Integrins Enhance Platelet-derived Growth Factor (PDGF)-dependent Responses by Altering the Signal Relay Enzymes That Are Recruited to the PDGF β Receptor

(Received for publication, February 8, 1999, and in revised form, March 25, 1999)

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Since the extracellular matrix (ECM) can promote platelet-derived growth factor (PDGF)-dependent responses, we hypothesized that the ECM mediates this effect by preventing the PDGF β receptor (βPDGFR) from associating with the negative regulator, RasGAP (the GTPase-activating protein of Ras). We found that binding of RasGAP to the wild-type βPDGFR was decreased; the activation of Ras and Erk was enhanced, and [3H]thymidine uptake was better in cells cultured on fibronectin than in cells cultured on polylysine. To investigate the mechanism by which culturing cells on fibronectin diminished the recruitment of RasGAP to the βPDGFR, we focused on SHP-2 since it dephosphorylates the βPDGFR at the phosphotyrosine required for binding of RasGAP. Culturing cells on fibronectin increased the amount of SHP-2 that associated with the βPDGFR. Furthermore, cells expressing receptor mutants that failed to associate with SHP-2 were insensitive to fibronectin. The ECM enhances PDGF-dependent responses by increasing the association of SHP-2 with the βPDGFR, which in turn decreases the time that RasGAP interacts with the receptor. Thus, fibronectin changes PDGF-dependent signaling and biological responses by altering the signal relay enzymes that are recruited to the receptor.

Upon exposure of cells to platelet-derived growth factor (PDGF), the PDGF receptor (PDGFR) dimerizes and autophosphorylates on a number of intracellular tyrosine residues. One of the consequences of tyrosine phosphorylation of the PDGFR is the generation of binding sites for various SH2 domain-containing proteins. Some of the proteins that associate with the βPDGFR include Src family members, phosphatidylinositol 3-kinase (PI3K), the GTPase-activating protein of Ras (RasGAP), the tyrosine phosphatase (SHP-2), and phospholipase Cγ1 (PLCγ) (reviewed in Ref. 1). Evidence suggests that different signaling enzymes are required for initiating different cellular responses. By using receptor mutants that bind only PI3K or only PLCγ, it has been demonstrated that PI3K and/or PLCγ act as positive regulators of PDGF-dependent DNA synthesis (2, 3), cellular transformation (4), vesicle trafficking (5), and cell migration (6, 7). Micro-injection of reagents that interfere with the action of the receptor-associated signal relay enzymes also prevents PDGF-dependent entry into S phase (8–10). In contrast, RasGAP is a negative regulator of βPDGFR signaling. Analysis of βPDGFR mutants indicates that RasGAP prevents activation of PLCγ (11) and PI3K (12). Furthermore, PDGF triggers activation of Ras better in cell lines that do not express RasGAP as compared with the corresponding control cell lines (13). A similar role for RasGAP has emerged in signaling initiated by other receptor tyrosine kinases. For instance, the RasGAP locus negatively regulates the Sevenless receptor tyrosine kinase during Drosophila eye development (14). Furthermore, the strength of Torso signaling is modulated by RasGAP and has dramatic consequences on terminal structure development in Drosophila (15, 16). Hence, these studies indicate that RasGAP is a negative regulator of several different receptor tyrosine kinases.

Given that RasGAP is a negative regulator of the βPDGFR, it is somewhat puzzling that the wild-type βPDGFR associates with RasGAP and is able to efficiently drive biochemical and biological responses. One explanation is that all activated βPDGFRs do not associate with RasGAP. This idea is supported by the observation that the receptor is not stoichiometrically phosphorylated at tyrosine 771, which is required for binding of RasGAP (17). This suggests that in an activated cell, the population of receptors are heterogeneous with respect to which sites are phosphorylated and consequently which signaling molecules are present in a receptor dimer. We hypothesize that changing the composition of signal relay enzymes that are recruited to the βPDGFR will alter the signal relay pathways that the receptor will activate.

Since changes in the extracellular environment alter the way in which cells respond to PDGF, this variable may influence which signal relay enzymes associate with the PDGFR. One extracellular component that could potentially play a role in changing the composition of signal relay enzymes that associate with the PDGFR are integrins. Integrins are heterodimeric receptors that mediate attachment to the extracellular matrix (ECM) and cell-cell adhesive interactions (reviewed in Ref. 18). Several lines of evidence suggest that integrins can modulate growth factor receptor function. Adherence to the extracellular matrix is critical for stimulation of cell proliferation by growth factors (19) and growth factor-stimulated Erk activation (20). Furthermore, association of integrins with activated insulin and PDGF receptors correlates with enhanced mitogenicity and chemotaxis (21, 22).

