The Interaction of Src and RACK1 Is Enhanced by Activation of Protein Kinase C and Tyrosine Phosphorylation of RACK1*

Received for publication, February 13, 2001
Published, JBC Papers in Press, March 8, 2001, DOI 10.1074/jbc.M101375200

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RACK1 is an intracellular receptor for the serine/threonine protein kinase C. Previously, we demonstrated that RACK1 also interacts with the Src protein-tyrosine kinase. RACK1, via its association with these protein kinases, may play a key role in signal transduction. To further characterize the Src-RACK1 interaction and to analyze mechanisms by which cross-talk occurs between the two RACK1-linked signaling kinases, we identified sites on Src and RACK1 that mediate their binding, and factors that regulate their interaction. We found that the interaction of Src and RACK1 is mediated, in part, by the SH2 domain of Src and by phosphotyrosines in the sixth WD repeat of RACK1, and is enhanced by serum or platelet-derived growth factor stimulation, protein kinase C activation, and tyrosine phosphorylation of RACK1. To the best of our knowledge, this is the first report of tyrosine phosphorylation of a member of the WD repeat family of proteins. We think that tyrosine phosphorylation of these proteins is an important mechanism of signal transduction in cells.

The Src family of intracellular protein-tyrosine kinases participates in diverse signaling pathways that regulate cell growth, differentiation, adhesion, and architecture (reviewed in Ref. 1). Identification of Src-binding proteins has led to better understanding of Src regulation and has provided clues about the function of Src in normal and transformed cells. For example, characterization of the interaction between Src and polypodia middle T antigen led to discovery of a fundamental mechanism by which the cellular Src protein is converted to a transforming protein (by dephosphorylation at Tyr-527) and defined the requirement of Src for polyoma transformation (2–7). Thus, characterization of a single Src-binding protein contributed substantially to our understanding of both RNA and DNA tumor biology.

Recently, using the unique domain/SH2/SH3 domain of Src as bait, and a human lung fibroblast cDNA library as prey, we identified RACK1, a known intracellular receptor for activated C kinase (RACK), as a Src-binding protein (8). We found that overexpression of RACK1 inhibited the specific activity of Src tyrosine kinases (as measured in vitro) and the growth of NIH 3T3 cells. RACK1 exerted its effect on growth, in part, by prolonging the G0/G1 phase of the cell cycle.

RACK1 was the first of a group of proteins (collectively called RACKs) to be identified and characterized by Mochly-Rosen and co-workers (reviewed in Refs. 9–11). RACK1 has sequence homology with the β subunit of heterotrimeric G proteins. RACK1 and Gβ are both members of an ancient family of regulatory proteins made up of highly conserved repeating units usually ending with Trp-Asp (WD) (reviewed in Refs. 12 and 13). WD repeat proteins are functionally diverse, although all seem to be regulatory and few are enzymes. The WD repeats in RACK1 are conserved from Chlamydomonas to human (reviewed in Ref. 13). Thus, the function of RACK1 was probably fixed before the evolutionary divergence of plants and animals.

Protein kinase C (PKC) is a family of serine/threonine kinases whose activity depends upon phospholipid, diacylglycerol, and in some cases on calcium (reviewed in Refs. 9–11 and 14). Upon stimulation with tumor promoter phorbol esters or hormones that increase intracellular concentrations of diacylglycerol, PKCs become activated and translocate to new subcellular sites where they phosphorylate isozyme-specific substrates. Individual, activated, PKC isozymes are translocated to distinct compartments, suggesting that they mediate distinct cellular functions (9–11, 15).

RACKs interact only with activated forms of PKCs, suggesting that PKC binding to RACK occurs after cell stimulation, to localize the active enzyme to the RACK site (reviewed in Refs. 9–11). Moreover, there are isozyme-specific RACKs, which presumably anchor each PKC isozyme close to its physiologic substrate. For example, in cardiac myocytes RACK1 is specific for βIIIPKC, whereas RACK2 is specific for εPKC (15–17). Thus, it appears that the specificity of PKC function may be determined, in part, by the different locations of isozyme-specific RACKs.

The observation that RACK1 interacts with two, distinct, cytoplasmic protein kinases raises interesting questions about the role of RACK1 in orchestrating the intersection of tyrosine and serine/threonine kinase signaling pathways. The purpose of this study was to further characterize the Src-RACK1 interaction and to begin to analyze the mechanism by which cross-talk occurs between two RACK1-linked signaling protein kinases. We found that the interaction of Src and RACK1 is mediated by the SH2 domain of Src and phosphotyrosines in the sixth WD repeat of RACK1, and is enhanced by serum, PDGF stimulation, PKC activation, and tyrosine phosphorylation of RACK1.
**EXPERIMENTAL PROCEDURES**

**Cell Culture—** NIH 3T3 cells overexpressing the β-platlet-derived growth factor (PDGF) receptor (a gift from Sara Courtneidge, Sugen, San Francisco, CA; Ref. 18) or wild-type or Y527F chicken c-Src (6) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% calf serum (Sigma), and maintained in G418 (200 mg/ml) (Life Technologies, Inc.). CHO cells (American Type Culture Collection [ATCC], Rockville, MD) were cultured in Ham’s F-12 medium (Mediatech) supplemented with 10% fetal bovine serum (FBS) (Sigma). HeLa cells (ATCC) were cultured in DMEM supplemented with 10% FBS.

**Plasmids—** pGEX-3X plasmids containing the SH2 domain(s) of phospholipase Cγ1 (PLCγ), Shp-2, Shp-1, p85, Ab1, Grb2, rasGAP, Csk, or Src were gifts from Lewis Cantley (Harvard University, Boston, MA) (19). pGEX-3X-RACK1, pGEX-3X-GRK, and pGEX-3X-OGF were constructed as described (8). pGEX plasmids were used for transient protein expression assays. pGEMc, a gift from Tony Hunter (Salk Institute, La Jolla, CA), was used to generate in vitro translated Src (8).

