Platelet-derived growth factor-dependent association of the GTPase-activating protein of Ras and Src

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Here we report that the platelet-derived growth factor β receptor (βPDGFR) is not the only tyrosine kinase able to associate with the GTPase-activating protein of Ras (RasGAP). The interaction of non-βPDGFR kinase(s) with RasGAP was dependent on stimulation with platelet-derived growth factor (PDGF) and seemed to require tyrosine phosphorylation of RasGAP. Because the tyrosine phosphorylation site of RasGAP is in a sequence context that is favoured by the Src homology 2 ('SH2') domain of Src family members, we tested the possibility that Src was the kinase that associated with RasGAP. Indeed, Src interacted with phosphorylated RasGAP fusion proteins; immunodepletion of Src markedly decreased the recovery of the RasGAP-associated kinase activity. Thus PDGF-dependent tyrosine phosphorylation of RasGAP results in the formation of a complex between RasGAP and Src. To begin to address the relevance of these observations, we focused on the consequences of the interaction of Src and RasGAP. We found that a receptor mutant that did not activate Src was unable to efficiently mediate the tyrosine phosphorylation of phospholipase Cγ (PLCγ). Taken together, these observations support the following hypothesis. When RasGAP is recruited to the βPDGFR, it is phosphorylated and associates with Src. Once bound to RasGAP, Src is no longer able to promote the phosphorylation of PLCγ. This hypothesis offers a mechanistic explanation for our previously published findings that the recruitment of RasGAP to the βPDGFR attenuates the tyrosine phosphorylation of PLCγ. Finally, these findings suggest a novel way in which RasGAP negatively regulates signal relay by the βPDGFR.

Key words: phospholipase Cγ, phosphorylation, platelet-derived growth factor receptor.

INTRODUCTION

Ligand stimulation of the platelet-derived growth factor β receptor (βPDGFR) leads to receptor dimerization, activation of the kinase activity of the receptor and phosphorylation of the receptor at numerous intracellular tyrosine residues. Molecules containing Src homology 2 (SH2) domains are then recruited to the βPDGFR where they act as signalling enzymes and/or as adapter molecules [1,2]. Examples of signalling enzymes include the GTPase-activating protein of Ras (RasGAP), phospholipase Cγ (PLCγ) and Src family members; the adapter proteins include Shc, Shb, Grb2 and Nck [1,2].

The βPDGFR is essential to mammalian development, as shown by the prenatal death of βPDGFR-deficient mice; these findings indicate that at least a subset of the signalling pathways downstream of the βPDGFR are critical for development. Other studies reveal that the βPDGFR is involved in the causation and/or progress of disease. For example, v-Sis, the transforming protein of simian sarcoma virus, is functionally identical with platelet-derived growth factor (PDGF) B, a tel-βPDGFR fusion protein is involved in a subgroup of patients with chronic myelomonocytic leukaemia, and transformation mediated by type 1 bovine papilloma virus E5 protein requires functional βPDGFR. In addition, neointimal accumulation of smooth-muscle cells associated with the formation of atherosclerotic plaques seems to involve signalling of the βPDGFR [1,2]. These examples of disease are correlated with the transforming effect of constitutive activation of the PDGF pathway in cultured cells [3–5] and indicate that tight control of PDGF signalling pathways is essential for the growth and health of higher organisms.

The importance of specific effectors of the βPDGFR in mitogenic signal transduction has been extensively studied. Our previous studies with βPDGFR mutants showed that PLCγ activation is sufficient, but not necessary, for most βPDGFR induced-DNA synthesis in fibroblasts and epithelial cells [6,7]. An earlier study also found that PLCγ is not necessary for PDGF-induced DNA synthesis [8]; however, microinjecting reagents that inhibit the function of the SH2 domains of PLCγ inhibited PDGF-dependent DNA synthesis [9]. Purified PLCγ seems to be able to induce DNA synthesis when microinjected into quiescent fibroblasts [10], however, this induction might be independent of phospholipase activity and dependent on the SH3 domain of PLCγ [11,12]. Although these studies have not reached a consensus on the necessity of PLCγ in the mitogenic response to PDGF, PLCγ has been shown to be necessary for mammalian development because mice generated with homologous deletions of PLCγ died by embryonic day 9 [13].

RasGAP inactivates Ras and thereby participates in a variety of signalling systems. There is evidence that RasGAP function extends beyond its ability to modulate Ras. RasGAP contains several domains that are not required for promoting the GTPase activity of Ras. Recent genetic studies indicate that RasGAP contributes to the regulation of growth in a Ras-independent fashion [14].

We have previously found that the recruitment of RasGAP suppresses the phosphoinositol 3-kinase (PI3K) and PLCγ path-

Abbreviations used: GST, glutathione S-transferase; KI, kinase insert; PDGF, platelet-derived growth factor (AA and BB indicate different isoforms of PDGF); PDGFR, PDGF receptor; αPDGFR, PDGF α receptor; βPDGFR, PDGF β receptor; PI3K, phosphoinositol 3-kinase; PH, pleckstrin homology; PLCγ, phospholipase Cγ; RasGAP, GTPase-activating protein of Ras; SH2, Src homology 2; WT, wild-type.

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ways [15,16]. For PI3K, the mechanism involves decreasing the levels of active Ras. RasGAP blocks PLCγ by a different route, by preventing the tyrosine phosphorylation of PLC. The existing knowledge of how RasGAP participates in signal relay pathways does not immediately suggest a molecular mechanism for the attenuation of PLCγ tyrosine phosphorylation by RasGAP.

