MOLECULAR AND CELLULAR BIOLOGY, Jan. 1994, p. 509–517
0270-7306/94/$04.00+0

Vol. 14, No. 1

A New Function for a Phosphotyrosine Phosphatase: Linking GRB2-Sos to a Receptor Tyrosine Kinase

WEI LI,[1]* RIKO NISHIMURA,[1] ADAM KASHISHIAN,[2] ANDREAS G. BATZER,[1] WILLIAM J. H. KIM,[1] JONATHAN A. COOPER,[2] AND JOSEPH SCHLESSINGER[1]

Department of Pharmacology, New York University Medical Center, New York, New York 10016,[1] and Fred Hutchinson Cancer Research Center, Seattle, Washington 98104[2]

Received 13 August 1993/Returned for modification 4 October 1993/Accepted 8 October 1993

Autophosphorylated growth factor receptors provide binding sites for the *src* homology 2 domains of intracellular signaling molecules. In response to epidermal growth factor (EGF), the activated EGF receptor binds to a complex containing the signaling protein GRB2 and the Ras guanine nucleotide-releasing factor Sos, leading to activation of the Ras signaling pathway. We have investigated whether the platelet-derived growth factor (PDGF) receptor binds GRB2-Sos. In contrast with the EGF receptor, the GRB2 does not bind to the PDGF receptor directly. Instead, PDGF stimulation induces the formation of a complex containing GRB2; 70-, 80-, and 110-kDa tyrosine-phosphorylated proteins; and the PDGF receptor. Moreover, GRB2 binds directly to the 70-kDa protein but not to the PDGF receptor. Using a panel of PDGF β-receptor mutants with altered tyrosine phosphorylation sites, we identified Tyr-1009 in the PDGF receptor as required for GRB2 binding. Binding is inhibited by a phosphopeptide containing a YXNX motif. The protein tyrosine phosphatase Syp/PTP1D/SHPTP2/PTP2C is approximately 70 kDa, binds to the PDGF receptor via Tyr-1009, and contains several YXNX sequences. We found that the 70-kDa protein that binds to the PDGF receptor and to GRB2 comigrates with Syp and is recognized by anti-Syp antibodies. Furthermore, both GRB2 and Sos coimmunoprecipitate with Syp from lysates of PDGF-stimulated cells, and GRB2 binds directly to tyrosine-phosphorylated Syp in vitro. These results indicate that GRB2 interacts with different growth factor receptors by different mechanisms and the cytoplasmic phosphotyrosine phosphatase Syp acts as an adapter between the PDGF receptor and the GRB2-Sos complex.

The Ras family of low-molecular-weight guanine nucleotide-binding proteins is implicated in the control of cell growth and differentiation (5). Two lines of experimental evidence indicate that Ras is a crucial downstream component of the signaling pathways of growth factor receptors with protein tyrosine kinase activity. First, microinjection of monoclonal anti-Ras antibody (Y13-259) prevents NIH 3T3 cells from entering S phase in response to serum and blocks transformation by tyrosine kinase receptor-like oncogenes (34, 52). Second, expression of a dominant negative Ras mutant (Asn-17) blocks growth factor-stimulated DNA synthesis and cell proliferation in NIH 3T3 cells, morphological differentiation of PC12 cells, and Raf-1 and mitogen-activated protein kinase activation in both cell types (7, 56, 57, 61). Furthermore, expression of oncogenic Ras mutants activates extracellular signal-regulated kinases and triggers cell proliferation in the absence of growth factors (2, 26, 61).

Ras exists in two states in cells, the active GTP-bound state and the inactive GDP-bound state. The two states are interconverted by the Ras GTPase activating proteins, which enhance the intrinsic GTPase activity of Ras and therefore change GTP-bound Ras to GDP-bound Ras, and by the activity of guanine nucleotide-releasing factors, which stimulate the exchange of GDP to GTP of Ras (42). The ratio of GTP-bound to GDP-bound forms is low in quiescent cells and becomes elevated in growth factor-stimulated cells (20, 45). Growth factor stimulation probably increases the ratio of GTP-bound Ras to GDP-bound Ras by either activating or recruiting guanine nucleotide-releasing factors (27, 63).

Phosphorylation of tyrosine kinase receptors on tyrosine residues provides high-affinity binding sites for cellular *src* homology 2 (SH2)-containing signaling molecules. SH2 domains have been identified for a wide range of molecules, which can be divided into two classes. One class contains proteins which contain functional or enzymatic domains such as *src* family tyrosine kinases, phospholipase Cγ, GTPase activating protein, two protein tyrosine phosphatases (PTP1C and Syp/PTP1D/SHPTP2/PTP2C), transcription factor ISGF-3, and the putative GDP-GTP exchanger Vav. The second class of proteins lacks any known catalytic domain and is composed virtually exclusively of SH2 and SH3 domains. Members of this group include Crk, GRB2, and Nck. It now seems that some of these proteins function as adapters to link receptor tyrosine kinases to a downstream effector molecule(s) (38).

GRB2 is ubiquitously expressed and entirely composed of one SH2 domain and two SH3 domains (30, 32). Microinjection of GRB2 protein potentiates the effect of Ras on cellular mitogenesis (30). GRB2 is the mammalian homolog of Sem-5 in *Caenorhabditis elegans* and Drk in *Drosophila melanogaster* (11, 30, 36). Genetic analysis indicates that Sem-5 and Drk act downstream of protein tyrosine kinase receptors and upstream of Ras during vulval development in *C. elegans* and eye development in *D. melanogaster*, respectively (11, 36, 49). Recently, it has been demonstrated that GRB2 acts as an adapter molecule by binding to the activated epidermal growth factor (EGF) receptor via its SH2 domain and to guanine nucleotide-releasing factor, Sos, through its SH3 domains, leading to an increase in the proportion of Ras bound to GTP (6, 10, 15, 18, 28, 36, 38, 43, 48, 49).

* Corresponding author. Present address: The Ben May Institute, The University of Chicago, 5841 South Maryland Avenue, MC 6027, Chicago, IL, 60637. Phone: (312) 753-1408. Fax: (312) 702-6260.

How the specificity of each growth factor receptor is controlled is poorly understood. Depending on both the identity of the tyrosine kinase receptor and the nature of the target cell, a given receptor can elicit proliferative, differentiative, or metabolic responses in the cell. One level of control may lie in the specificity of the receptor-signaling protein complex formation. This is determined, at least in part, by the structures of individual SH2 domains and the local sequences surrounding phosphorylation sites in the receptors (8, 33, 37, 46). Additional factors may limit the interactions in the cell (13).

We have compared the interaction of GRB2 with the EGF receptor and platelet-derived growth factor (PDGF) receptor (PDGFR). We found that, while GRB2 binds to the EGF receptor directly via its SH2 domain, its association with the PDGFR appears to be indirect, through the previously identified protein tyrosine phosphatase Syp/PTP1D/SHPTP2/PTP2C (1, 16, 17, 60). This result indicates that different growth factor receptors can interact with the same downstream signaling molecule by different mechanisms and that the Syp phosphatase is multifunctional.

