO-K1 cells (supplemental Fig. S1) confirming the observation that redistribution of PS in the cell promotes targeting of Rac1 to these compartments. Stimulation of K186A/R187A Rac1-transfected cells with PS showed no plasma membrane translocation, instead the protein remained mainly in the cytoplasmic puncta (Fig. 4H). Overexpression of the isolated PBM of Rac1 co-localizes with lipid-enriched regions in HL60 cells (24), supporting the idea that integrity of the PBM is critical for Rac1 targeting to PS-enriched regions at the plasma membrane. Nevertheless, PS stimulation of cells harboring the PS-binding mutant induced less prominent filopodia but no membrane ruffles, similar to inactive Rac1 distribution (Fig. 4G). Thus, we propose that the Rac1-PS interaction is involved in cytoskeletal rearrangements characteristic of migratory cells.

Activated Rac1 interacts with several downstream effectors and elicits various cellular responses. When bound to

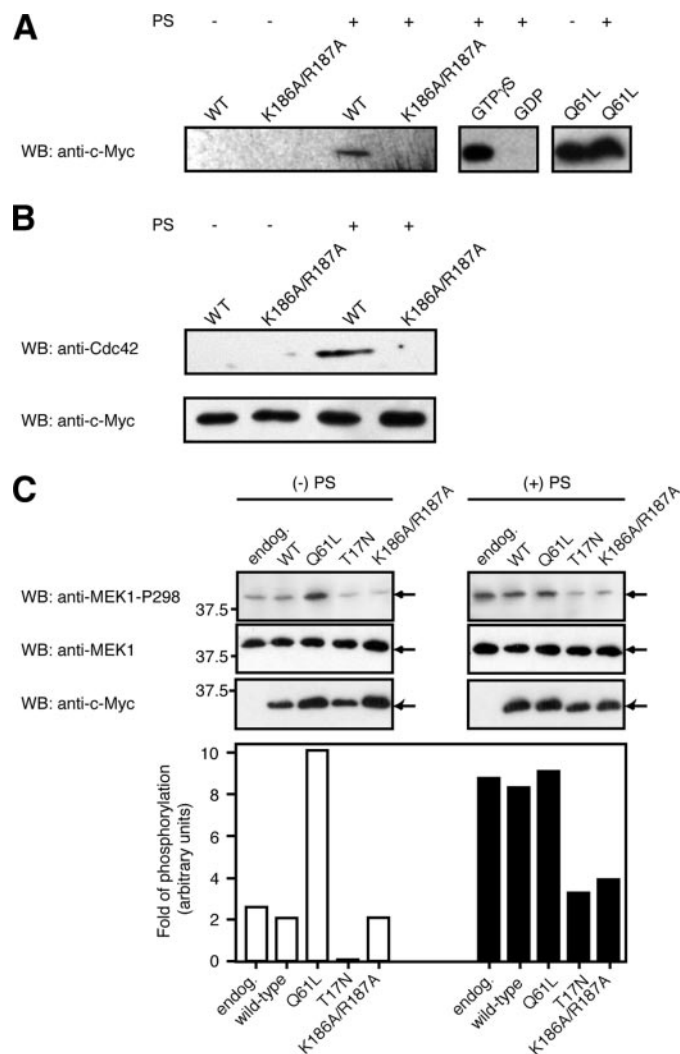


FIGURE 5. PS-stimulated CHO-K1 cells induce GTP loading and MEK-1 phosphorylation. A, PAK1 CRIB pull-down assay of lysates of Rac1 (wild type (WT) or K186A/R187A mutant form) transfected CHO-K1 cells with or without PS stimulation. Controls using GTP- γ S or constitutively activated Q61L Rac1 are shown on the right. B, PAK1 CRIB pull-down assay of the transfected cells described above were monitored for Cdc42 activation. C, CHO-K1 cells were transfected with Rac1 constructs, lysed, and immunoblotted with antibodies to MEK1 and phospho-MEK1 (Ser²⁹⁸) (left panels) or transfected as before and further stimulated with PS/PC liposomes (right panels). Densitometry of the phosphorylated proteins is shown below. The quantification is representative of two independent experiments. WB, Western blot.