In the present study we investigated (1) the mechanism by which PLCγ is tyrosine-phosphorylated after stimulation of the βPDGFR and (2) the mechanism by which RasGAP attenuates the ability of the receptor to mediate the tyrosine phosphorylation of PLCγ. Our findings indicate that the receptor is probably not phosphorylating PLCγ and that Src family members or a Src-activated kinase accomplish this task. In addition, the results suggest a model of how RasGAP inhibits the tyrosine phosphorylation of PLCγ. When RasGAP is phosphorylated, it binds Src family members and functionally sequesters them from substrates such as PLCγ. These observations reveal a previously unknown role for RasGAP in signalling by receptor tyrosine kinases and a novel mechanism for the regulation of Src family members.

EXPERIMENTAL

Cell lines and PDGF receptor (PDGFR) mutants

HepG2 cells expressing approx. 5×10^5 receptors per cell and TRMP cells expressing approx. 10^6 receptors per cell were cultured exactly as described previously [6]. The Patch cell line (Ph), which naturally express approx. 10^5 βPDGFRs per cell, but no PDGF α receptors (αPDGFRs), were a gift from Dan Bowen-Pope (University of Washington, Seattle, WA, U.S.A.). The Ph cells were obtained from embryos of *Ph*/*Ph* mice that have a deletion including the αPDGFR gene. They were maintained exactly as described previously [7].

Construction of the F5, Y771, Y1021 [6], Y21/71 [15], and N2WT chimaeras and the N2F72/74 chimaera [17] have been described previously.

Antibodies

The PDGFR and RasGAP antisera used in these studies were crude polyclonal rabbit antisera raised against glutathione S-transferase (GST) fusion proteins; they have been described previously [15,18]. The antibody (58.5) used to purify the fusion proteins from bacterial cell lysates was a crude rabbit polyclonal antibody recognizing GST. The PLCγ antisera used for immunoprecipitation was a mixture of crude polyclonal rabbit antisera raised against a GST fusion protein including residues 550–850 of rat PLCγ. The mixture of PLCγ monoclonal antibodies used for Western blotting and the anti-phosphotyrosine antibody (4G10) were purchased from Upstate Biotechnology and used in accordance with the manufacturer's directions. Src-2 is a rabbit polyclonal antibody; it was purchased from Santa Cruz Biotechnology and was used in accordance with the manufacturer's directions.

Western blot analysis

The Western blot shown in Figure 1(B) was performed exactly as described previously [19]. All other Western blots were performed with an identical procedure, except that after the primary antibody, blots were probed with a horseradish-peroxidase-conjugated secondary antibody. The blots were then developed with Western blot detection reagents [enhanced chemiluminescence (Amersham) or Renaissance (NEN)].

Kinase assay *in vitro*

The kinase activity of βPDGFR immunoprecipitates was analysed exactly as described previously [18]. Immunoprecipitate from approx. 10^5 cells were used in a kinase assay containing 1.0 μg of GST–PLCγ (residues 550–850 of rat PLCγ) as an exogenous substrate. The kinase activity in RasGAP immunoprecipitates was assayed similarly except that immunoprecipitates from approx. 10^6 cells were subjected to the analysis. The kinase activity of proteins purified with Sepharose-coupled peptides or glutathione–agarose-coupled fusion proteins was measured as for the immunoprecipitates. Peptides or fusion proteins (10 μg) were incubated with the lysate from approx. 2×10^6 cells. Treatment of SDS/PAGE gels with alkali was performed by incubating the gel in 1 M KOH for 2 h at 56 °C.

Fusion proteins

The kinase insert (KI) fusion protein has been described previously [18]. The SHP-2 fusion protein contains the C-terminal 44 residues of SHP-2. The RasGAP-fusion protein (residues 444–477 of human RasGAP) was amplified by PCR with the introduction of *Eco*R1 and *Bam*H1 restriction sites. This permitted in-frame subcloning into the pGEX-3X expression vector. The fusion proteins were phosphorylated by infecting bacteria expressing the fusion proteins with bacteriophage encoding the receptor tyrosine kinase Elk1, as described previously [20]. Phosphorylation was assessed by anti-phosphotyrosine Western blot analysis (results not shown). Fusion proteins were purified from bacterial cell lysates on a glutathione–agarose affinity column, or by immunoprecipitation with an antibody (58.5) that recognizes GST.

Peptides

Purified tyrosine-phosphorylated and unphosphorylated peptides corresponding to residues 454–468 of human RasGAP were synthesized by the Macromolecular Resources Center (Colorado State University, Fort Collins, CO, U.S.A.). Tyrosine phosphorylation was assessed by anti-phosphotyrosine Western blot analysis of the peptide solution spotted on nitrocellulose (results not shown). Y988 peptide is RVDSDNAY^{988}IGVTYK, which corresponds to the sequence surrounding Tyr-988 of human αPDGFR. Peptides were coupled to Sepharose by treating CNBr-Sepharose with 1 mM HCl, washing it with 0.1 M $NaHCO_3$, then incubating 5 mg of peptide with Sepharose in 0.1 M $NaHCO_3$ for 8 h at room temperature. The Sepharose was then centrifuged and the A_{280} of the supernatant was read to assess the efficiency of coupling. Sepharose was then incubated with 1 M ethanolamine, pH 8.0, for 1.5 h at room temperature, washed four times with 0.2 M acetate (36 mM sodium acetate/164 mM acetic acid)/0.5 M NaCl buffer (pH 4.0), then twice in PBS, and stored in PBS with 0.02 % sodium azide.

Phosphoamino acid analysis

Phosphorylated proteins were identified by autoradiography, excised from the SDS/PAGE gel, eluted and hydrolysed in 5.7 M HCl at 110 °C for 1 h. Phosphoamino acids were resolved in two dimensions electrophoretically, as described previously [21].