MATERIALS AND METHODS

HER14 cells (NIH 3T3 cell transfected with human EGF receptor) were cultured in Dulbecco modified Eagle medium containing 10% calf serum as previously described (21). Dog kidney epithelial cells (TRMP) expressing wild-type or mutant human PDGFRs type β were maintained as described previously (23, 24). Human recombinant PDGF-BB and EGF were purchased from INTERGEN (Purchase, N.Y.). Anti-human PDGFR antibodies were used as previously described (23). Polyclonal rabbit anti-human GRB2 and anti-phosphotyrosine (PY) antibodies were generated and used as previously described (30, 35). Rabbit anti-mouse Syp antibody was kindly provided by Gen-Sheng Feng and Tony Pawson (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). Generation and production of glutathione *S*-transferase (GST) fusion proteins of GRB2 and Nck was as previously described (29, 30).

In vitro binding assays. GST-GRB2, GST-GRB2–SH2, and GST-Nck fusion proteins were freshly prepared for the in vitro binding experiments. Stimulation of cells with PDGF for 5 min and EGF for 2 min at 37°C, under which the maximum protein tyrosine phosphorylation was achieved, was used throughout the study. Lysates of HER14 cells stimulated with or without PDGF (50 ng/ml) were incubated with GST-GRB2 or GST-GRB2–SH2 proteins immobilized on glutathione-agarose beads for 1 h at 4°C. For binding competition experiments, the GST-GRB2 or GST-Nck beads were preincubated with the phosphopeptides for 1 h at 4°C prior to incubation with the cell lysate. The beads or immunocomplexes were washed three times with the lysis buffer (29) and one time with radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl [pH 7.4], 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) and boiled in 1× electrophoresis sample buffer. The supernatants were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane, and immunoblotted with anti-PDGFR antibodies. The PDGFR was visualized by incubation with ^{125}I-protein A and autoradiography.

To dissociate and denature the preexisting protein complexes, cells stimulated in the presence or absence of PDGF-BB (50 ng/ml) or EGF (250 ng/ml) were lysed in a small volume (10^7 cells in 100 μl) of lysis buffer (29), but the 1% Triton X-100 was replaced with 0.2% Triton X-100 and 1% SDS. After centrifugation (14,000 × *g* for 15 min at 4°C), the supernatants were heated to 95°C for 5 min, cooled on ice, and then diluted with the Triton X-100 lysis buffer (35) so that the final concentration of SDS was 0.1%. Under these conditions, preexisting protein complexes were dissociated and denatured (35; data not shown). The samples were incubated either with GST-GRB2 or with GST-Nck fusion proteins immobilized on agarose beads or with antibodies against GRB2 or Syp and analyzed by SDS-PAGE and Western blot (immunoblot) with ^{125}I-protein A and by autoradiography.

For the assays of protein-protein interaction on nitrocellulose membrane, proteins were transferred to nitrocellulose membrane, which was blocked with bovine serum albumin (BSA), incubated with purified GST-GRB2 or GST-Nck proteins for 1 h at room temperature, and washed four times in Tris-buffered saline–Triton X-100 buffer and one time in Tris-buffered saline without Triton X-100. The GST-GRB2- or GST-Nck-bound protein bands were visualized by using a secondary anti-GST antibody and then incubated with ^{125}I-protein A and autoradiographed.

Detection of GRB2-PDGFR complex in lysates of PDGF-stimulated cells. HER14 or TRMP cells were grown to 80% confluency in 6-cm tissue culture dishes and incubated in medium containing 0.5% fetal calf serum for 16 h. Cells were then treated with or without human recombinant PDGF (50 ng/ml for HER14 and 400 ng/ml for TRMP) at 37°C for 5 min, concentrations at which maximum protein tyrosine phosphorylation was detected (data not shown). Cells were washed three times with ice-cold phosphate-buffered saline buffer and solubilized in lysis buffer. The supernatants of the clarified cell lysates were incubated with anti-GRB2 or Nck antibodies for 3 h at 4°C, and the immunocomplexes were precipitated by incubation with protein A-Sepharose beads for 45 min at 4°C. The beads were washed three times with lysis buffer without BSA and boiled in 1× electrophoresis sample buffer containing 0.5 M β-mercaptoethanol. The supernatants were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with anti-PDGFR antibodies. The PDGFR was visualized by labeling with ^{125}I-protein A followed by autoradiography. The amount of PDGFR was determined from the radioactive content of the excised bands.

Two-dimensional gel electrophoresis analysis. Cells labeled with $^{32}P_i$ and incubated in the presence of PDGF were solubilized in lysis buffer. The supernatants were first denatured as described above and then either immunoprecipitated with anti-Syp antibody or incubated with GST-GRB2 beads as described above. The immunocomplexes or the beads were washed six times with RIPA buffer prior to two-dimensional electrophoresis analysis. Samples of 15 μl were analyzed on isoelectric focusing gels as described previously and were then subjected to second-dimension SDS-PAGE (12, 19). The gels were dried and subjected to autoradiography.

RESULTS

PY-containing proteins complexed with GRB2 in PDGF-treated cells. HER14 cells (NIH 3T3 cells overexpressing the human EGF receptor [21]) were stimulated in the presence or absence of PDGF or EGF and solubilized in the lysis buffer (Materials and Methods), and their clarified postnuclear fractions were immunoprecipitated with rabbit anti-GRB2 polyclonal antibodies (30). The immunocomplexes were resolved by SDS-PAGE, transferred to a nitrocellulose

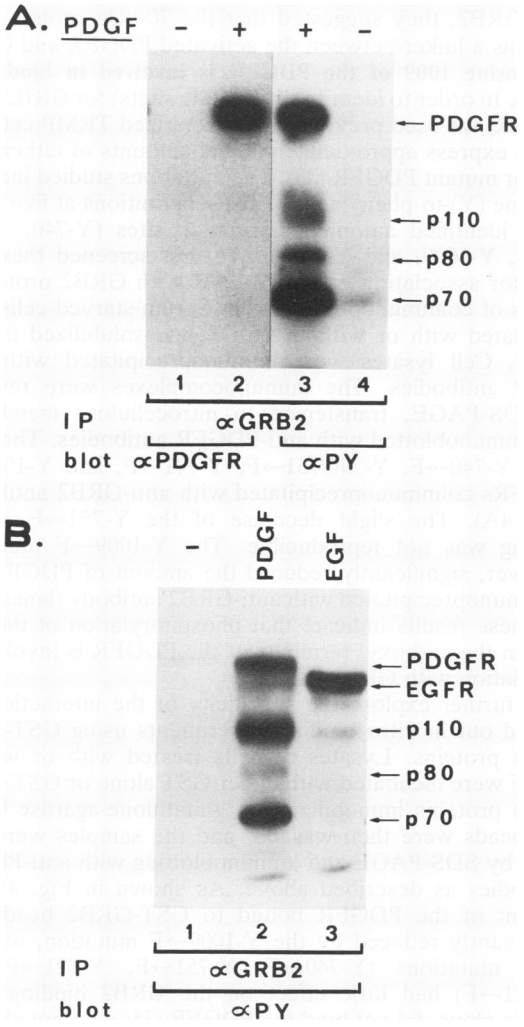

FIG. 1. Comparison of GRB2-bound PY proteins in EGF- or PDGF-stimulated cells. Serum-starved HER14 cells stimulated in the presence or absence of human recombinant PDGF-BB (50 ng/ml) (A and B) or EGF (250 ng/ml) (B) at 37°C for 5 min or 2 min, respectively, were solubilized and immunoprecipitated with anti-GRB2 antibodies. The samples were analyzed by Western blot analysis with either anti-PDGF (αPDGFR) (A) or anti-PY (αPY) (A and B) antibodies. Protein bands were visualized by using ^{125}I-protein A and autoradiography. IP, immunoprecipitation.