GTP, Rac1 induces phosphorylation of PAK1 (25), which in turn phosphorylates Thr²⁹² and Ser²⁹⁸ of MEK1 *in vivo* (26). Stimulation of CHO-K1 cells with PS induced GTP loading into Rac1 as monitored by the PAK1 CRIB pull-down assay (Fig. 5A). This suggests that PS promotes the activation of Rac1 by guanine nucleotide-exchange (GEF) proteins. Acti-

FIGURE 4. Loading PS into cell membrane induces changes in Rac1 localization and stimulates filopodia and membrane ruffles formation. CHO-K1 cells transfected with vectors expressing wild-type (*myc*)₆-tagged Rac (or its mutants) were immunostained with anti-*myc* antibody (red). Phalloidin (green) stained actin stress fibers, filopodia, and membrane ruffles. Nuclei were stained with 4',6-diamino-2-phenylindol (DAPI) (blue). The merge reveals the co-localization of Rac1 with the actin-related subcellular compartments. A, Rac1 is predominantly cytosolic. B, Rac1 (Q61L) induced a morphological change and localized to membrane ruffles (white arrowheads) at the plasma membrane. C, Rac1 (T17N) was cytosolic and punctuate around the nucleus. D, Rac1 (K186A/R187A) was punctuate around the nucleus. E-H, CHO-K1 cells were stimulated with PS/PC liposomes. E, Rac1-transfected cells with membrane ruffles (white arrowheads) and filopodia formation. F, subcellular localization of Rac1 (Q61L) after PS loading showed membrane ruffles (white arrowheads) and filopodia formation. Note the accumulation of the protein in the direction of the membrane ruffles. G, Rac1 (T17N) was cytosolic, punctuate, and filopodia are less evident upon PS stimulation. H, Rac1 (K186A/R187A) was still punctuate and no obvious ruffles and few filopodia are visible. A-D, bar, 10 μ m. E-H, bar, 20 μ m.

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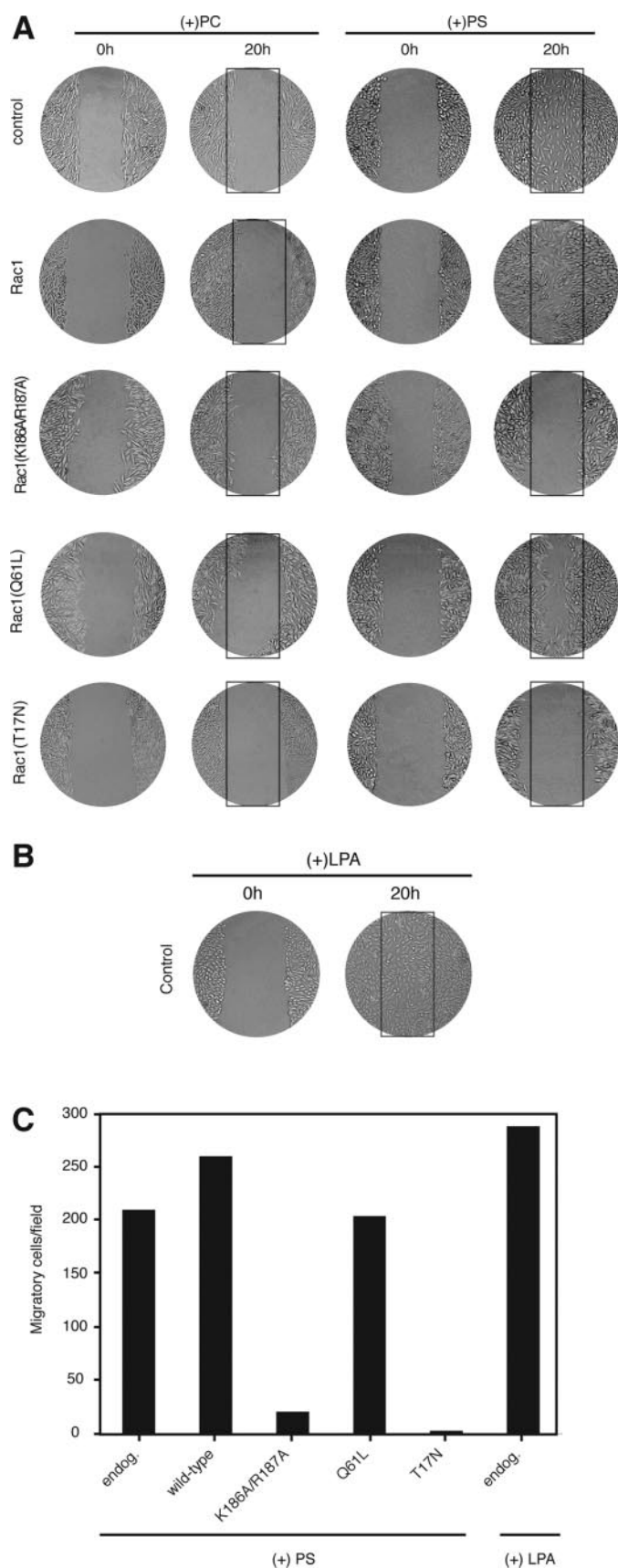