RasGAP-associated kinase activity

HepG2 cells expressing the wild-type (WT) βPDGFR were grown to 80 % confluence, serum-starved in Dulbecco's modified

Eagle's medium and then exposed to buffer (10 mM acetic acid/2 mg/ml BSA) or stimulated with 40 ng/ml PDGF BB for 5 min at 37 °C. The cells were washed, lysed in an extraction buffer and centrifuged to remove the insoluble debris, as described previously [22]. Lysates representing 1.2×10^6 cells were immunodepleted of RasGAP by three successive rounds of immunoprecipitation with an antibody (70.3) against RasGAP. The lysates that were not immunodepleted were subjected to mock immunoprecipitation in which the antibody was omitted. The resulting lysates were incubated with approx. 2.5 μg of the fusion proteins immunoprecipitated from the bacterial cell lysates at 4 °C for 1.5 h and washed, as described previously [19]. Immunoprecipitates representing approx. 10^5 cells were subjected to a kinase activity *in vitro* in the presence of an exogenous substrate, GST–PLCγ, as described previously [18], resolved with SDS/PAGE and detected by autoradiography. The bands were quantified with a PhosphorImager and Imagequant software (Molecular Dynamics).

RESULTS

RasGAP associates with a kinase in PDGF-stimulated cells

To test the idea that RasGAP associates with tyrosine kinase activity, we immunoprecipitated RasGAP from resting or PDGF-stimulated cells and subjected the samples to a kinase assay *in vitro*. Kinases were present in the RasGAP immunoprecipitates from PDGF-stimulated cells, as evidenced by the phosphorylation of the exogenous substrate (GST–PLCγ), RasGAP itself and a number of other proteins (Figure 1A). The kinase activity was not detected in RasGAP immunoprecipitates prepared from resting cells. Because PDGF promotes the association of RasGAP with the βPDGFR, it is possible that the kinase present in the RasGAP immunoprecipitate was the βPDGFR. Indeed, Western blot analysis of the RasGAP immunoprecipitates demonstrated that the βPDGFR was present in the stimulated samples (Figure 1B). However, the amount of receptor that was present in the RasGAP immunoprecipitate did not fully account for the amount of kinase activity. Receptor immunoprecipitates with even more receptor than was present in the RasGAP samples displayed only 25–30 % of the kinase activity. Consequently it seemed that the kinase activity present in the RasGAP immunoprecipitate was not solely due to the associating βPDGFR. There was no receptor present in lane 3 of Figure 1(B) because RasGAP does not bind the βPDGFR at detectable levels in a resting cell. To test whether the polyclonal anti-βPDGFR serum interfered with kinase activity in the kinase assay *in vitro*, we immunoprecipitated the receptor with a polyclonal antiserum directed to a different region of the receptor