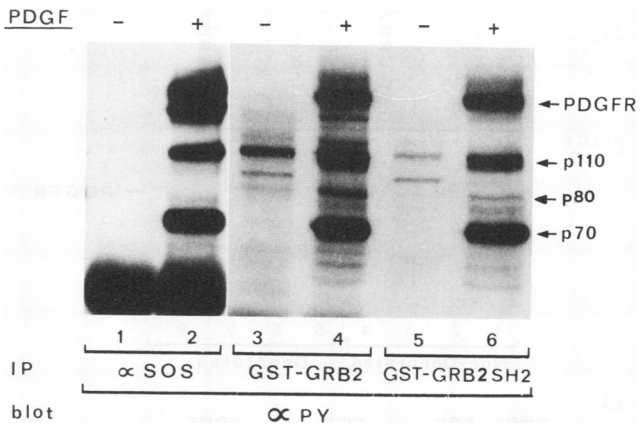

FIG. 2. SH2 domain of GRB2 is involved in the complex formation. The lysates of HER14 cells stimulated with or without PDGF (50 ng/ml) for 5 min at 37°C were either immunoprecipitated with anti-Sos antibodies (αSOS; lanes 1 and 2) or incubated with GST-GRB2 (lanes 3 and 4) or GST-GRB2–SH2 (lanes 5 and 6) fusion protein beads. Samples were resolved in SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-PY (αPY) antibodies. Protein bands were visualized by incubating with ^{125}I-protein A and autoradiography. IP, immunoprecipitation.

membrane, and immunoblotted with either anti-PDGFR or anti-PY antibodies. Figure 1A shows that GRB2 coimmunoprecipitated with the PDGFR from lysates of PDGF-stimulated (lane 2) but not unstimulated (lane 1) cells. When the samples were immunoblotted with anti-PY antibodies (lanes 3 and 4), the 190-kDa PDGFR and additional bands of 110, 80, and 70 kDa were detected. As shown previously (30), the activated EGF receptor immunoprecipitated together with GRB2 from EGF-treated cells. However, the 70-, 80-, and 110-kDa PY-containing proteins were not detected in anti-GRB-2 immunoprecipitate from EGF-treated HER14 cells (Fig. 1B, lane 3).

We tested whether the PDGFR and 110-, 80-, and 70-kDa phosphoproteins could associate with GRB2 in vitro by adding a GST-GRB2 fusion protein to lysates of control and PDGF-stimulated cells and detecting bound proteins by using anti-PY antibodies. The 190-kDa PDGFR and 70-, 80-, and 110-kDa proteins complexed with GST-GRB2 in vitro (Fig. 2, lanes 3 and 4). This binding was mediated by the SH2 domain of GRB2, because a GST-GRB2–SH2 fusion protein also bound the three proteins, with a decrease in the intensity of the 80-kDa band (Fig. 2, lanes 5 and 6). However, this experiment does not address whether the SH2 domain of GRB2 binds to a complex of the three proteins or binds directly to each of the three proteins.

GRB2 binds directly to a 70-kDa tyrosine-phosphorylated protein in PDGF-treated cells. The association of GRB2 with the EGF receptor or PDGFR could be direct or indirect. To test for direct binding, we used two approaches. First, we assayed GRB2 binding to the EGF receptor or PDGFR from heat- and SDS-denatured cell lysates. Cells, unstimulated or stimulated with PDGF, were lysed and heat denatured at 95°C in the presence of SDS. The lysates were then incubated with GST-GRB2 fusion protein immobilized on glutathione beads. The unbound material was removed, and the beads were washed prior to Western blot analysis with anti-PDGFR antibodies. GST-NCK fusion protein was included as a positive control, since it binds to both the undenatured and denatured PDGFR (35). As shown in Fig. 3A, both GST-GRB2 and GST-NCK bound to the tyrosine-phosphorylated PDGFR from the undenatured cell lysate (lanes 2 and 4). The nature of the lower-molecular-weight bands in lane 4 is unknown; possibly it's some degraded species of the PDGFR. However, GST-GRB2 failed to bring down the PDGFR from the denatured cell lysate (lane 8), while GST-NCK beads were still able to bind (lane 6). In a similar experiment, but stimulated with EGF, GRB2 bound to the EGF receptor from both nondenatured and denatured cell lysates (data not shown). These results suggest that one or more proteins which are associated with the PDGFR under nondenaturing conditions are required for GRB2 binding, whereas NCK is able to bind the PDGFR directly. To further test this notion, we used a second approach. Proteins in the total lysates of either EGF- or PDGF-stimulated or unstimulated cells were resolved by SDS-PAGE, transferred

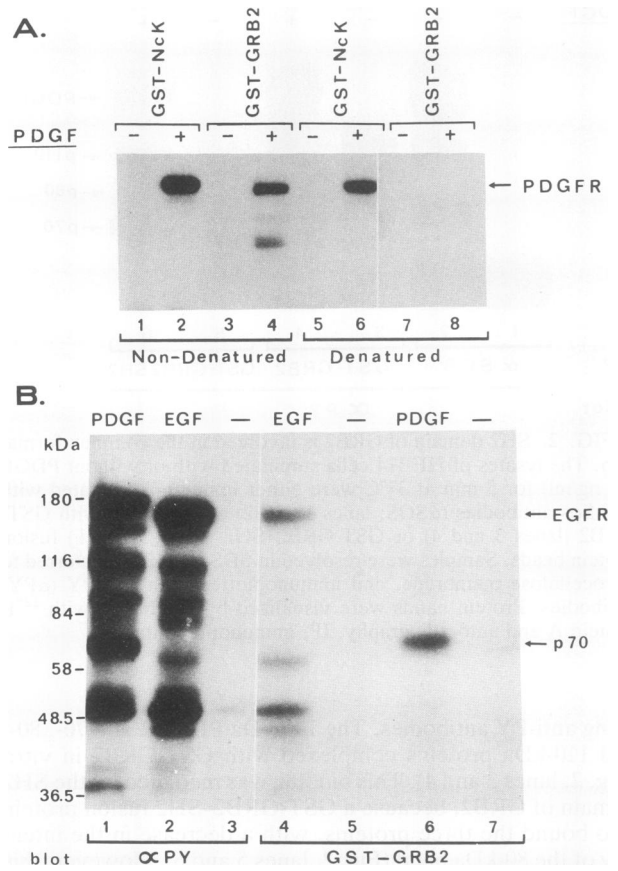

FIG. 3. GRB2 binds to EGF receptor (EGFR) directly but to PDGFR indirectly. The lysates of HER14 cells treated with or without PDGF (A and B) or EGF (B) as described in the legend to Fig. 1 were either denatured (A, lanes 5 to 8) by heating at 95°C for 5 min in the presence of SDS (1%) or not denatured (A, lanes 1 to 4, and B) prior to incubation with GST-GRB2 or GST-Nck fusion protein beads (A) or immunoprecipitation by anti-PY antibodies (B, lanes 1 to 3) or directly resolved by SDS-PAGE (B, lanes 4 to 7). Samples were analyzed by Western blot analysis with either anti-PDGFR (A) or anti-PY antibodies (αPY; B, lanes 1 to 3), followed by incubating with ^{125}I-protein A, or incubation with purified GST-GRB2 fusion protein (B, lanes 4 to 7), followed by incubation with anti-GST antibody and ^{125}I-protein A. The results were visualized by autoradiography.

to nitrocellulose membranes, and probed with purified soluble GST-GRB2 fusion protein. The proteins on the membrane which bound to the soluble GST-GRB2 were visualized by using anti-GST antibodies and then subjected to incubation with ^{125}I-protein A and autoradiography (Materials and Methods). Figure 3B shows that GRB2 binds directly to the tyrosine-phosphorylated EGF receptor and to three low-molecular-weight proteins on the membrane from EGF-stimulated cell lysate (lane 4), which most likely represent the three tyrosine-phosphorylated species of Shc oncogene products (44). GRB2 did not bind to the PDGFR on membrane; instead it bound to a 70-kDa protein (lane 6). This protein comigrates with a major PY protein in PDGF-, but not EGF-, stimulated cells (lanes 1 versus 2). These data suggest that GRB2 does not bind to the PDGFR directly but binds to a 70-kDa protein that is phosphorylated on tyrosine in response to PDGF. Although these results do not directly address the question of how PDGFR coimmunoprecipitate with GRB2, they suggested that the 70-kDa protein could serve as a linker between the activated PDGFR and GRB2.