**Antibodies—** For Src antibodies, 1) monoclonal antibody (mAb) 327 (21) was used (unless otherwise stated) for immunoprecipitation and immunoblot analyses; 2) anti-peptide antibody N16 or R7) and protein complexes were collected with the addition of 30 mM NaCl, and 20 mM glutathione and dialyzed four times against TBS.

**GST Fusion Proteins—** Purified GST fusion proteins (1–5 mg) were obtained from Pansorbin, (immobilized glutathione beads, washed four times in buffer containing 0.5% Nonidet P-40, 20 mM Tris, pH 8.0, 100 mM NaCl, and 1 mM EDTA) and boiled in sodium dodecyl sulfate (SDS) sample buffer. Proteins were resolved by SDS-PAGE and detected by fluorography (see above). GST fusion proteins were eluted by the addition of 100 mM Tris, pH 8.0, 120 mM NaCl, and 20 mM glutathione and dialyzed four times against TBS.

**Fluorescence Polarization Assay—** Samples were excited at 485 nm and emission was detected at 535 nm using an Accumet microscope (Analytical Spectral Devices, Boulder, CO) equipped with a 40× objective lens.

**Immunoprecipitation Assays—** For immunoprecipitation assays, 1) monoclonal antibody (mAb) 327 ascites (2 mg/ml), affinity-purified mAb 327 ascites (2 mg/ml), mAb PY20 (0.8 mg/ml), or anti-peptide antibody R7, was incubated with GST fusion proteins for 3 h at 4 °C as described (8). Briefly, 2 × 10⁷ cells were seeded in six-well plates in Ham’s F-12 medium containing 10% FBS. 24 h later, transfections were performed using 0.5–1 µg of plasmid DNA and 10 µl of Lipofectamine in serum-free media. 5 h later, cells were placed in fresh media containing 10% FBS. Cells were lysed 48 h after transfection.

**Protein Extractions, Immunoprecipitations, and In Vitro Protein Kinase Assays—** Cells were washed three times with ice-cold TBS and lysed in modified RIPA buffer (0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 10 mM sodium phosphate, pH 7.0, 100 µM sodium fluoride, and 1 µg/ml leupeptin, 1% aprotinin, 2 mM EDTA, and 1 mM dithiothreitol) (6, 8, 23–27). Lysates were centrifuged at 14,000 × g for 1 h at 4 °C. Protein concentrations were measured by the BCA protein assay (Pierce), and samples were standardized to equal amounts of total cellular protein (6, 8, 23–27). Lysates were incubated for 3 h at 4 °C with excess antibody (1 µg of mAb 327 or PY20, or anti-peptide N16 or R7) and protein complexes were collected with the addition of 30 µl of protein A/G-Sepharose beads (Amersham Pharmacia Biotech). Protein kinase assays were performed by incubating mAb 327 immunoprecipitates (of Y527F Src-overexpressing NIH 3T3 cell lysates) for 10 min at 30 °C in 0 µl of kinase buffer containing 50 mM piperezine-N,N’-bis (2-ethanesulfonic acid), pH 7.0, 10 mM magnesium chloride, 10 mM dithiothreitol, 1 µg of GST-RACK1 or GST, and, where indicated, 1 mM ATP [α-32P] (6, 8, 23–27). Lysates were detected by autoradiography. Protein complexes were collected with the addition of radiolabeled, in vitro translated SRC (see below). Immunoprecipitation Assays—Src or PY20 immunoprecipitates were resolved on 10% SDS-polyacrylamide gels (acylamide-bisacrylamide, 29:0.8). Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) in transverse buffer containing Tris-HCI, pH 7.4, 192 mM glycine, and 15% methanol using a Trans-Blot apparatus (Bio-Rad) for 2 h at 60 V (6, 8, 23–27). Protein binding sites on the membranes were blocked by incubating membranes over-night in TNT buffer (10 mM Tris-HCI, pH 7.5, 100 mM sodium chloride, 0.1% (v/v) Tween 20 (Sigma)) containing 3% nonfat, powdered milk (blocking buffer). Membranes were incubated with mAb RACK1 (0.08 mg/ml), affinity-purified mAb 327 ascites (2 µg/ml), mAb PY20 (0.8 mg/ml), or polyclonal anti-GST (2 µg/ml) for 1 h, washed in TNT buffer with changes every 5 min for 30 min, and incubated with horseradish peroxidase-conjugated donkey anti-mouse Ig (Zymed Laboratories Inc., San Francisco, CA) for RACK1 blots, goat anti-mouse IgG (Bio-Rad) for mAb 327 or PY20 blots, or goat anti-rabbit IgG (Bio-Rad) for anti-peptide blots (6, 23–27). Proteins were detected by ECL (see above).

**In Vitro Translation of Proteins—** pGEMc (2 µg) was transcribed and translated in vitro using a TnT coupled rabbit reticulocyte lysate system (Promega, Madison, WI), as instructed by the manufacturer and as described (8). In vitro translated products labeled with Pro-Mix[35S]S (70% l-[35S]methionine and 30% l-[35S]cysteine; >1,000 Ci/mmol; Amersham Pharmacia Biotech) were diluted (1:100) in buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.2% Nonidet P-40) and incubated with 1 µg of purified GST or GST-RACK1 for 3 h at 4 °C as described above. 1/20 of the unbound translation reaction product was loaded directly on the gel as a marker for in vitro translated Src. Gels were treated with Fluoro-Hance (Research Products International Corp., Mount Prospect, IL), and radiolabeled proteins were detected by fluorography.

**Protein Phosphatase Assays—** Cell lysates, GST fusion proteins, or immunoprecipitates were incubated in phosphate buffer (50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA) with or without the addition of purified calf intestinal alkaline phosphatase (50 units) (Promega) for 30 min at room temperature. The reaction was stopped by heating the mixture to 75 °C for 15 min (27–30).