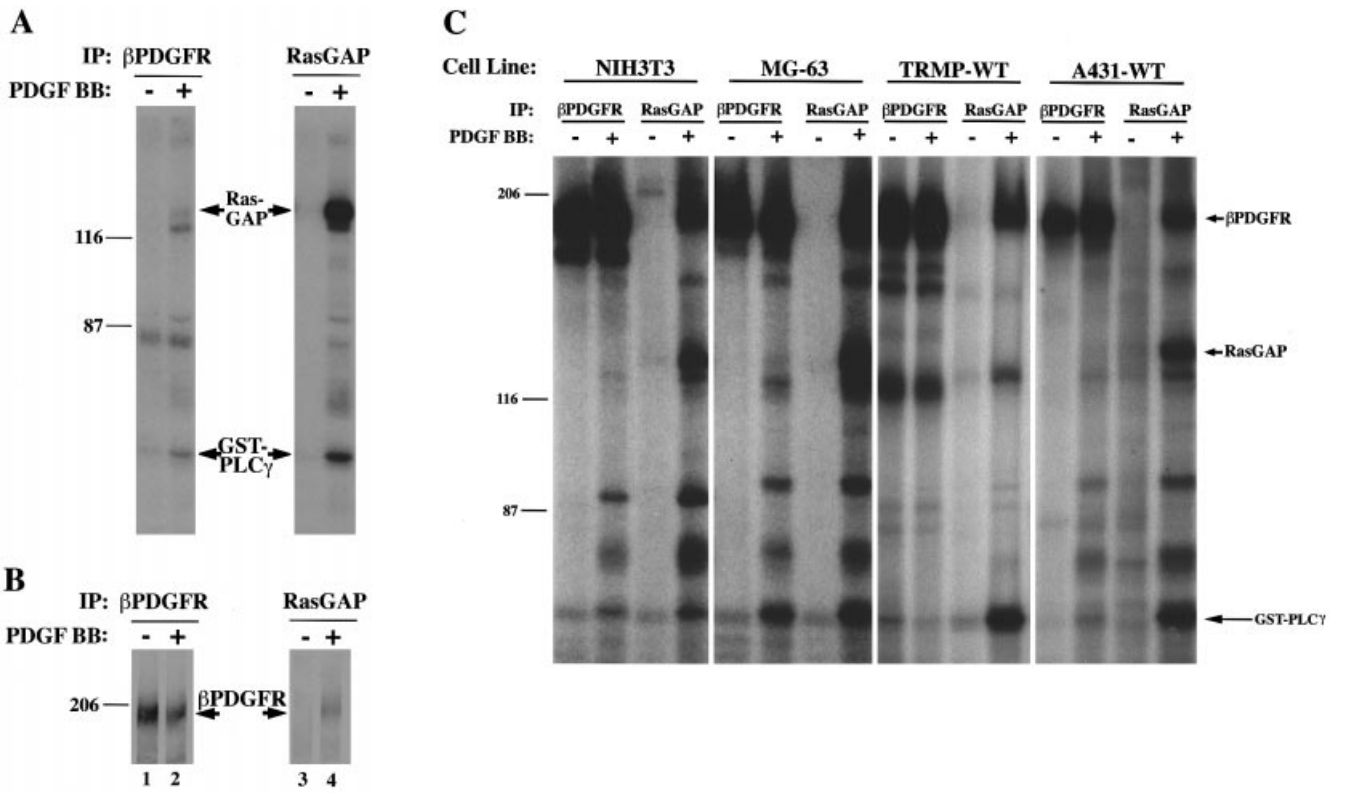

Figure 1 Kinase activity present in βPDGFR and RasGAP immunoprecipitates

(**A**) Confluent, quiescent cultures of HepG2 cells expressing the WT βPDGFR were left resting (−) or were stimulated (+) with 50 ng/ml PDGF BB for 5 min at 37 °C. The cells were lysed; the lysates were immunoprecipitated (IP) with either anti-βPDGFR serum (30A) or anti-RasGAP serum (70.3). βPDGFR immunoprecipitates from approx. 10^5 cells or RasGAP immunoprecipitates from approx. 10^6 cells were subjected to a kinase assay *in vitro* in the presence of an exogenous substrate (GST–PLCγ). SDS/PAGE was then used to resolve the samples. The portion of the gel below approx. 160 kDa was treated with alkali and subjected to autoradiography. RasGAP and the exogenous substrate (GST–PLCγ) are indicated with arrows. (**B**) The portion of the gel containing proteins greater than approx. 160 kDa was transferred to Immobilon and subjected to Western blot analysis with anti-βPDGFR serum (30A). Arrows point to the βPDGFR. (**C**) The same experiment as that described for HepG2 cells was performed with the indicated cell lines. MG-63 is a human osteosarcoma cell line that, like NIH 3T3 cells, naturally expresses both the αPDGFR and βPDGFR. TRMP is a dog kidney epithelial cell line; A431 is an epithelioid cell line. The human βPDGFR was expressed ectopically in both of these cell lines, as previously described [6,15,20]. The positions of molecular mass markers are indicated (in kDa) at the left.

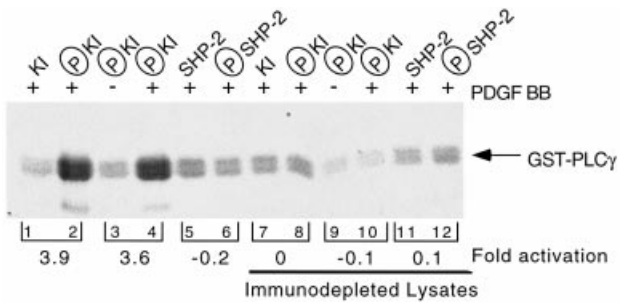

Figure 2 Kinase activity associated with the receptor–KI fusion protein

Confluent, quiescent cultures of HepG2 cells expressing the wild-type βPDGFR were left resting (−) or were stimulated (+) with 40 ng/ml PDGF BB at 37 °C for 5 min. The cells were washed and lysed; half of the lysates (representing approx. 1.2×10^6 cells) were immunodepleted of RasGAP and the other half were subjected to a mock immunodepletion as described in the Materials and methods section. The immunodepleted lysates and their native counterparts were incubated with 2.5 μg of phosphorylated (P) or unphosphorylated GST fusion protein that included the KI of the βPDGFR or the C-terminus of SHP-2 (SHP-2). The fusion proteins were recovered, washed and subjected to a kinase assay *in vitro* in the presence of an exogenous substrate (GST–PLCγ). The proteins were resolved by SDS/PAGE; the extent of substrate phosphorylation was detected by autoradiography. The bands were quantified with a PhosphorImager and are expressed as fold activation.

(the KI rather than the tail), and the samples immunoprecipitated with the RasGAP antiserum still had more kinase activity (results not shown).

To determine whether the RasGAP immunoprecipitates associated with kinase activity after stimulation with PDGF in cells other than the HepG2 cells harbouring introduced βPDGFRs, we assayed several additional cell types, including NIH 3T3 and MG63, which naturally express the αPDGFR and βPDGFR, and TRMP and A431 cells, into which we had introduced the receptor [15,23]. In all cell types we found that when RasGAP was isolated from PDGF-stimulated cells, the amount of kinase activity in the immunoprecipitates could not be fully accounted for by the presence of the receptor itself (Figure 1C). These studies suggested that PDGF stimulates the formation of a complex between RasGAP and one or more kinases other than the βPDGFR, and that these events are not restricted to a single cell type or to settings in which the receptor is expressed ectopically.

To begin to identify the non-PDGFR kinase that associated with RasGAP, we sought to recover RasGAP without the PDGFR and thereby to eliminate the receptor and proteins that might be associating via the receptor. To this end, a GST fusion protein was constructed in which GST was fused to the KI region (residues 698–797) of the WT human βPDGFR. The KI was chosen because it contains Tyr–771 as well as the surrounding sequences, which are important for RasGAP binding. Furthermore, when this fusion protein is tyrosine phosphorylated it is capable of binding RasGAP [20]. This fusion protein was phosphorylated by co-expression with an activated tyrosine kinase in bacteria, then either the phosphorylated or unphosphorylated fusion proteins were purified by immunoprecipitation with an antibody that recognized GST; the resulting immune complexes were then incubated with lysate from resting or PDGF-stimulated cells. After being washed, the samples were subjected to a kinase assay *in vitro* with GST–PLCγ as an exogenous substrate. As shown in lanes 1 and 2 of Figure 2, there was 3.9-fold more kinase activity associated with the phosphorylated fusion protein than with the unphosphorylated fusion protein. To obtain an idea of the selectivity of the kinase for phosphorylated fusion proteins, we did the same experiment with a GST fusion protein including the C-terminal 44 residues of human SHP-2. This region of SHP-2 has only one tyrosine residue, which is within a consensus Grb2-binding site (SARVY^{580}ENVG). This fusion protein bound to Grb2 when tyrosine phosphorylated (results not shown) but it was unable to recover an increased amount of kinase activity (Figure 2, lanes 5 and 6). Thus the KI, but not other tyrosine phosphorylated fusion proteins, was able to recover kinase activity from lysates of PDGF-stimulated cells.