Tyrosine 1009 of the PDGFR is involved in binding to GRB2. In order to identify the binding site(s) for GRB2 in the PDGFR, we used previously characterized TRMP cell lines which express approximately equal amounts of either wild-type or mutant PDGFR (23). The mutations studied included tyrosine (Y)-to-phenylalanine (F) substitutions at five previously identified autophosphorylation sites (Y-740, Y-751, Y-771, Y-1009, and Y-1021). We first screened these cell lines for association of the PDGFR with GRB2 protein by means of coimmunoprecipitation. Serum-starved cells were stimulated with or without PDGF and solubilized in lysis buffer. Cell lysates were immunoprecipitated with anti-GRB2 antibodies. The immunocomplexes were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-PDGFR antibodies. The wild-type, Y-740→F, Y-740/751→F, Y-771→F, and Y-1021→F PDGFRs coimmunoprecipitated with anti-GRB2 antibodies (Fig. 4A). The slight decrease of the Y-751→F mutant binding was not reproducible. The Y-1009→F mutation, however, significantly reduced the amount of PDGFR that coimmunoprecipitated with anti-GRB2 antibody (lanes 5 and 6). These results indicate that phosphorylation of the Tyr-1009 in the carboxyl terminus of the PDGFR is involved in association with GRB2.

To further explore the specificity of the interaction, we carried out in vitro binding experiments using GST-GRB2 fusion proteins. Lysates of cells treated with or without PDGF were incubated with either GST alone or GST-GRB2 fusion proteins immobilized on glutathione-agarose beads. The beads were then washed, and the samples were analyzed by SDS-PAGE and immunoblotting with anti-PDGFR antibodies as described above. As shown in Fig. 4B, the amount of the PDGFR bound to GST-GRB2 beads was significantly reduced by the Y-1009→F mutation, whereas other mutations (Y-740→F, Y-751→F, Y-771→F, and Y-1021→F) had little effect on the GRB2 binding. GST protein alone did not bind the PDGFR (35; data not shown). Furthermore, we observed that a phosphopeptide corresponding to the sequence around Y-1009 (from −3 to +9 of Y1009) inhibited GST-GRB2 association to the PDGFR in vitro (data not shown). Thus, phosphorylated Tyr-1009 in the PDGFR is a region for GRB2 association.

Various experiments have suggested that the SH2 domain of GRB2 has specificity for PY residues contained in the primary sequence pYXNX (pY refers to phosphorylated tyrosine), whereas the sequence around Tyr-1009 of the PDGFR is YTAV. GRB2 binds to Tyr-1068 (pYINQ) in the EGF receptor and to the insulin receptor substrate 1 (IRS-1), which has the consensus of YXNX at Y-727, Y-895, and Y-939 (5a, 51, 55). GRB2 also binds to Shc (44, 51), apparently through a phosphorylated YVNV motif. Screening a degenerate phosphopeptide library with the SH2 domain of GRB2–Sem-5 revealed a similar binding motif, Y(L/V/I/M)N(V/P) (53). To test whether the association of GRB2 with the PDGFR would be inhibited by synthetic phosphopeptides containing the YXN sequence, we used a phosphopeptide corresponding to the amino acid sequence of Y-939 (pYMNM) in IRS-1, and a control phosphopeptide, pY-751 (pYVPM), from the PDGFR as listed in Fig. 5B. GRB2 association to the PDGFR was inhibited by the synthetic phosphopeptide pY-939 in a dose-dependent manner (Fig. 5A, lanes 3 and 4), but not by phosphopeptide pY-751 (lane 5). As a control, we tested Nck binding: pY-939 did not affect Nck binding (lane 2), whereas pY-751 abol-

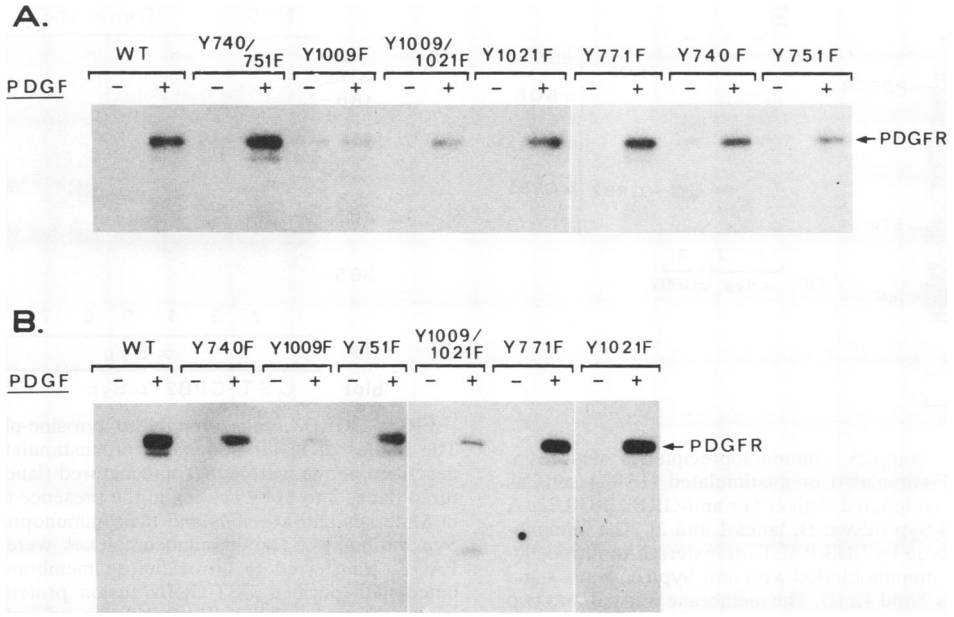

FIG. 4. Tyrosine 1009 is involved in the association of GRB2 to the PDGFR. TRMP cells expressing either wild-type or mutant PDGFR were incubated in the presence or absence of PDGF-BB (400 ng/ml) for 5 min at 37°C prior to solubilization in lysis buffer. The clarified cell lysates were either immunoprecipitated with anti-GRB2 antibodies (A) or incubated with GST-GRB2 fusion protein beads (B). The samples were analyzed by Western blot analysis with anti-PDGFR antibodies. These results represent four (A) and three (B) independent experiments. The slight reduction of Y-751→F mutant binding was not reproducible.