FIGURE 6. PS-induced cell migration. *A*, untransfected or transfected CHO-K1 cells with wild type, Q61L, T17N, and K186A/R187A Rac1 constructs were stimulated with either PC (*left panels*) or PS/PC liposomes (*right panels*), wounded, and photographed at 0 and 20 h after wounding. *B*, lysophospha-

tidic acid-induced cell migration. Untransfected CHO-K1 cells were stimulated with lysophosphatidic acid and treated as described in *A*. *C*, migratory cells in each of the panels were counted blindly in one field.

tion of Cdc42 by Rac1/PS signaling was also investigated. PS stimulation of Rac1-transfected CHO-K1 cells led to the formation of Cdc42-GTP, as monitored by the PAK1 CRIB pulldown binding assay (Fig. 5*B*). In contrast, mutation of Rac1 PS binding sites abrogated Cdc42 activation (Fig. 5*B*). These results correlate with the ability of prenylated Cdc42 to bind PS liposomes *in vitro* (Fig. 2*A*) and filopodia formation of Rac1-transfected PS-stimulated cells (Fig. 4, *E* and *F*). Thus, we propose that redistribution of PS promotes the activation of both Rac1 and Cdc42 GTPases.

We next examined MEK1 activation by Rac1/PS signaling. We found that only the Q61L Rac1 mutant induced substantial phosphorylation of endogenous MEK1 in naïve CHO-K1 cells consistent with previous observations (Fig. 5*C*) (26). A pulse of PS in these cells induced phosphorylation of MEK1 only when they were transfected with wild type or activated Q61L protein, but not with the inactive T17N Rac1 or the PS-binding mutant form. Thus, we propose that Rac1/PS/PAK1 signaling leads MEK1 phosphorylation, which is necessary for cell migration. Untransfected cells also showed phosphorylation of MEK1 after stimulation of PS, suggesting that higher levels of both T17N and K186A/R187A mutants exert a dominant negative effect over the endogenous Rac1 protein for MEK1 activation.

The effects of the Rac1 mutations on endogenous Rac1 activity can be explained based on observations taken from Ras studies (27). The S17N Ras, which acts similarly as T17N Rac1, is able to bind to GEFs and therefore competes with endogenous Ras for binding to these factors. Because S17N is in excess over the endogenous Ras and cannot interact with downstream proteins, it generates a dead-end by sequestering GEFs from endogenous Ras. Subcellular sequestration is another mechanism that regulates GEF activity (28). Because the Rac1 PBM is not critical for GEF binding (29), we propose that the K186A/R187A Rac1 mutant sequesters GEFs in the cytosol. This mutation on Rac1 impairs PS binding (Fig. 2*C*) and reduces GTP loading by GEFs (Fig. 5*A*). Thus, the K186A/R187A Rac1-GEF complex prevents GTP binding by the PS-stimulated endogenous Rac1. As Rac1/PAK signaling modulates MEK1 phosphorylation (30), sequestration of GEFs by the PS-binding Rac1 mutant prevents endogenous Rac1-mediated MEK1 activation.