Our hypothesis is that RasGAP is acting as an adapter protein between the GST–KI fusion protein and the kinase. One way of testing this idea was to determine whether the kinase activity could be recovered from cell lysates that had been immunodepleted of RasGAP. After three rounds of successive immunoprecipitation with a RasGAP antibody, the lysates contained less than 10 % of the starting amount of RasGAP (results not shown) and were poor sources of kinase activity (Figure 2, lanes 7–12). These experiments suggest that the kinase activity could be recovered from a cell lysate with a fragment of the βPDGFR and also that RasGAP served as a bridge between the receptor and the kinase.

Phosphorylation of RasGAP is required for the recovery of kinase activity in RasGAP immunoprecipitates

We next tested the idea that tyrosine phosphorylation of RasGAP is a prerequisite for its association with the kinase activity. RasGAP is poorly tyrosine-phosphorylated in resting cells; PDGF greatly stimulates this modification. We therefore compared the ability of RasGAP to associate with kinase activity in resting and PDGF-stimulated cells. We found that the phosphorylated GST–KI fusion protein was unable to recover the enhanced kinase activity from lysates of unstimulated cells (Figure 2, compare lanes 3 and 4). Further support for this idea came from a different line of investigation, in which we looked at the recovery of kinase activity in RasGAP immunoprecipitates from cells expressing mutant receptors. The F771 (in which Tyr-771 is replaced with Phe) and F5 (in which five tyrosine residues are replaced with Phe) βPDGFRs do not associate with RasGAP or trigger its tyrosine phosphorylation; we found that these receptors failed to mediate the PDGF-dependent increase in recovery of kinase activity in RasGAP immunoprecipitates seen in cells expressing the WT receptor (results not shown). These experiments support the idea that the PDGF-dependent increase in kinase activity in RasGAP-containing samples is contingent on the tyrosine phosphorylation of RasGAP.

If phosphorylation of RasGAP is indeed the trigger for its association with the kinase, then a phosphorylated RasGAP fusion protein would be capable of recovering kinase activity from cell lysates. One major tyrosine phosphorylation site of human RasGAP has been identified as Tyr-460 [24]; we constructed a GST fusion protein to include the region of human RasGAP surrounding Tyr-460 (residues 444–477, in which Tyr-460 was the only tyrosine residue). The fusion protein was expressed in both tyrosine-phosphorylated and unphosphorylated forms, incubated with cell lysates, washed and subjected to a kinase assay *in vitro*. The phosphorylated RasGAP fusion protein associated with readily detectable kinase activity, whereas much less kinase activity was recovered with the unphosphorylated fusion protein (Figure 3A). In contrast with the KI fusion protein, the increased association of kinase activity with the RasGAP fusion protein was independent of stimulation by PDGF. This latter observation further supports the idea that the

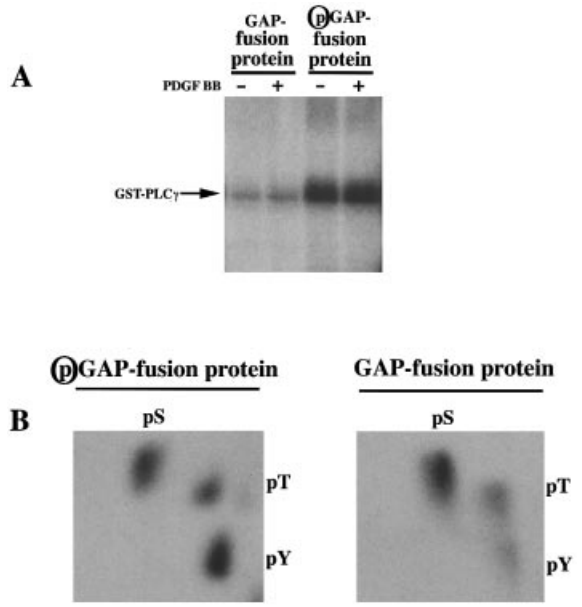

Figure 3 Kinase activity associated with the phosphorylated RasGAP fusion protein or RasGAP peptide

(**A**) Confluent, quiescent cultures of HepG2 cells expressing the WT βPDGFR were left resting (−) or were stimulated (+) with 50 ng/ml of PDGF BB for 5 min at 37 °C. The cells were lysed; lysates representing approx. 2×10^6 cells were incubated with 10 μg of either unphosphorylated or phosphorylated (P) RasGAP fusion protein. The fusion proteins were washed several times and the resulting samples were subjected to a kinase assay *in vitro* in the presence of an exogenous substrate (GST–PLCγ). The proteins were resolved by SDS/PAGE; the radiolabelled proteins were detected by autoradiography. (**B**) The phosphorylated substrate from lanes 1 and 3 of (**A**) was excised from the gel and subjected to phosphoamino acid analysis. Abbreviations: pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

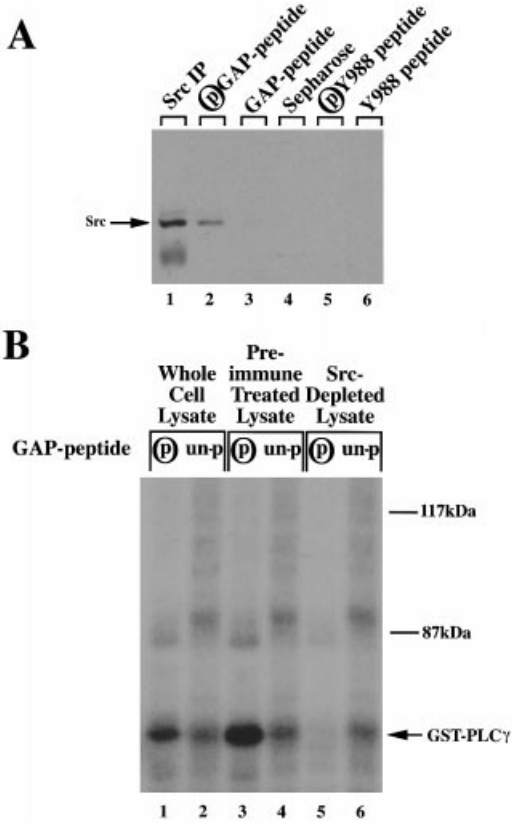

Figure 4 Src is the kinase activity associated with the phosphorylated RasGAP peptide