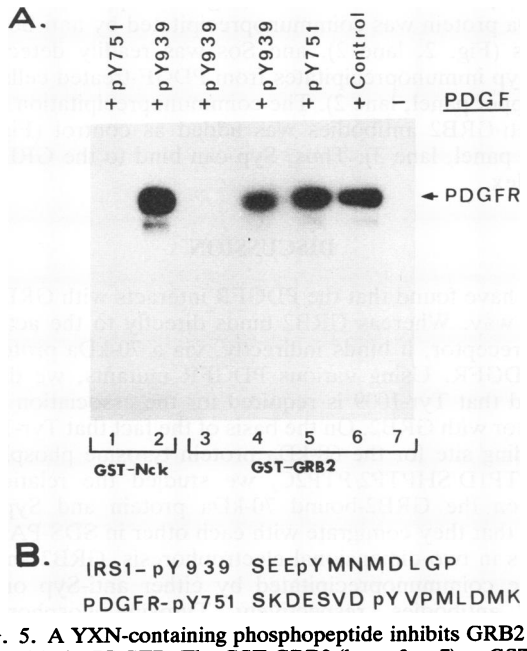

FIG. 5. A YXN-containing phosphopeptide inhibits GRB2 association with the PDGFR. The GST-GRB2 (lanes 3 to 7) or GST-Nck (lanes 1 and 2) protein beads were preincubated with (lanes 1 to 5) or without (lanes 6 and 7) the phosphopeptides (B; pY refers to phosphorylated tyrosine) for 1 h at 4°C at concentrations of either 10 μM (lane 4) or 50 μM (lanes 1, 2, 3, and 5). The lysates of PDGF-stimulated (lanes 1 to 6) or control (lane 7) HER14 cells as described above were then added to the beads for an additional hour prior to washing and Western blot analysis with anti-PDGFR antibodies.

ished the Nck binding completely (lane 1 [35]). These results indicate that association of GRB2 with the activated PDGFR can be blocked by phosphopeptides that bind tightly to the GRB2 SH2 domain but differ from the sequence around Tyr-1009, suggesting the involvement of another SH2-containing protein in linking GRB2 to the PDGFR.

GRB2 binds to a protein tyrosine phosphatase. Tyr-1009 is known to be a binding site for the 65- to 68-kDa protein tyrosine phosphatase, Syp/PTP1D/SHPTP2/PTP2C (25). Therefore, GRB2 binding to PDGFR mutants parallels the binding of Syp. We reasoned that the GRB2-bound 70-kDa protein might be related to Syp. Several observations are consistent with this. First, Syp is tyrosine phosphorylated and associated with PDGFRs in response to PDGF stimulation (16, 60). Syp also binds to activated EGF receptor but is not phosphorylated extensively in response to EGF (16, 60), consistent with the decreased binding of GST-GRB2 to the 70-kDa protein on immunoblots of EGF-treated relative to PDGF-treated lysates (Fig. 3B). Second, the 70-kDa GRB2-bound PY protein and Syp migrate similarly in SDS-PAGE. Finally, Syp has three potential GRB2 SH2 binding sites at Y-279, Y-304, and Y-546 (16). All of these sites are also conserved in PTP1C (41, 47, 62), and in the *Drosophila* homolog corkscrew (40). To test whether GRB2 binds to Syp, we utilized both coimmunoprecipitation and in vitro binding assays. The lysates of cells treated with or without PDGF were immunoprecipitated with either anti-GRB2 or anti-Syp antibodies. The immunocomplexes were resolved in SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Syp, anti-GRB2, or anti-PY antibodies. Figure 6A shows that Syp was coimmunoprecipitated by anti-GRB2 antibodies from PDGF-treated (lane 2) but not control (lane 1) cell lysates and comigrates on SDS-PAGE with the 70-kDa PY-containing protein (lane 4). Conversely, GRB2 was brought down by anti-Syp antibodies (Fig. 6B, lower panel; lane 2). Approximately 18% of the

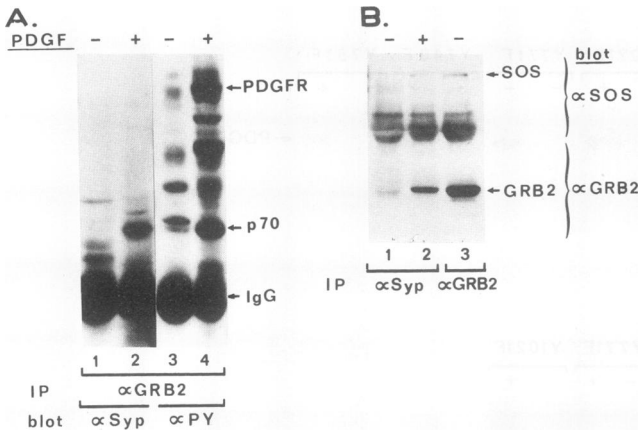

FIG. 6. GRB2-Sos complex coimmunoprecipitates with Syp. The lysates of PDGF-stimulated or unstimulated HER14 cells as above were immunoprecipitated with either anti-GRB2 (αGRB2; A and B, lane 3) or anti-Syp (αSyp; B, lanes 1 and 2). The immunocomplexes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-Syp (A, lanes 1 and 2) or anti-PY (A, lanes 3 and 4). (B) The membrane was cut into two pieces of upper and lower panels as indicated by the middle arrow, prior to blotting with anti-GRB2 (lower panel) or anti-Sos (αSOS; upper panel) antibodies. The protein bands were visualized by ^{125}I-protein A and autoradiography and quantitated by counting the excised bands in a gamma counter. IgG, immunoglobulin G; IP, immunoprecipitation.

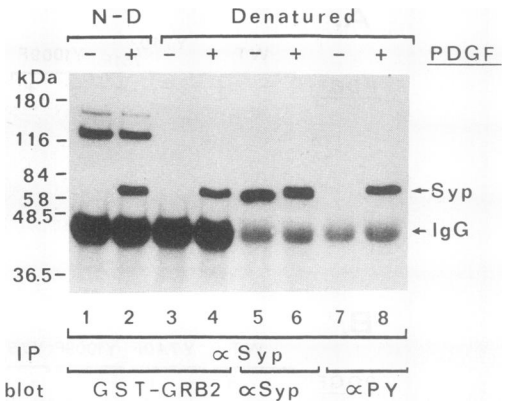

FIG. 7. GRB2 binds directly to tyrosine-phosphorylated Syp. The lysates of PDGF-stimulated or unstimulated HER14 cells as described above were either undenatured (lanes 1 and 2) or denatured (lanes 3 to 8) by heating in the presence of SDS as described in Materials and Methods and then immunoprecipitated with anti-Syp antibodies. The immunocomplexes were resolved in SDS-PAGE, transferred to nitrocellulose membrane, and either incubated with purified GST-GRB2 fusion protein (lanes 1 to 4) or immunoblotted with anti-Syp (αSyp; lanes 5 and 6) or anti-PY (αPY; lanes 7 and 8) antibodies. Protein bands were visualized by incubation with ^{125}I-protein A and by autoradiography. IgG, immunoglobulin G; IP, immunoprecipitation.

total anti-GRB2 immunoprecipitable GRB2 protein (lane 3) was coimmunoprecipitated by the anti-Syp antibodies (lane 2). Since the efficiencies of the anti-GRB2 and anti-Syp immunoprecipitations were 16 and 48%, respectively (data not shown), the actual Syp-bound GRB2 is ~6% of GRB2 protein in the total cell lysate. To test whether GRB2 can bind directly to the tyrosine-phosphorylated Syp, anti-Syp immunocomplexes from lysates of PDGF-stimulated or unstimulated cells were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with purified, soluble GST-GRB2 as previously described. GRB2 was able to bind directly to the tyrosine-phosphorylated Syp which was immobilized on the membrane (Fig. 7, lane 2). To further make sure that the 70-kDa protein immunoprecipitated by anti-Syp antibody is Syp PTPase and not some Syp-associated but unrelated protein which has the same molecular weight, protein complexes in the cell lysates were dissociated and denatured by 95°C heat and SDS treatment (Materials and Methods) prior to anti-Syp immunoprecipitation (Fig. 7, lanes 3 to 8). GRB2 was still able to bind to the tyrosine-phosphorylated Syp protein (lane 4) which was isolated from denatured cell lysates.