**Synthesis and Purification of RACK1 Peptides—** Tyrosine-phosphorylated (or identical unphosphorylated) peptides corresponding to the sequence surrounding each of the 6 tyrosines of RACK1 were synthesized by the Protein Structure Core Facility of the Digestive Disease Center, Stanford University (Director, Gary Schoolnik), on an automated Millipore PeptidSynthesizer using FMC-NovasyN KA resin (NovBioChem, San Diego, CA) (31–33). Phosphotyrosine was incorporated as Fmoc (N-(9-fluorenylethoxycarbonyl)-Tyr(PO3H2)-OH. The crude peptides were purified using reverse phase high performance liquid chromatography (HPLC) (3.9 × 300 mm C18 column) and a linear gradient containing 0.5% trifluoroacetic acid in 15–65% acetonitrile. The purity of the HPLC-purified products was confirmed using mass spectrometry. The purified phosphopeptides (Tyr-52, TRDTEYNYP,P30G4PHQ; Tyr-140, TLKVCRYIP0,TYQD; Tyr-195, HIGHTGYIP3, lNTV; Tyr-228, NEKRHLYIP3,TLD; Tyr-246, CFSPNYIP3,WLCA; Tyr-302, QTLFAGYIP3,TDLN), or the identical unphosphorylated peptides, were used for peptide competition assays.

**Phosphopeptide Competition Assays—** CHO cells were treated with phorbol-12-myristate-13-acetate (PMA) (Life Technologies, Inc.) (10 ng/ml) at 37 °C for 10 min prior to lysis in RIPA buffer. Lysate containing 200 µg of total cellular protein was incubated with peptide (100 µM) and GST-Src-SH2 (500 nm) or GST for 1 h at 4 °C (5, 34). Glutathione-agarose beads (30 µl) were added, and the mixture was incubated with gentle rocking for 2 h at 4 °C. Proteins were eluted from the beads, resolved by SDS-PAGE, and subjected to immunoblot analysis with anti-RACK1, as described above.

**Phosphorylation of GST Fusion Proteins in vitro in Presence of Tyrosines in RACK1—** Oligonucleotide-directed mutagenesis was used to substitute phenylalanine for tyrosine at residues 52, 140, 194, 228, 246, or 302 of RACK1, utilizing the Transformer site-directed mutagenesis kit according to the manufacturer’s protocol (CLONTECH, Palo Alto, CA) and the following oligonucleotides: Y52F oligo, GATGAGACCAACTTTGAATCTTCA; Y195F oligo, CACACAGGCTTCTGAACACCGTG; Y228F oligo, GCCAACAACCTTTTACCGT.
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AGAT; Y246F oligo, CCTAACCCTTCTTGCCGTGGTCT, Y302F oligo, CTGGTTGCTCCTCTCGACGAC.

The sequence of each RACK1 mutant was confirmed by automated DNA sequencing (Protein and Nucleic Acid Facility, Stanford University, Stanford, CA). Mutant RACK1 genes were inserted into pcDNA3 to create pcDNA3-HA-RACK1(Y152F, Y140F, Y199F, Y229F, Y246F, or Y302F) as described (8).

Treatment of Cells—CHO cells were transfected with pcDNA3 plasmids as described above. 18 h later after transfection, cells were placed in fresh media containing 0.5% FBS. 24 h later, cells were treated for various time periods with PMA, which was dissolved in dimethyl sulfoxide (MeSO) and used at a concentration of 10 ng/ml (35, 36). In some cases, cells were pre-treated with a PKC inhibitor: GF109203X, chol- erythrine, or calphostin C (each was dissolved in MeSO and used at a concentration of 0.1 μM) (Calbiochem, La Jolla, CA) for 30 min prior to PMA stimulation. Control cells were treated with MeSO alone. NIH 3T3 cells that were stably overexpressing the PDGFR were maintained in 0.5% serum for 48 h before treatment with PDGF-BD (Sigma) (10 ng/ml) for various time periods. For immunoblot analysis with anti-phosphotyrosine PY20, cells were treated with 100 ng/ml Me2SO alone. NIH 3T3 cells stably overexpressing c-Src were grown subconfluently on coverslips in DMEM supplemented with 10% FBS for 24 - 48 h and then in fresh media containing 0.5% FBS for 72 h. Cells were treated with PMA (10 ng/ml) or MeSO for various time periods prior to fixation in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and permeabilization in 0.4% Triton X-100/PBS for 20 min at room temperature (37). Non-specific sites were blocked with 10% bovine serum albumin (BSA) in PBS containing 0.2% BSA, for 1 h. Cells were then incubated with primary antibodies, anti-RACK1 (1:500) and anti-Src (mAb 327) (1:100) in PBS containing 5% goat serum and 0.2% BSA for 1 h. After washing three times in PBS containing 0.2% BSA, cells were incubated with secondary antibodies, FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and lis- samine-rhodamine conjugated goat anti-mouse IgM (Jackson Immuno- research), which were diluted 1:100 in PBS containing 5% goat serum and 0.2% BSA, for 40 min in the dark. Coverslips were washed three times in PBS containing 0.2% BSA and a fourth time in PBS containing bisbenzimide. Src and RACK1 immunostaining were visualized with FITC and Texas Red filters, respectively. Control experiments demonstrated that cross-reactivity did not occur between the two secondary antibodies. Cells were photographed using Nikon TE 300 eclipse lenses on a Bio-Rad MRC 1024 confocal microscope. Confocal image analysis was performed using a Bio-Rad MRC600 laser confocal scanner, and final digital images were processed using Adobe Photoshop 5.1 (Adobe System, San Jose, CA). Layers comprised a thickness of 0.2 μm.