Quiescent cultures of TRMP cells expressing WT βPDGFR were lysed. (**A**) Lysates from approx. 2×10^6 cells were immunoprecipitated with the anti-Src antibody SRC 2, incubated with 10 μg of the indicated peptides or incubated with Sepharose alone. Complexes were resolved by SDS/PAGE, transferred to Immobilon and immunoblotted with anti-Src antibody. Lane 1 is a Src immunoprecipitate (IP) prepared from approx. 2×10^6 cells, and is included as a positive control. (**B**) Lysates from approx. 2×10^6 cells were either mock-immunoprecipitated (using no antiserum or preimmune serum) or subjected to two rounds of immunoprecipitation with an antibody, SRC 2, that recognizes Src, Yes and Fyn. The resulting lysates were then incubated with 10 μg of Sepharose-coupled RasGAP peptide or phosphorylated RasGAP peptide. The fusion proteins were washed and the resulting samples were subjected to a kinase assay *in vitro* in the presence of GST–PLCγ. The proteins were then resolved by SDS/PAGE and the radiolabelled proteins were detected by autoradiography. Similar results were obtained in four independent experiments, although a comparable amount of kinase activity was usually present in lanes 1 and 3. Abbreviations: P, phosphorylated; un-p, unphosphorylated. The positions of molecular mass markers are indicated (in kDa) at the right.

key PDGF-dependent event is the tyrosine phosphorylation of RasGAP.

To determine whether the kinase that associated with the phosphorylated RasGAP fusion protein was a serine/threonine kinase or a tyrosine kinase, the phosphorylated substrates were subjected to phosphoamino acid analysis. Figure 3(B) shows that there was much more phosphotyrosine in the substrate from the phosphorylated RasGAP fusion protein than from the unphosphorylated fusion protein. This analysis demonstrates that tyrosine kinase activity associates with RasGAP and that this event seems to require phosphorylation of Tyr-460.

Src is the kinase that associates with RasGAP in PDGF-stimulated cells

Our next step was to identify the kinase that associated with phosphorylated RasGAP. We made peptides, in both tyrosine phosphorylated and unphosphorylated forms, that corresponded to residues 454–468 of human RasGAP; as with the RasGAP fusion protein, Tyr-460 was the only tyrosine residue present. To characterize these RasGAP peptides, they were immobilized on Sepharose and tested for their ability to recover kinase activity from cell lysates. As with the GST RasGAP fusion proteins, the phosphorylated RasGAP peptide bound to kinase activity, whereas the unphosphorylated RasGAP peptide associated with a greatly decreased level of kinase activity (results not shown). When the kinase assay shown in Figure 3(A) was overexposed, we observed a band of approx. 60 kDa in the samples with enhanced kinase activity (lanes 3 and 4, and results not shown). This is the approximate size of Src family members, previously shown to bind to RasGAP via several molecular interactions in artificial systems [25–27]. The sequence after Tyr-460 of RasGAP is Tyr^{460}-Asn-Thr-Ile, whereas the optimal binding sequence for the SH2 domain of Src, as predicted from a phosphotyrosine peptide library, is pTyr, Glu/Asp/Thr, Glu/Asn/Asp, Ile/Val/Met/Leu, with a strong preference for isoleucine at the third position [28,29]. Because the RasGAP sequence downstream of Tyr-460 contains isoleucine at the +3 position, we tested whether Src family members associated with the phosphorylated RasGAP peptide. The phosphorylated and unphosphorylated RasGAP peptides were incubated in cell lysates then washed; the proteins were eluted, resolved by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with an antisera (Src-2) that detects all three Src family members (Src, Yes and Fyn) expressed in fibroblasts. Figure 4(A) shows that the phos-

phorylated RasGAP peptide bound at least one of the Src family members. Tyrosine-phosphorylated and unphosphorylated control peptides corresponding to the sequence surrounding Tyr-988 of the αPDGFR, as well as Sepharose alone, did not bind Src family members (Figure 4A).

Because Src family members associated with the phosphorylated RasGAP peptide, it is likely that they accounted for at least a portion of the kinase activity that was recovered with phosphorylated RasGAP. In addition, we found that the GST–PLCγ substrate was readily phosphorylated by Src immunoprecipitates in a kinase assay *in vitro* (results not shown). To assess the relative contribution of Src family members to the activity bound to the RasGAP peptide, cell lysates were first immunodepleted of Src family members by two successive rounds of immunoprecipitation with the anti-(Src-2) sera. Western blotting of the resulting lysates indicated that Src family members had been decreased by 90–95% (results not shown). These immunodepleted lysates were then used in peptide pull-down experiments similar to those described in Figure 4(A). Figure 4(B) shows that when the lysates were immunodepleted with the anti-(Src-2) sera, the amount of kinase activity recovered with the phosphorylated RasGAP peptide was decreased markedly. Mock immunodepletion (with preimmune serum) did not diminish the recovery of kinase activity (Figure 4B, lanes 3 and 4). In this particular experiment we detected a slight increase in the amount of kinase activity in mock-immunodepleted samples in comparison with lysates that were not subjected to immunodepletion (compare lanes 1 and 3). Replicate experiments did not show this difference consistently. These experiments demonstrated that the elimination of the majority of Src family members from the lysate eliminated the bulk of the kinase activity that could be recovered with the phosphorylated RasGAP peptide. It is therefore likely that Src family members account for most of the kinase activity bound to phosphorylated RasGAP. We have not yet determined whether all three of the Src family members found in fibroblasts are equal contributors to the kinase activity that we find associated with phosphorylated RasGAP.