These data indicated that the 70-kDa protein is related to the protein tyrosine phosphatase Syp and that Syp from PDGF-treated cells is able to bind directly to GRB2. We also carried out two-dimensional gel electrophoresis analysis of the GRB2-bound 70-kDa and anti-Syp antibody-immunoprecipitated p70 from denatured cell lysates. These two molecules showed similar isoelectrofocusing values and molecular weights (data not shown). Taken together, these results indicate that the 70-kDa protein which acts as an adapter between GRB2 and the PDGFR is antigenically and physically indistinguishable from the protein tyrosine phosphatase Syp.

If Syp binds to GRB2, and provided that binding of Syp to GRB2 does not interfere with binding of GRB2 to Sos, then Syp should also bind to Sos. Anti-Sos or anti-Syp immunoprecipitates from PDGF-stimulated and control cells were immunoblotted and probed with either anti-PY (Fig. 2, lanes 1 and 2) or anti-Sos antisera (Fig. 6B, upper panel). The 70-kDa protein was coimmunoprecipitated by anti-Sos antibodies (Fig. 2, lane 2), and Sos was readily detected in anti-Syp immunoprecipitates from PDGF-treated cells (Fig. 6B, upper panel, lane 2). The coimmunoprecipitation of Sos by anti-GRB2 antibodies was added as control (Fig. 6B, upper panel, lane 3). Thus, Syp can bind to the GRB2-Sos complex.

DISCUSSION

We have found that the PDGFR interacts with GRB2 in a novel way. Whereas GRB2 binds directly to the activated EGF receptor, it binds indirectly, via a 70-kDa protein, to the PDGFR. Using various PDGFR mutants, we demonstrated that Tyr-1009 is required for the association of the receptor with GRB2. On the basis of the fact that Tyr-1009 is a binding site for the 68-kDa protein tyrosine phosphatase Syp/PTP1D/SHPTP2/PTP2C, we studied the relationship between the GRB2-bound 70-kDa protein and Syp. We found that they comigrate with each other in SDS-PAGE as well as in two-dimensional electrophoresis. GRB2 and Syp can be coimmunoprecipitated by either anti-Syp or anti-GRB2 antibodies, respectively. Tyrosine-phosphorylated Syp that was immunoprecipitated from heat- and SDS-denatured cell lysates, in which any preassociated proteins were dissociated, was able to bind directly to purified GRB2. These results indicate that the GRB2-bound 70-kDa tyrosine-phosphorylated protein is related to or identical with the 68-kDa Syp.

Three other ways by which GRB2 interacts with tyrosine kinases are known. First, GRB2 binds directly to the EGF receptor (Fig. 3B) (28, 30), and the *Drosophila* GRB2 ho-

molog, Drk, binds directly to the Sev receptor (36, 49). Second, GRB2 binds to phosphorylated Shc, a protein with a single SH2 domain that is phosphorylated on a tyrosine residue in cells transformed by v-Src or stimulated by EGF (39, 44, 50, 51). Like GRB2, Shc overexpression can drive cells to proliferate, presumably by activating Sos and the Ras pathway. Shc binds to the activated EGF receptor (39) and so would bring GRB2-Sos to the receptor. Third, the membrane protein IRS-1 is phosphorylated on several tyrosine residues in insulin-stimulated cells (55). It is then able to bind the p85 subunit of phosphatidylinositol 3-kinase, Syp, and GRB2 (3, 4, 50, 51, 58). The binding of GRB2 to Syp resembles these last two situations, in that phosphorylation of a third protein by an activated receptor tyrosine kinase generates a binding site for GRB2. In all three cases, the effect may be to recruit the GRB2-Sos complex to the membrane, where the substrate for Sos, Ras, is located. Either the translocation of Sos or an allosteric effect on Sos activity may then cause an increase in the amount of GTP-bound Ras.

The role of Syp as an adapter between the activated PDGFR and the GRB2-Sos complex in the regulation of PDGF signal transduction awaits further investigation. However, Valius and Kazlauskas found that Tyr-1009 in the PDGFR was sufficient, in the absence of other known tyrosine phosphorylation sites, for a PDGF-dependent increase in the GTP-bound Ras (59). This suggests that binding of GRB2-Sos to the PDGFR-Syp complex may activate Ras. They also showed that this interaction was unnecessary for Ras activation, suggesting Syp-independent pathways. It is possible that additional docking proteins will be involved in recruitment of GRB2-Sos to PDGF and other growth factor receptors. In this regard, it is interesting that 110- and 80-kDa PY proteins also coimmunoprecipitated with GRB2 from PDGF-treated cells, although these proteins appeared not to bind GRB2 directly.

Syp is closely related (76%) to the *Drosophila* corkscrew protein (Csw), which is maternally required for normal determination of cell fates at the termini of the embryo. Csw functions downstream of the Torso receptor tyrosine kinase, which is structurally analogous to PDGFR (9, 54) and acts as a positive regulator along the pathway (40). This pathway also requires Sos1 and Ras1 (14, 31). The position of Csw relative to Ras is not clear, but overexpression of an activated Ras1 allele can compensate for the lack of Csw, suggesting that Csw may regulate Ras in *Drosophila* embryos (31).

The ability of a PY phosphatase, Csw, to act positively on a pathway triggered by a tyrosine kinase, Torso, has been a mystery. One possibility is that the phosphatase activates a Src family kinase. Feng et al. showed that Syp becomes constitutively tyrosine phosphorylated in v-Src-transformed Rat-2 cells (16). If Syp dephosphorylates Tyr-527 of Src, or its equivalent in other members of Src family, then those kinases would be activated. However, our studies suggest a second function for Csw, Syp, and their relatives, in addition to their functions as PY phosphatases. Such adapter function may be more relevant to their positive role in signaling. Syp can act as a positive regulator along the PDGFR signaling pathway by linking the GRB2-Sos complex, which activates Ras, to the activated PDGFR in mammals. This would then activate the cascade of kinases initiated by Raf. The complex genetic relationships between the genes encoding Csw and Raf in *D. melanogaster* (40) suggest that either Drk can also bind directly to the Torso receptor or that Csw has a second signaling function, perhaps involving its phosphatase activity.

The mechanism(s) by which each growth factor receptor controls its specificity of signaling is still poorly understood. We proposed previously that the activity of a given receptor tyrosine kinase equals the sum of the activities of the signaling proteins that interact with its tyrosine-phosphorylated form (46). The notion presented in this paper, in which the GRB2-Sos complex is linked to the PDGFR via an adapter protein that has its own catalytic activity, indicates the complexity of interactions involved.