RESULTS

RACK1 Associates, in Vitro, with the SH2 Domains of Src, PLCγ, and rasGAP—Previously, we showed that RACK1 binds to the SH2 domain of Src in vitro (8). To determine whether RACK1 binds to the SH2 domain of other signaling proteins, we incubated GST fusion proteins containing the SH2 domain of Src, PLCγ, Shp-2, Shp-1, p85, Abl, Grb2, rasGAP, Csk, or Src together with HeLa cell lysates; collected protein complexes on glutathione-agarose beads; and assayed for RACK1 binding by immunoblot analysis with anti-RACK1 (Fig. 1, upper panel). We observed that RACK1 bound to the SH2 domain of Src (lane 1), to the N-terminal SH2 domain of PLCγ (lane 3) and of rasGAP (lane 10), and not to the SH2 domains of the other proteins tested. When the membrane was stripped of antibody and re-probed with GST antibody (lower panel), a similar amount of GST-SH2 fusion protein was present in each lane except for the lanes containing the C-terminal SH2 domain of p85, where less protein was present (lane 6). Thus, RACK1 interacted specifically with the SH2 domain of Src and the N-terminal SH2 domain of PLCγ and rasGAP, and not with the SH2 domains of Shp-1, Shp-2, p85, Abl, Grb2, Csk, or Src.

RACK1 Associates, in Vivo, with the SH2 Domain of Src—To determine whether the SH2 domain of Src mediates binding of Src and RACK1 in vivo, we utilized a Src mutant that contains a 3-amino acid deletion (Arg-155, Arg-156, and Gly-157) in the SH3 domain and that lacks 80 amino acids in the SH2 domain. Neither RACK1 nor Src proteins were detected in control IgG immunoprecipitates on glutathione-agarose beads, and performed immunoblot analysis with anti-GST (bottom panel). For proteins containing two SH2 domains, C represents the C-terminal and N the N-terminal SH2 domain. Data are representative of three independent experiments.

Fig. 1. Binding of RACK1 to GST-SH2 fusion proteins. RIPA lysates of HeLa cells (containing 300 μg of total cellular protein) were incubated with 2 μg of purified GST fusion protein containing an SH2 domain of Src (lane 1), PLCγ (lanes 2 and 3), Shp-2 (lane 4), Shp-1 (lane 5), p85 (lanes 6 and 7), Abl (lane 8), Grb2 (lane 9), rasGAP (lane 10), Csk (lane 11), or Src (lane 12). Protein complexes were collected on glutathione-agarose beads. Proteins bound to GST-SH2 fusions were recovered, resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and subjected to immunoblot analysis with a monoclonal antibody specific for RACK1 (top panel). The membrane was stripped of antibody and re-blotted with anti-GST (bottom panel). For proteins containing two SH2 domains, C represents the C-terminal and N the N-terminal SH2 domain.
Immunoprecipitation of Src in each lane (data not shown). Analysis with anti-Src revealed an equivalent amount of immunoprecipitate (compare photyrosine followed by immunoblot analysis with anti-RACK1 buffer and tested for binding of RACK1 to Src (Fig. 3C). We transiently expressed HA-RACK1 and Src in CHO cells, treated Src immunoprecipitates with alkaline phosphatase or phosphatase buffer and subjected aliquots to immunoblot analysis with anti-RACK1. Lanes 1, lysate containing 5 μg of total cellular protein was loaded directly on the gel prior to transfer and immunoblot analysis with anti-RACK1. B, tyrosine phosphorylation of RACK1 by Src increases binding of RACK1 to in vitro translated Src, and dephosphorylation of the tyrosine-phosphorylated RACK1 decreases binding. Y527F Src immunoprecipitates were incubated with 1 μg of GST-RACK1 (lanes 1, 2, and 4–6) or GST alone (lane 7) in the presence (+) or absence (−) of cold ATP (1 μM) and in vitro protein kinase reactions were performed. Aliquots from the reaction mixture containing both GST-RACK1 and ATP (lane 2) or GST-RACK1 without the addition of ATP (lane 1) were subjected to immunoblot analysis with anti-RACK1. Lane 1, lysate containing 5 μg of total cellular protein was loaded directly on the gel prior to transfer and immunoblot analysis with anti-RACK1. Data are representative of at least three independent experiments.

To further analyze the effect of tyrosine phosphorylation of RACK1 on the interaction of RACK1 with Src's SH2 domain, we transiently expressed HA-RACK1 and Src in CHO cells, treated Src immunoprecipitates with alkaline phosphatase or phosphatase buffer and tested for binding to wild-type Src and not to mutant Src that contains a deletion in the SH2 domain. CHO cells were transiently transfected with vector alone (lanes 3 and 7), HA-RACK1 (lanes 4 and 8), or HA-RACK1 together with wild-type Src (lanes 2, 6, and 10) or a Src mutant containing a deletion in the SH2 domain (dl155 Src; lanes 1, 5, and 9). A, proteins were immunoprecipitated with anti-Src (lanes 3–6) or mouse IgG (lanes 7–10) from RIPA lysates containing 100 μg of total cellular protein, resolved by SDS-PAGE, and subjected to immunoblot analysis with anti-RACK1. Lanes 1 and 2, lysate containing 5 μg of total cellular protein was loaded directly on the gel prior to transfer and immunoblot analysis with anti-RACK1. B, the membrane shown in A was stripped of antibody and re-blotted with anti-Src. Data are representative of four independent experiments.

This result suggested that phosphorylation of RACK1 enhances the binding of RACK1 to Src.

To determine whether it is specifically tyrosine phosphorylation of RACK1 that enhances binding, we incubated GST-RACK1 with constitutively active Y527F Src (4–7) in the presence or absence of cold ATP, performed an in vitro kinase reaction, and subjected aliquots to immunoblot analysis with anti-phosphotyrosine (Fig. 3B, lanes 1 and 2). We observed increased tyrosine phosphorylation of GST-RACK1 by Src when ATP was added to the reaction mixture. We treated the tyrosine-phosphorylated or unphosphorylated GST-RACK1 with alkaline phosphatase or phosphatase buffer and assayed for binding to [35S]methionine/cysteine-labeled, in vitro translated Src (lanes 3–7). We observed more binding of Src to tyrosine-phosphorylated GST-RACK1 (lane 5) than to dephosphorylated GST-RACK1 (lane 6) or to unphosphorylated GST-RACK1 (lane 4). When we repeated the experiment using potato acid phosphatase instead of alkaline phosphatase, we observed similar results (data not shown).