RasGAP sequesters Src to prevent PDGF-dependent tyrosine phosphorylation of PLCγ

We have previously reported that binding of RasGAP to the βPDGFR attenuates PDGF-dependent PLCγ tyrosine phosphorylation [15]. The finding that RasGAP recruits Src offers a mechanistic explanation of our previous findings. We speculate that in PDGF-stimulated cells, it is Src and not the receptor that mediates phosphorylation of PLCγ. Furthermore, when RasGAP is recruited to the receptor, it sequesters Src and thereby prevents the phosphorylation of PLCγ. An important component of this hypothesis is the unusual idea that Src contributes to the phosphorylation of proteins that are typically considered direct substrates of the receptor. Our recent studies indicate that the receptor mutants that fail to activate Src but retain full kinase activity induce a submaximal phosphorylation of some of the proteins that are phosphorylated in a PDGF-stimulated cell [17,31]. These studies did not examine the importance of Src for the phosphorylation of PLCγ; this was therefore our next undertaking. We compared the tyrosine phosphorylation of PLCγ in cells expressing a WT or mutant PDGFR that was selectively defective in its ability to bind and activate Src family members. Because mutating the tyrosine residues required for Src to associate with the βPDGFR severely impaired the PDGF-stimulated kinase activity of the receptor [32,33], we could not use the βPDGFR for the experiments

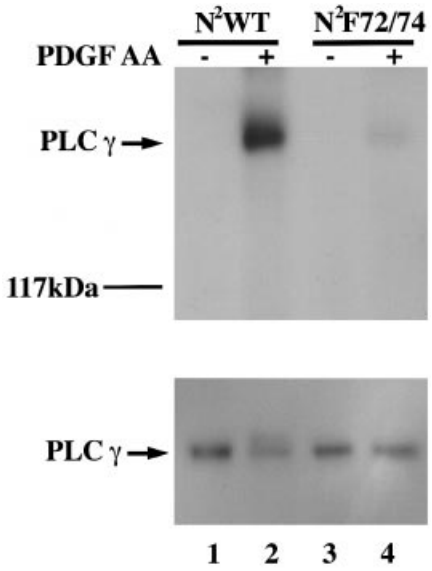

Figure 5 PLCγ is not efficiently tyrosine-phosphorylated in cells expressing a chimaeric PDGFR mutant that does not bind or activate Src family members

Quiescent cultures of Patch cells expressing the WT (N^2WT) or mutant (N^2F72/74) chimaeric PDGFR were left resting (−) or were stimulated (+) with 50 ng/ml PDGF AA for 5 min at 37 °C. The cells were lysed; the lysates were immunoprecipitated with the anti-PLCγ serum 36A. Immunoprecipitates representing approx. 2.0×10^6 cells were resolved by SDS/PAGE, transferred to nitrocellulose and immunoblotted with the anti-phosphotyrosine antibody 4G10 (upper panel). The nitrocellulose was stripped and re-probed with the anti-PLCγ antibody UBI (lower panel).

described above. Instead, we used a chimaeric receptor consisting of the αPDGFR extracellular, transmembrane and juxtamembrane regions fused to the intracellular portion of the βPDGFR. Tyr → Phe mutations at Tyr-572 and Tyr-574, which are required for the stable binding of Src family members to the αPDGFR, do not affect the kinase activity of the αPDGFR [31,34]. This mutant does not recruit or activate Src family members [17]. The WT and mutant chimaeras were expressed in Patch cells, an NIH 3T3-like cell line that is derived from mouse embryos homozygous for the *Ph* deletion that includes the αPDGFR [35]. The introduced chimaera can be selectively activated with PDGF AA [17]. As shown in Figure 5, the WT chimaera triggered robust phosphorylation of PLCγ after stimulation of the cells with PDGF AA. In contrast, tyrosine phosphorylation of PLCγ was greatly impaired in the cells expressing mutant chimaera. Both receptors associated with a comparable amount of PLCγ in response to stimulation with PDGF; the extent of receptor tyrosine phosphorylation was similar [17]. RasGAP, SHP-2 and Shc were also phosphorylated less well in cells expressing the mutant receptor [17]. These results strongly suggest that Src family members are required for the efficient phosphorylation of PLCγ. In addition, they support the idea that when RasGAP associates with Src family members, Src family members are functionally sequestered, and this prevents them from mediating the tyrosine phosphorylation of PLCγ.

DISCUSSION

We have found (1) that RasGAP associates with a tyrosine kinase, (2) that this association is dependent on the PDGF-stimulated phosphorylation of RasGAP and (3) that the kinase

is Src or a related family member. These observations offer an explanation of our previous findings that RasGAP attenuates PDGF-dependent tyrosine phosphorylation of PLCγ.

Negative regulation of the signalling cascades

RasGAP is on the growing list of proteins that negatively regulate signalling. The RasGAP locus was identified as a negative regulator of the Sevenless receptor tyrosine kinase in genetic experiments in the *Drosophila* system [36]; subsequent studies have shown that this locus includes a RasGAP [14]. For the βPDGFR, we have previously identified RasGAP as a negative regulator of signalling by PI3K [16] as well as PLCγ [15]. For PI3K, RasGAP seems to prevent the activation of PI3K by preventing the accumulation of active Ras, which is required, at least in some systems, for the maximal activation of PI3K [37,38]. Indeed, RasGAP was originally purified for its ability to inactivate Ras *in vitro*. Whether RasGAP has a similar role *in vivo* has been more difficult to demonstrate; however, recent studies support this idea. For instance, βPDGFR mutants that fail to associate with RasGAP are better able to activate Ras than the WT receptor [39]. In addition, cells derived from homozygous RasGAP knock-out embryos have a normal basal level of active Ras but a much increased level of GTP-bound Ras after stimulation by PDGF [40]. Other studies have shown that overexpression of RasGAP inhibits the proliferation induced by activated Ras and that overexpression of c-Ras or c-Src rescues such cells [41–44].