ACKNOWLEDGMENTS

W.L. and R.N. made equal contributions to this work.

We thank Gen-Sheng Feng and Tony Pawson for providing anti-Syp antiserum and Janice Small and Jenny Torgerson for secretarial assistance. We thank Andrius Kazlauskas and Ben Neel for interesting discussions.

This work was supported by a grant from SUGEN Inc. to J.S., NIH grant CA-54786 to J.A.C., and ACS institutional grant IRG-41-34 to W.L.

REFERENCES

1. **Ahmad, S., D. Banville, Z. Zhao, E. H. Fischer, and S.-H. Shen.** 1993. A widely expressed human protein-tyrosine phosphatase containing src homology 2 domains. Proc. Natl. Acad. Sci. USA **90**:2197–2201.
2. **Alida, M., M. de Vries-Smits, B. M. T. Burgering, S. L. Leevers, C. J. Marshall, and J. L. Bos.** 1992. Involvement of p21ras in activation of extracellular signal-regulated kinase 2. Nature (London) **357**:602–605.
3. **Backer, J. M., M. G. Myers, S. E. Shoelson, O. J. Chin, X.-J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. D. Skolnick, J. Schlessinger, and M. F. White.** 1992. Insulin receptor substrate IRS-1 associates with and activates Ptdins-3-kinase. EMBO J. **11**:3469–3479.
4. **Baltensperger, K., L. M. Kozma, A. D. Cherniack, J. K. Klarlund, A. Chawla, U. Banerjee, and M. P. Czech.** 1993. Binding of the Ras activator son of Sevenless to insulin receptor substrate-1 signaling complexes. Science **260**:1950–1953.
5. **Barbacid, M. A.** 1987. Ras oncogenes. Annu. Rev. Biochem. **56**:779–827.

5a. **Batzer, A., et al.** Submitted for publication.

6. **Buday, L., and J. Downward.** 1993. Epidermal growth factor regulates p21 ras through the formation of a complex of receptor, Grb2 adaptor protein, and Sos nucleotide exchange factor. Cell **73**:611–620.
7. **Cai, H., J. Szeberenyi, and G. M. Cooper.** 1990. Effect of a dominant inhibitory Ha-*ras* mutation on mitogenic signal transduction in NIH 3T3 cells. Mol. Cell. Biol. **10**:5314–5323.
8. **Cantley, L. L., K. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64**:281–302.
9. **Casanova, J., and G. Struhl.** 1989. Localized surface activity of Torso, a receptor tyrosine kinase specifies terminal body pattern in Drosophila. Genes Dev. **3**:2025–2038.
10. **Chardin, P., J. Camonis, W. L. Gale, L. Van Aelst, J. Schlessinger, M. H. Wigler, and D. Bar-Sagi.** 1993. Human Sos1: a guanine nucleotide exchange factor for Ras that binds to GRB2. Science **260**:1338–1343.
11. **Clark, S. G., M. J. Stern, and H. R. Horvitz.** 1992. C. elegans cell-signaling gene sem-5 encodes a protein with SH2 and SH3 domains. Nature **356**:340–344.
12. **Cooper, J., and T. Hunter.** 1985. Major substrate for growth factor-activated protein tyrosine kinases is a low-abundance protein. Mol. Cell. Biol. **5**:3304–3309.
13. **Cooper, J. A., and A. Kashishian.** 1993. In vivo binding properties of SH2 domains from GTPase activating protein and phosphatidylinositol 3-kinase. Mol. Cell. Biol. **13**:1737–1745.
14. **Doyle, H. J., and J. M. Bishop.** 1993. Torso, a receptor tyrosine kinase required for embryonic pattern formation, shares sub-