Similar to Rac1, the Raf-1 kinase binds to PS-containing liposomes through its cysteine-rich domain (31). Raf-1 is able to act synergistically with both Rac and Cdc42 to activate the extracellular signal-regulated protein kinases (ERKs) through mechanisms involving PAK1 phosphorylation of MEK1 at Ser²⁹⁸ (32). Surprisingly, the PS-dependent MEK1 signaling does not appear to be ERK-related, because no PS-dependent phosphorylation of ERK1/ERK2 could be detected (data not shown). We envision at least three possible scenarios that would explain our observations. First, sub-

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cellular re-localization triggered by PS/Rac1 signaling may impede MEK-dependent ERK activation by altering segregation of either component. Second, ERK phosphorylation exists but is transient, and it is due to the activation of a specific PS/Rac1-dependent phosphatase. Last, substrates other than ERK are targeted by MEK in a Rac1/PS-dependent fashion. In this regard, mitotic-specific activation of MEK1 has been shown to be uncoupled from ERK activation by a regulatory mechanism mediated by cyclin B/Cdc2 (33). Alternative MEK1-dependent, ERK-independent mechanisms have been also suggested in both insulin stimulation of the SOS protein kinase (34) and during the hyperosmotic response in Hep-G2 cells (35).

Given the role of Rac1 in cell migration (21), we tested whether CHO-K1 cell migration into wounds was stimulated by PS (Fig. 6, A and C). Cell migration control was monitored by stimulation with the well established chemotactic agent lysophosphatidic acid (Fig. 6B) (36). The transfection of wild type and Q61L Rac1 proteins similarly induced migration of CHO-K1 cells into wounds (Fig. 6, A and C). Interestingly, activated Q61L Rac1 did not close wounds in the absence of phospholipid stimulation (Fig. 6, A and C). This is in agreement with the hypothesis that PS activates Cdc42 to stimulate filopodia formation, which is required for cell migration (21). Thus, loading of GTP into Rac1 is not sufficient for cell migration and PS may be needed to recruit Rac1 to specific regions at the plasma membrane for migratory signaling. In contrast, expression of mutant Rac1, which is either compromised in PS interaction (K186A/R187A) or constitutively inactive (T17N), blocked cell migration (Fig. 6, A and C). This result concurs with the observation that overexpression of the isolated PBM inhibits cell migration (24). Thus, the cell migratory function of Rac1 is PS-dependent and is mediated by its unique PBM.

Membrane phospholipids such as PS play important roles not only structurally but also as signal transducers both inside and outside of cells. The distribution of PS is determined by the action of specific transporters that promote the active shuttling of PS across of the membrane bilayer. In addition to the well known role of PS in early stages of apoptosis (37), transient PS redistribution at the plasma membrane has been reported in other circumstances, including modulation of the activities of membrane proteins in lymphocytes (38), sperm capacitation (39), and blood coagulation cascade (40). Moreover, it has been recently reported that the PS-binding protein tyrosine phosphatase-MEG2 is transiently translocated to the plasma membrane after PS stimulation (11), reflecting the PS redistribution. As Rac1 GTPase activity is independent of PS (16), it is plausible that PS recognition by Rac1 exerts a regulatory function by subcellular localization rather than nucleotide exchange. In this study, we provide evidence that PS recognition by the PBM of activated Rac1 is required for cell morphological changes that underpin cell migration. Because the role of Rac1 in cancer involves stimulation of cell migration (41), further mechanistic studies of PS-mediated functions of Rac1 may provide new opportunities to inhibit metastasis.

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