Several lines of evidence support the possibility that RasGAP has roles in signalling other than as a negative regulator of Ras. The observation that RasGAP binds the effector domain of Ras suggested that RasGAP might be an effector of Ras [45,46]. Indeed, germinal vesicle breakdown stimulated by active Ras is inhibited by inactivating the SH3 domain of RasGAP. In addition, the SH2 and SH3 domains of RasGAP inhibit the inactivation of muscarinic-receptor-activated K^+ channels [47]. Expressing the N-terminal region or RasGAP, which contains the SH2, SH3 and pleckstrin homology (PH) domains, induces a variety of responses such as disrupting the cytoskeleton and decreasing cell adhesion [48], inducing oncogenic transformation [48,49], increasing c-Fos expression [50] and inducing neuronal differentiation [51]. Our findings that RasGAP acts as a negative adapter protein further underscores the numerous roles of RasGAP in regulating cell homoeostasis.

Interaction of RasGAP with signalling proteins

RasGAP interacts with several distinct signalling proteins such as p190RhoGAP [52,53] and the adapter protein p62Dok [54,55]. In addition, several studies have shown that Src family members bind to RasGAP. In cells overexpressing Src or Src mutants, RasGAP co-immunoprecipitated with Src; this interaction depended on the phosphorylation of Tyr-527 and the presence of an intact SH2 domain in Src [25,56]. With the use of baculoviral reconstitution systems, a large portion of RasGAP, including its SH2, SH3 and PH domains, have been shown to be important for the interaction with Src [27]. The SH3 domain of the Src family member Hck seems to bind to a proline-rich region of RasGAP [26]. These studies demonstrate an interaction between these two signalling molecules in artificial systems. In the more physiological setting of thrombin-activated platelets, Lyn, Fyn and Yes, but not Src or Hck, co-immunoprecipitated with RasGAP [57]. These earlier studies did not demonstrate the physiological consequences of interaction between RasGAP and Src family members.

We found that the interaction between RasGAP and Src family members occurs as a result of PDGF stimulation and only when RasGAP is phosphorylated at Tyr-460. One caveat of our findings is that we demonstrated the PDGF-dependent interaction between RasGAP and Src with systems that were primarily *in vitro*. This was necessary because RasGAP associates with the βPDGFR in PDGF-stimulated cells; under these conditions the receptor itself associates with Src. Consequently it is difficult to design experiments demonstrating the interaction of Src family members with RasGAP in a PDGF-stimulated cell.

One way in which the binding of RasGAP to Src family members could attenuate PLCγ phosphorylation by Src family members is by inactivating Src's kinase activity. However, the data in Figures 1–4 strongly suggest that when Src binds to RasGAP it retains its kinase activity. Consistent with this idea is our finding that the phosphorylated RasGAP peptide was not able to inhibit Src activity *in vitro* (T. K. Schlesinger and A. Kaslauskas, unpublished work). Therefore RasGAP is not attenuating Src by turning off its kinase activity. It is possible that moving Src from the receptor (where it associates with the receptor's juxtamembrane domain) to phosphorylated RasGAP is sufficient to prevent the phosphorylation of PLCγ. Furthermore, it is possible that moving Src from the receptor to RasGAP might redirect Src to different cellular substrates and might thereby alter the amplitude and/or the nature of signals emanating from the activated βPDGFR.

Phosphorylation of PLCγ in a PDGF-stimulated cell

There is strong evidence supporting the idea that the βPDGFR directly phosphorylates PLCγ. Meisenhelder et al. [58] showed that exposure of NIH 3T3 cells to PDGF led to a rapid increase in PLCγ tyrosine phosphorylation. The tyrosine residues of PLCγ that were the sites *in vivo* were also phosphorylated in a kinase assay *in vitro* with purified βPDGFR and purified PLCγ. These results, along with the co-immunoprecipitation of PLCγ and the βPDGFR [58], suggested that PLCγ was the direct substrate of the βPDGFR. However, subsequent studies *in vitro* have shown that Src family members are also able to phosphorylate PLCγ at the major *in vivo* phosphorylation sites [59]. Because PDGF activates both of the kinases that phosphorylate PLCγ, it is not clear which is responsible for the phosphorylation of PLCγ in the intact cell. Because it is very difficult to demonstrate unequivocally which kinase is phosphorylating a substrate in an intact cell, a considerable degree of uncertainty will always persist. Thus although our results are strongest in ruling out the receptor as the primary kinase for PLCγ *in vivo*, we cannot be certain whether it is Src family members, a kinase activated by Src family members (such as c-Abl [60]) or some other kinase that associates with the PDGFR via the same sites as Src family kinases, or a combination of the above candidates that phosphorylate PLCγ in a PDGF-stimulated cell. Note that, although our studies clearly indicate a requirement for Src, in other systems PDGF-dependent phosphorylation of proteins is Src-independent [61]. The basis for these differences is not obvious at present.

Our investigation of the mechanism by which RasGAP attenuates the PDGF-dependent phosphorylation of PLCγ has led to the following model. Binding of RasGAP to the βPDGFR and the phosphorylation of RasGAP result in the association of Src family members with RasGAP; as a result, Src is no longer able to phosphorylate PLCγ directly or indirectly. This model includes a number of relatively novel concepts. First, RasGAP can function as a negative adapter protein, which binds and sequesters but does not inactivate Src family members. Secondly,

the kinase that phosphorylates PLCγ in PDGF-stimulated cells is not the βPDGFR but some other PDGF-activated kinase, which might be one of the Src family members.

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