strates with the Sevenless and EGF-R pathways in Drosophila. Genes Dev. 7:633–646.
15. **Egan, S. E., B. W. Giddings, M. W. Brooks, L. Buday, A. M. Sizeland, and R. Weinberg.** 1993. Association of Ras exchange protein Sos with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature (London) **363:**45–51.
16. **Feng, G. S., C. C. Hui, and T. Pawson.** 1993. SH2-containing phosphotyrosine phosphatase as a target of protein tyrosine kinases. Science **259:**1607–1610.
17. **Freeman, R. M., Jr., J. Plutzky, and B. G. Neel.** 1992. Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of Drosophila corkscrew. Proc. Natl. Acad. Sci. USA **89:**11239–11243.
18. **Gale, W. N., D. Kaplan, E. J. Lowenstein, J. Schlessinger, and D. Bar-Sagi.** 1993. Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. Nature (London) **363:** 88–92.
19. **Garrels, J.** 1979. Two-dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. J. Biol. Chem. **254:**7961–7977.
20. **Gibbs, J. B., M. S. Marshall, E. M. Scolnick, R. A. F. Dixon, and U. S. Vogel.** 1990. Modulation of guanine nucleotides bound to Ras in NIH 3T3 cells by oncogenes, growth factors, and the GTPase activating protein (GAP). J. Biol. Chem. **265:**20437–20442.
21. **Honegger, A. M., T. J. Dull, S. Felder, E. Van Obberghen, F. Bellot, D. Szapary, A. Schmidt, A. Ullrich, and J. Schlessinger.** 1987. Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. Cell **5:**199–209.
22. **Kashishian, A., and J. A. Cooper.** 1993. Phosphorylation sites at the C-terminus of the platelet-derived growth factor receptor bind phospholipase Cγ1. Mol. Biol. Cell **4:**49–57.
23. **Kashishian, A., A. Kazlauskas, and J. A. Cooper.** 1992. Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase in vivo. EMBO J. **11:**1373–1382.
24. **Kazlauskas, A., and J. A. Cooper.** 1989. Autophosphorylation of the PDGF receptor in the kinase insert region regulates interaction with cellular proteins. Cell **58:**1121–1133.
25. **Kazlauskas, A., G. S. Feng, T. Pawson, and M. Valius.** 1993. The 64 kDa protein that associates with the PDGF receptor subunit via tyrosine 1009 is the SH2-containing phosphotyrosine phosphatase Syp/SHPTP2/PTP1D/SHPTP3. Proc. Natl. Acad. Sci. USA. **90:**6939–6942.
26. **Leevers, S. J., and C. J. Marshall.** 1992. Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoprotein. EMBO J. **11:**569–574.
27. **Li, B., D. Kaplan, H. Kung, and T. Kamata.** 1992. Nerve growth factor stimulation of the Ras-guanine nucleotide exchange factor and GAP activities. Sciences **256:**1456–1459.
28. **Li, N., A. Batzer, R. Daly, V. Yajnik, E. Skolnik, P. Chardin, D. Bar-Sagi, B. Margolis, and J. Schlessinger.** 1993. Guanine nucleotide releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signaling. Nature (London) **363:**85–88.
29. **Li, W., P. Hu, E. Y. Skolnick, A. Ullrich, and J. Schlessinger.** 1992. SH2 and SH3 containing Nck protein is oncogenic and common target for different surface receptors. Mol. Cell. Biol. **12:**5824–5833.
30. **Lowenstein, E. J., R. J. Daly, A. G. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, and J. Schlessinger.** 1992. The SH2 and SH3-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell **70:**431–442.
31. **Lu, X., T.-B. Chou, N. G. Williams, T. Roberts, and N. Perrimon.** 1993. Control of cell fate determination by p21ras/Ras1, an essential component of torso signaling in Drosophila. Genes Dev. **7:**621–632.
32. **Matuoka, K., M. Shibata, A. Yamakawa, and T. Takenawa.** 1992. Cloning of ASH, a ubiquitous protein composed of one Src homology region (SH) 2 and two SH3 domains, from human and rat cDNA libraries. Proc. Natl. Acad. Sci. USA **89:**9015–9019.
33. **Mayer, B. J., and D. Baltimore.** 1993. Signaling through SH2 and SH3 domains. Trends Cell Biol. **3:**8–13.
34. **Mulcahay, L. S., M. R. Smith, and D. W. Stacey.** 1985. Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature (London) **313:**241–243.
35. **Nishimura, R., W. Li, A. Kashishian, A. Mondino, M. Zhou, J. Cooper, and J. Schlessinger.** 1993. Two signaling molecules share a phosphotyrosine-containing binding site in the platelet-derived growth factor receptor. Mol. Cell. Biol. **13:**6889–6896.
36. **Olivier, J. P., T. Raabe, M. Henkemyer, B. Dickson, G. Mbamalu, B. Margolis, J. Schlessinger, E. Hafen, and T. Pawson.** 1993. A Drosophila SH2-SH3 adapter protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange Sos. Cell **73:**179–191.
37. **Pawson, T., and D. D. Gish.** 1992. SH2 and SH3 domains; from structure to function. Cell **72:**359–362.
38. **Pawson, T., and J. Schlessinger.** 1993. SH2 and SH3 domains: protein modules that determine specificity of protein interaction in signal transduction. Curr. Biol. **3:**434–442.
39. **Pelicci, G., L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Forni, I. Nicoletti, F. Grignani, T. Pawson, and P. G. Pelicci.** 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. Cell **70:**93–104.
40. **Perkins, L. A., I. Larsen, and N. Perrimon.** 1992. Corkscrew encodes a putative protein tyrosine phosphatase that functions to transduce the terminal signal from the receptor tyrosine kinase torso. Cell **70:**225–236.
41. **Plutzky, J., B. G. Neel, and R. D. Rosenberg.** 1992. Isolation of a src homology 2-containing tyrosine phosphatase. Proc. Natl. Acad. Sci. USA **89:**1123–1127.
42. **Polakis, P., and F. McCormick.** 1993. Structural requirements for the interaction of $p21^{ras}$ with GAP, exchange factors, and its biological effector target. J. Biol. Chem. **268:**9157–9160.
43. **Rozakis-Adcock, M., R. Fernley, S. Wade, T. Pawson, and D. Bowtell.** 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF-receptor to the Ras activator mSos. Nature (London) **363:**83–85.
44. **Rozakis-Adcock, M., J. McGlade, G. Mbamalu, G. Pelicci, R. Dely, W. Li, A. Batzer, S. Thomas, J. Brugge, P. G. Pelicci, J. Schlessinger, and T. Pawson.** 1992. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. Nature (London) **360:**689–692.
45. **Satoh, T., M. Endo, M. Nakafuku, S. Nakamura, and Y. Kaziro.** 1990. Platelet-derived growth factor stimulated formation of active p21ras GTP in Swiss mouse 3T3 cells. Proc. Natl. Acad. Sci. USA **87:**5993–5997.
46. **Schlessinger, J., and A. Ullrich.** 1992. Growth factor signaling by receptor tyrosine kinases. Neuron. **9:**383–391.
47. **Shen, S.-H., L. Bastein, B. I. Posner, and P. Chretein.** 1991. A protein-tyrosine phosphatase with sequence similarity to the SH2 domains of protein-tyrosine kinases. Nature (London) **352:**726–739.
48. **Simon, M. A., D. D. L. Bowtell, G. S. Dodson, T. R. Laverty, and G. M. Rubin.** 1991. Ras1 and putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. Cell **67:**701–716.
49. **Simon, M. A., G. S. Dodson, and G. M. Rubin.** 1993. An SH3-SH2-SH3 protein is required for p21Ras1 activation and binds to Sevenless and Sos proteins in vitro. Cell **73:**169–177.
50. **Skolnik, E. Y., A. Batzer, N. Li, C. H. Lee, E. Lowenstein, M. Mohammadi, B. Margolis, and J. Schlessinger.** 1993. The function of Grb2 in linking the insulin receptor to Ras signaling pathways. Science **260:**1953–1957.
51. **Skolnik, E. Y., C.-H. Lee, A. Batzer, L. M. Vicentini, M. Zhou, R. Daly, M. J. Myers, Jr., J. M. Backer, A. Ullrich, M. F. White, and J. Schlessinger.** 1993. The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of ras signalling. EMBO J. **12:**1929–1936.
52. **Smith, M. R., S. J. DeGudicubus, and D. W. Stacey.** 1986. Requirement for c-ras proteins during viral oncogene transfor-

mation. Nature (London) **320**:540–543.

53. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider, B. G. Neel, R. B. Birge, J. E. Fajardo, M. M. Chou, H. Hanafusa, B. Schaffhausen, and L. C. Cantley.** 1993. SH2 domains recognize specific phosphopeptide sequences. Cell. **72**:767–778.
54. **Sprenger, F., L. M. Stevens, and C. Nusslein-Volhard.** 1989. The Drosophila gene Torso encodes a putative receptor tyrosine kinase. Nature (London) **338**:478–483.
55. **Sun, X.-J., P. Rothenberg, C. R. Kahn, J. M. Backer, E. Araki, P. A. Wilden, D. A. Cahill, J. Goldstein, and M. F. White.** 1991. Structure of the insulin receptor substrate IRS-1 defines an unique signal transduction protein. Nature (London) **352**:73–77.
56. **Szeberenyi, J., H. Cai, and G. M. Cooper.** 1990. Effect of a dominant inhibitory Ha-*ras* mutation on neuronal differentiation of PC12 cells. Mol. Cell. Biol. **10**:5324–5332.
57. **Thomas, S. M., M. DeMarco, G. D'Arcangelo, S. Halegoua, and J. Brugge.** 1992. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of Map kinases. Cell **68**:1030–1040.
58. **Tobe, K., K. Matuoka, H. Tamemoto, K. Ueki, Y. Kaburagi, S. Asai, T. Noguchi, M. Matsuda, S. Tanaka, S. Hattori, Y. Fukui, Y. Akanuma, Y. Yazaki, T. Takenawa, and T. Kadowaki.** 1993. Insulin stimulates association of insulin receptor substrate-1 with the protein abundant Src homology/growth factor receptor-bound protein 2. J. Biol. Chem. **268**:1167–1171.
59. **Valius, M., and A. Kazlauskas.** 1993. Phospholipase C-γ1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. Cell **73**:321–334.
60. **Vogel, W., R. Lammers, J. Houng, and A. Ullrich.** 1993. Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. Science **259**:1611–1614.
61. **Wood, K. W., C. Sarnecki, T. M. Roberts, and J. Blenis.** 1992. Ras mediates nerve growth factor receptor modulation of three signal-transducing molecules: Map kinase, Raf-1, and Rsk. Cell **68**:1041–1050.
62. **Yi, T., J. L. Cleveland, and J. N. Ihle.** 1992. Protein tyrosine phosphatase containing SH2 domain: characterization, preferential expression in hematopoietic cells and localization to human chromosome 12p12p-13. Mol. Cell. Biol. **12**:836–846.
63. **Zhang, K., A. G. Papageorge, and D. R. Lowy.** 1992. Mechanistic aspects of signaling through ras in NIH 3T3 cells. Science **257**:671–674.