To further analyze the effect of tyrosine phosphorylation of RACK1 on the interaction of RACK1 with Src, we transiently expressed HA-RACK1 and Src in CHO cells, treated Src immunoprecipitates with alkaline phosphatase or phosphatase buffer and tested for binding of RACK1 to Src (Fig. 3C). We used two Src antibodies for immunoprecipitation: one that recognizes the unique domain of Src (N16) and another that recognizes the C terminus of Src (R7). When we immunoprecipitated Src with either antibody and tested for RACK1 binding by immunoblot analysis with anti-RACK1, we observed decreased binding of RACK1 and Src when alkaline phosphatase was added to the immunoprecipitate (Fig. 3C, compare lanes 7 and 8 or lanes 9 and 10). Immunoblot analysis with anti-phosphotyrosine followed by immunoblot analysis with anti-RACK1 demonstrated that lysate RACK1 was tyrosine-phosphorylated (lane 11) and that tyrosine phosphorylation on RACK1 decreased with the addition of alkaline phosphatase to the immunoprecipitate (compare lanes 11 and 12). Immunoblot analysis with anti-Src revealed an equivalent amount of immunoprecipitated Src in each lane (data not shown). Additional studies showed that RACK1 is phosphorylated on more than one tyrosine, and that the RACK1 antibody recognizes both phosphorylated and unphosphorylated forms of RACK1 equally well (data not shown). Together, these results suggested that phosphorylation of RACK1 on tyrosine enhances binding of RACK1 to Src.

Phosphoryrosines in the Sixth WD Repeat of RACK1 Mediate the Interaction of RACK1 with Src's SH2 Domain—Together, the results of the binding assays shown in Figs. 2 and 3 suggested that a phosphorylated tyrosine(s) on RACK1 might mediate the interaction of RACK1 with Src's SH2 domain of Src. Thus, we searched for tyrosines in the RACK1 sequence that, when phosphorylated, could potentially interact with the SH2 domain of Src. Analysis of the amino acid sequence of RACK1
revealed six tyrosines (Fig. 4A): Tyr-52 (located at the boundary of WD repeat 1 and 2), Tyr-140 (in repeat 4), Tyr-194 (in repeat 5), Tyr-228 and Tyr-246 (in repeat 6), and Tyr-302 (in repeat 7).

To determine which phosphotyrosine(s) on RACK1 might interact with Src's SH2 domain, we first performed phosphopeptide competition assays. To do so, we synthesized tyrosine-phosphorylated or unphosphorylated peptides corresponding to the sequence surrounding each tyrosine of RACK1 and tested the peptides for their ability to compete with RACK1 from HeLa cell lysate for binding to a GST fusion protein containing Src's SH2 domain (GST-SH2). We incubated lysates with GST, GST-SH2, or GST-SH2 together with the tyrosine-phosphorylated RACK1 peptide or the corresponding unphosphorylated peptide, collected protein complexes on glutathione-agarose beads, and performed immunoblot analysis with anti-RACK1. Peptides correspond to the sequence surrounding each tyrosine of RACK1. Lower panel, the membrane shown in the upper panel was stripped of antibody and re-blotted with anti-Src. Data are representative of three independent experiments.

Phosphorylation of RACK1 and the Interaction of RACK1 and Src—Phosphorylation of RACK1 might mediate, at least in part, the interaction of RACK1 with Src's SH2 domain. Together, the results from the peptide competition assays and the mutational analyses indicate that phosphotyrosines in the sixth WD repeat of RACK1 mediate the interaction of RACK1 with Src's SH2 domain.

Serum, PDGF, and Activation of PKC Enhance the Tyrosine Phosphorylation of RACK1 and the Interaction of RACK1 and Src—RACK1 is known to be an intracellular receptor for activated PKC (reviewed in Refs. 9–11). To determine whether PKC activation influences the interaction of RACK1 and Src and the tyrosine phosphorylation of RACK1, we treated cells with mitogens that activate PKC (the tumor promoter PMA or serum) and analyzed their effect. To do so, we expressed vector alone or HA-RACK1 together with Src in CHO cells, treated serum-starved quiescent cells with PMA (10 ng/ml) or 10% serum, immunoprecipitated proteins with anti-Src or anti-
phosphotyrosine, and performed immunoblot analysis with anti-RACK1 (Fig. 6A). We and others have been unsuccessful in attempts to generate antibodies that immunoprecipitate RACK1 efficiently from cell lysates. Thus, to study tyrosine-phosphorylated RACK1, we immunoprecipitated proteins with anti-phosphotyrosine and performed immunoblot analysis with anti-RACK1. After treating cells for 15 min with either PMA or serum, we observed a striking increase in the amount of RACK1 bound to Src (compare lanes 4 and 5 or lanes 4 and 7, respectively) and a striking increase in tyrosine phosphorylation of RACK1 (compare lanes 10 and 11 or lanes 10 and 13, respectively). The effect of PMA was transient; after sustained treatment for 60 min, binding of RACK1 and Src and tyrosine phosphorylation of RACK1 had returned to base-line levels (compare lanes 5 and 6 or lanes 11 and 12, respectively). When the blot was stripped of antibody and re-probed with Src antibody (Fig. 6B), similar amounts of Src protein were present in all Src immunoprecipitates of Src-transfected cells (compare lanes 4–7) and similar amounts of tyrosine-phosphorylated Src were present in all anti-phosphotyrosine immunoprecipitates of Src-transfected cells (compare lanes 10–13). These results indicated that treatment of cells with serum or PMA increases the tyrosine phosphorylation of RACK1 and the binding of RACK1 to Src.

To determine whether PDGF, a growth factor in serum that can indirectly activate PKC through PLCγ (39), influences the association of RACK1 and Src, we treated serum-starved fibroblasts that overexpress the β-PDGF receptor with purified PDGF-BB (10 ng/ml) for various time periods, immunoprecipitated proteins with anti-Src, and performed immunoblot analysis with anti-RACK1 (Fig. 7). We observed an increase in RACK1 bound to Src after 2 and 5 min of PDGF treatment (compare lanes 1–3 of Fig. 7, A and lanes 2 and 3 of Fig. 7B). The effect of PDGF was transient; after treatment for 10, 30, or 120 min, binding of RACK1 and Src had returned to base-line levels (Fig. 7A, compare lanes 4–6 with lane 3). When the blot was stripped of anti-RACK1 and re-probed with anti-Src, similar amounts of Src protein were present in all Src immunoprecipitates (compare lanes 7–12). Immunoblot analysis of lysate proteins with anti-RACK1 revealed that equivalent amounts of RACK1 protein were present in all lanes (compare lanes 13–18). The effect of PDGF on the RACK1-Src interaction was concentration-dependent, with the maximal effect achieved with 10 ng/ml (data not shown; Ref. 18).

To determine whether PDGF enhances the tyrosine phosphorylation of RACK1, we treated serum-starved cells that overexpress the PDGF receptor with PDGF for 5 min, immunoprecipitated lysate proteins with anti-phosphotyrosine, and performed immunoblotting with anti-RACK1 (Fig. 7B). We observed increased tyrosine phosphorylation on RACK1 following PDGF treatment (compare lanes 3 and 4). Thus, PDGF stimulation enhances the tyrosine phosphorylation of RACK1 and the binding of RACK1 to Src.

To determine the time course of PMA effect on RACK1-Src binding, we treated CHO cells that were transiently expressing HA-RACK1 and Src for varying time periods with PMA, immunoprecipitated proteins with anti-Src, and performed immunoblot analysis with anti-RACK1. We observed that the maximum binding of RACK1 and Src occurred after 15 min of PMA treatment (Fig. 6A, lanes 2–5 and 7). Again, immunoblot analysis with anti-Src revealed that similar amounts of Src protein were present in all Src immunoprecipitates (lanes 9–14). Likewise, immunoblot analysis of lysate proteins with anti-RACK1 revealed that similar amounts of RACK1 protein were expressed in all cells (lanes 15–20). RACK1 was not detected in IgG immunoprecipitates of PMA-treated cells (Fig. 8B, lane 9). Moreover, when we co-expressed Src and a RACK1 mutant (Y246F) that does not bind Src, we did not detect RACK1 in Src immunoprecipitates of PMA-treated cells (Fig. 8B, lane 8).
Fig. 8. Effect of PKC inhibitors on PMA-induced tyrosine phosphorylation of RACK1 and binding of RACK1 to Src. A, CHO cells were transiently transfected with HA-RACK1 and Src, and serum-starved as described in the legend to Fig. 6. Cells were left untreated (lane 1), treated with PMA for 5 min (lane 2) or 15 min (lanes 3, 6, 8, and 9), or treated with the PKC inhibitor GF109203X (0.1 μM) prior to PMA treatment (lanes 4, 5, 7, 10, and 11). Lanes 1 and 8, cell lysate containing 5 μg of total cellular protein was loaded directly on the gel prior to transfer and subjected to immunoblot analysis with anti-RACK1 (lanes 1–3) or with anti-Src (lanes 4–7) or with another inhibitor of PKC activity, chelerythrine (0.1 μM) prior to PMA treatment (lanes 6, 10, and 12). Lanes 7–9 were loaded with 100 μg of total cellular protein and subjected to immunoblot analysis with anti-RACK1 (lanes 7–9). Immunoblot analyses of lysate proteins with anti-RACK1 showed that equivalent amounts of RACK1 protein were present in all lysates (lanes 10–12). These results confirmed that PKC activation is required for PMA-enhanced tyrosine phosphorylation of RACK1 and binding of RACK1 to Src. In a complementary approach to analyze the effect of PMA on the interaction of RACK1 and Src, we treated serum-starved, NIH 3T3 cells that were stably overexpressing c-Src (6) with PMA (10 ng/ml) for varying time periods and localized Src and RACK1 using confocal immunofluorescence microscopy (Fig. 9). In quiescent cells (row 1), we observed both Src (column 1) and RACK1 (column 2) in the cytoplasm (particularly in the perinuclear region) but there was little colocalization of the two proteins: yellow regions in the double immunofluorescence-labeled cells (column 3), and white regions in the computer-generated images of the double immunofluorescence-labeled cells (column 4). After treatment of cells with PMA for 2 hr (row 2) or 5 hr (row 3) min, a subpopulation of Src molecules (column 1) translocated to the cell periphery (presumably to the plasma membrane), whereas most RACK1 molecules (column 2) remained more centrally located. After treatment of cells with PMA for 10 min (rows 4 and 5), a subpopulation of RACK1 molecules translocated to the cell periphery (column 2) and appeared to colocalize with Src: yellow regions in the double immunofluorescence-labeled cells (column 3), and white regions in the computer-generated images of the double immunofluorescence-labeled cells (column 4). These findings are internally consistent with those of our biochemical studies; both show that PMA stimulation enhances the association of RACK1 and Src. In addition, the immunofluorescence studies suggested that the association of the two proteins occurs at the cell periphery, presumably at the plasma membrane.

DISCUSSION

This study shows that RACK1 interacts with the SH2 domain of Src and several other intracellular signaling molecules. The Src-RACK1 interaction is mediated, at least in part, by the SH2 domain of Src and by phosphotyrosines in the sixth WD repeat of RACK1, and is enhanced by PKC activation and tyrosine phosphorylation of RACK1.

Evidence that RACK1 interacts with the SH2 domains of PMA on the RACK1-Src interaction was concentration-dependent, with the maximal effect achieved with 10 ng/ml (data not shown; Refs. 35 and 36). Together, these results showed that the effect of PMA on the interaction of RACK1 and Src is both time- and dose-dependent.

To confirm that activation of PKC is necessary for PMA-enhanced binding of RACK1 and Src, we pre-treated cells with inhibitors of PKC and analyzed the effect of PMA on the Src-RACK1 interaction (Fig. 8). When we pre-treated cells with GF109203X (a bisindoylmaleimide I derivative), at a concentration that inhibits PKC and not Src activity (0.1 μM; Refs. 40 and 41), we detected little increase in binding of RACK1 and Src after treating cells with PMA for 15 min (Fig. 8, A (lane 6) and B (lanes 4 and 7)). Similarly, when we pre-treated cells with another inhibitor of PKC activity, chelerythrine (0.1 μM) (10), we observed little enhancement of binding of RACK1 and Src after treating cells with PMA (Fig. 8B, lane 5). Treatment of cells with a third PKC inhibitor, calphostin C (0.1 μM) (10), also blocked the PMA-induced enhancement of RACK1-Src binding (data not shown). These results confirmed that PKC activation is required for PMA-enhanced binding of RACK1 and Src. To confirm that activation of PKC is necessary for PMA-enhanced tyrosine phosphorylation of RACK1, we pre-treated cells with inhibitors of PKC activity prior to PMA treatment, immunoprecipitated proteins with anti-phosphotyrosine and performed immunoblot analysis with anti-RACK1 (Fig. 8C). As we observed previously, PMA treatment for 15 min resulted in a marked increase in tyrosine phosphorylation of RACK1 (compare lanes 1 and 2). When we pre-treated cells with the PKC inhibitor GF109203X (0.1 μM) prior to PMA treatment, we did not detect an increase in tyrosine phosphorylation of RACK1 (lane 3). Similar results were seen with other PKC inhibitors (data not shown). As we showed previously, PMA treatment increased binding of Src and RACK1 (compare lanes 4 and 5), whereas pretreatment with GF109203X did not (lane 6). When the blot shown in lanes 4–6 was stripped of antibody and re-probed with Src antibody, we observed that Src protein levels were equivalent in all Src immunoprecipitates (lanes 7–9). Immunoblot analyses of lysate proteins with anti-RACK1 showed that equivalent amounts of RACK1 protein were present in all lysates (lanes 10–12). These results confirmed that PKC activation is required for PMA-enhanced tyrosine phosphorylation of RACK1 and binding of RACK1 to Src.
multiple signaling molecules in vitro is that GST fusions containing an SH2 domain of Src, PLCγ1, or rasGAP bind to RACK1 from HeLa cell lysate (Fig. 1). Evidence that the Src-RACK1 interaction is mediated by the SH2 domain of Src in vivo is that a Src mutant, which contains a 3-amino acid deletion in the phosphotyrosine-binding pocket of the SH2 domain (dl155), does not bind to RACK1 (Fig. 2). In addition, in vitro studies show that RACK1 binds to a Src mutant that contains...
an intact SH2 domain and has an 80-amino acid deletion in the SH3 domain, yet does not bind to d155 Src (data not shown), indicating that it is specifically the SH2 and not the SH3 domain that mediates Src’s interaction with RACK1. Evidence that the Src-RACK1 interaction is mediated by phosphorylases in the sixth WD repeat of RACK1 is that a phosphopeptide corresponding to the sequence surrounding Tyr-246 or Tyr-228 can compete with RACK1 from HeLa cell lysate for binding to GST-Src-SH2 (Fig. 4), and that a mutant of RACK1 that contains a phenyalanine substitution for tyrosine at residue 246 does not bind Src (Fig. 5). Evidence that the Src-RACK1 interaction is enhanced by tyrosine phosphorylation of RACK1 is that: 1) phosphorylation of RACK1 on tyrosine by Src in an in vitro kinase assay increases binding of RACK1 to Src, and phosphatase treatment of the tyrosine-phosphorylated RACK1 decreases binding (Fig. 3B); 2) phosphatase treatment of endogenous RACK1 decreases tyrosine phosphorylation of RACK1 and binding of RACK1 to Src (Fig. 3C); and 3) treatment of cells with agents that enhance the tyrosine phosphorylation of RACK1 (serum, PDGF, or PMA) also enhance the binding of RACK1 to Src (Figs. 6–8). Finally, evidence that the Src-RACK1 interaction is enhanced by PKC activation is that treatment of cells with specific activators of PKC increases the association of RACK1 and Src (as shown by both biochemical and immunofluorescence studies; Figs. 8 and 9, respectively), and pre-treatment of cells with specific inhibitors of PKC decreases the association (Fig. 8).

Although RACK1 has been reported to interact with the PKC family of serine/threonine kinases (reviewed in Refs. 9–11), this is the first report of RACK1 interacting with a tyrosine kinase and of RACK1 being tyrosine-phosphorylated. Moreover, although serine/threonine phosphorylation of members of the WD repeat family of proteins has been observed (42), to the best of our knowledge, this is the first report of tyrosine phosphorylation of a member of this family. We believe that tyrosine phosphorylation of RACK1 and other WD repeat proteins may be important “switches” that link these molecules to other signaling molecules and relays signals across multiple pathways. Because each WD repeat protein contains multiple WD domains and multiple tyrosines, the “switches” may be many, and the signals may be amplified and diverse.

A clear correlation exists between enhanced tyrosine phosphorylation of RACK1 and enhanced binding of RACK1 to Src (Figs. 3, 4, and 6). Thus, it is tempting to suggest that the two are linked, that RACK1 is a substrate for the Src tyrosine kinase, and that signals (like PKC or PDGF activation) which bring Src and RACK1 in close proximity to each other result in phosphorylation of RACK1 by Src and, in turn, enhanced binding of RACK1 to Src’s SH2 domain. However, Src is only one of many tyrosine kinases that could potentially phosphorylate RACK1 in cells, and factors in addition to tyrosine phosphorylation of RACK1 may regulate the interaction of RACK1 and Src. Nonetheless, we believe that both tyrosine phosphorylation of RACK1, and the interaction of Src and RACK1 are important mechanisms of signal transduction in cells.

Our finding that the intracellular localization of RACK1 in NIH 3T3 cells changes in response to PKC activation (moving from a perinuclear to another intracellular site; Fig. 9) confirms recent findings of Ron et al. (15). Moreover, our finding that PKC activation induces the co-localization of RACK1 and Src (Figs. 6, 8, and 9) is similar to the finding of Ron and co-workers that PKC activation induces the co-localization of RACK1 and β1PPKC. Together, these findings suggest that RACK1 is involved in the intracellular trafficking of protein kinases from one intracellular site to another, perhaps bringing the enzymes into close proximity with their specific substrates. RACK1 has been shown to interact with at least six different proteins: PKC (16), PLC-γ (43), Src (8), integrin β subunit (44), cAMP-specific phosphodiesterase PDE4δ isoform (45), and p65 synaptotagmin (46). The interaction of RACK1 and PKC, Src, or integrin β subunit is inducible by PMA treatment (44), and in the case of Src and PKC, specifically by PKC activation. PKC activation appears to occur before RACK1 binds to Src because blocking PKC activation prevents most binding. PKC activation may induce a post-translational modification of RACK1 (other than serine/threonine phosphorylation) that allows RACK1 to interact with other signaling proteins. Here we report that one modification induced, indirectly, by PKC activation is tyrosine phosphorylation of RACK1 (Figs. 6 and 8). Others have shown that PKA activation results in tyrosine phosphorylation of several proteins including Src and ErbB2 (35). Perhaps PKC activation brings RACK1 and other signaling proteins into close proximity with tyrosine kinases. Here we show that Src is one tyrosine kinase that associates with RACK1 following PKC activation (Figs. 6, 8, and 9). Alternatively, PKC activation may induce a conformational change in RACK1 that exposes an otherwise inaccessible tyrosine for phosphorylation. Because PKC does not directly phosphorylate RACK1 (16), the simplest explanation for how activated PKC might induce a conformational change in RACK1 is by directly binding to RACK1. Whether activated PKC is required to bind RACK1 before and/or in order that Src or integrin β subunit bind RACK1, is unknown (see below).

Interestingly, Src, PLC-γ, and rasGAP are all known to associate via their SH2 domains with the PDGF receptor (PDGF) (18, 39). Here we report that Src, PLC-γ, and rasGAP also associate via their SH2 domains (at least in vitro) with RACK1 from HeLa cell lysate (Fig. 1) and that PDGF treatment of cells enhances both the tyrosine phosphorylation of RACK1 and the binding of RACK1 to Src (Fig. 7). This suggests intriguing similarities, and possibly a link between PDGF and RACK1 signaling; perhaps RACK1 is involved in the recruitment, assembly, and/or regulation of signaling molecules at the PDGF receptor. Here we show that RACK1 interacts with the SH2 domains of some signaling proteins (Fig. 1). Rodriguez et al. (47) have shown that RACK1 interacts with the pleckstrin homology domain of other signaling proteins.

Overall, RACK1 appears to serve as a scaffold, anchor, or adaptor protein that is involved in the recruitment, assembly, and/or regulation of a variety of different signaling molecules (48). In the case of the PKC and Src protein kinases, RACK1 may help to recruit substrates or regulatory proteins and/or to stabilize the kinases in protein complexes. Examples of other proteins that work in similar ways include protein kinase A-anchoring proteins which interact with PKC, protein kinase A, and protein phosphatase 2B (49).

Of the large family of WD repeat proteins to which RACK1 belongs, RACK1 most closely resembles Gβ subunit, which interacts with the β-adrenergic receptor kinase, and like RACK1, has seven WD repeats (50, 51). Interestingly, β-adrenergic receptor kinase associates with Src after stimulation of β-arrestin (52, 53) although the crystal structure of RACK1 has not been determined, it is very likely similar to that of Gβ subunit (54). Gβ folds into a symmetric “propeller” structure with seven “propeller blades,” each corresponding to one of the seven WD repeats (reviewed in Refs. 12, 13, and 55). The blades of the propeller are thought to be the sites of interaction of Gβ subunit with other proteins. Each blade may be specialized for interacting with specific proteins. Moreover, there is an insert of 3 amino acids into repeat 6 of Gβ that helps generate a very hydrophobic region that runs from the top of the propeller down
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The SH2 domain (19, 60, 61), they are highly conserved evolutionally to Src's SH2 domain. Although the phosphotyrosine 228 represents a minor, secondary site on PLC-1 and RACK1 in vitro (Fig. 1). It is possible that there are regions of PLC-1 in addition to the SH2 domain (such as the C2 domain) that also mediate the binding of PLC-1 to RACK1.

In summary, we have shown that RACK1 interacts with the SH2 domain of Src and several other intracellular signaling molecules. In the case of Src, binding is mediated by phosphotyrosines in the sixth WD repeat of RACK1, and enhanced by PKC activation and by tyrosine phosphorylation of RACK1. We believe that tyrosine-phosphorylated RACK1 plays an important role in protein-protein interactions and signal transduction pathways, perhaps by serving as a scaffold, anchor, or adaptor protein that helps to recruit, assemble, and/or regulate signaling molecules.

Acknowledgments—We thank Rachel Harte for assistance with data analysis and figure preparation. We thank Lewis Cantley for pGEX plasmids containing the SH2 domains of PLCg, Shp-1, Shp-2, p85, Ab1, Grb2, rasGAP, Csk, and Shc; Sara Courtenidge for NIH 3T3 cells overexpressing the PDGFR; Sarah Parsons for pM5H-src and pMdi15sc-src; Tony Hunter for pGEMsrc; and Anson Lowe for antibodies to PKC antibodies. We are grateful to Rosemary Fernandez and Gary Schoolnik for synthesis and purification of RACK1 peptides and phosphopeptides (Protein Structure Core Facility, Stanford Digestive Disease Center), and Evelyn Resurreccion, Eugene Butcher, Jou Tsu-Shu, and James Nelson for assistance with immunofluorescence studies (Cell Biology Core Facility, Stanford Digestive Disease Center). We thank Darin Moehly-Rosen, Anson Lowe, and Bishr Omary for critical review of the data and Darin Moehly-Rosen for critical review of the manuscript.

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