Most of the data describing integrin-dependent modulation

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of growth factor receptor function have been obtained by investigating integrin-dependent signaling in cells that have been replated on ECM-coated dishes or beads. Although this commonly used system is an excellent approach to study signaling downstream of integrins, we were interested in how long term culturing of cells on ECM alters growth factor signaling. Consequently, after replating cells on an ECM-coated dish, we waited until the integrin-dependent signaling subsided (12–14 h) and then stimulated the cells with PDGF. We found that recruitment of RasGAP to the wild-type βPDGFR was decreased in cells cultured on fibronectin; the duration of PDGFB-dependent Ras and Erk activation was prolonged, and the DNA synthesis response was enhanced. The decreased binding of RasGAP was tightly correlated with an increase in the recruitment of SHP-2 to the wild-type βPDGFR. In addition, mutation of the binding site on the βPDGFR for SHP-2 negated the effect of culturing cells on fibronectin. These findings suggest that long term engagement of integrins alters PDGFB-dependent signaling by changing the amount of individual signaling molecules that associate with the wild-type βPDGFR, and the net effect is enhanced DNA synthesis. Finally, it appears that SHP-2 is one of the enzymes that acts as a liaison between the ECM and the βPDGFR.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**

The Ph cell line was derived from mouse embryos homozygous for the Ph/Ph deletion that includes the αPDGFR gene (23) and was kindly provided by Dan Benedict-Pope (University of Washington). They are mouse embryo fibroblasts that express endogenous βPDGFR at approximately 1 × 10⁵ receptors per cell and no αPDGFRs. Ph cells were maintained in Dulbecco’s modified Eagle’s (DME) medium supplemented with 5% calf serum, and 1 mg/ml G418 was added to cultures grown out in medium containing 200 μg/ml hygromycin. This cell line was a monoclonal antiserum raised against a synthetic phosphotyrosine peptide corresponding to amino acid residues 196–209 of human p44 Erk. It recognizes both tyrosine-phosphorylated and threonine/tyrosine doubly phosphorylated p42 and p44 Erk and was used at a 1:500 dilution. The goat-anti-mouse IgG antibody used for the immunoblot analysis was a monoclonal antibody available from Zymed Laboratories Inc. and was used at a 1:2500 dilution.

**Ras Assay**

The Ras assay is a modified version of the protocol previously described (31).

**GST-Raf 50–150 Isolation**—A construct containing a GST fusion with amino acids 50–150 of Raf was transformed into DH5α cells. A 20-ml starter culture of LB + 100 μg/ml carbenicillin was inoculated and grown overnight at 37 °C. The starter culture was diluted 1:100 with LB + carbenicillin and incubated at 37 °C until the A₆₀₀ was 0.6–0.8, at which time expression of the fusion protein was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. The cultures were incubated at 37 °C for an additional 2 h and centrifuged; the pellets were washed once with ice-cold H/S (32), and then the pellet was resuspended in HSE (20 mM HEPES, pH 7.5, 120 mM NaCl, 10% glycerol, 2 mM EDTA) + 10 μg/ml leupeptin and aprotinin, sonicated, centrifuged to remove the insoluble debris, and Nonidet P-40 added to a final concentration of 0.5%. The supernatant was saved for 30 min on ice with 50 μg/ml of proteinase inhibitor beads and washed eight times with HSE + 0.5% Nonidet P-40 + 10 μg/ml leupeptin and aprotinin. The purified fusion proteins were then left attached to the GST beads and used immediately to purify activated Ras.

**Purification of Active Ras**—Ph cells expressing the various receptor constructs were trypsinized and plated on polylysine (5 mg/ml) or fibronectin-coated plates (Falcon) in DME + 10% calf serum. After the cells had attached and begun spreading (20–30 min at 37 °C), the cells were washed twice with PBS and placed in serum-free DME for 12 h. The cells were then exposed for 5–30 min to 50 ng/ml PDGF AA or buffer (2 mM acetic acid and 10 mg/ml BSA), washed 2× in H/S (32), lysed, and then incubated with approximately 20–30 μg of GST-Raf fusion protein for 30 min at 4 °C. The beads were washed three times in 0.1% IgG buffer; samples from buffer (32) were used as negative controls and the beads were resolved on a 15% SDS-PAGE gel. The proteins were transferred to Immobilon in a methanol-containing transfer buffer (150 mM glycine, 25 mM NaCl, 10% CH₃OH) and subjected to a Ras Western blot except that Blotto without azide was used in place of Blotto.

**Erk Activation**

Parental Ph cells or Ph cells expressing a chimeric PDGFR were grown to 85–90% confluence, serum-starved in DME + 0.1% calf serum for 18–24 h, left resting (−) or stimulated with 50 ng/ml PDGF AA (Ph cells expressing a chimeric PDGFR) or 40 ng/ml PDGF BB (parental Ph cells) for 5–30 min at 37 °C. The cells were washed with H/S (32), lysed in EB (32) without BSA, and centrifuged to remove the insoluble debris. The amount of protein in the lysates was determined by the BCA protein assay (Pierce), and equal amounts of protein were resolved on a 15% SDS-PAGE gel. The resolved proteins were transferred to Immobilon in a methanol-containing transfer buffer (150 mM glycine, 25 mM NaCl, 10% CH₃OH) and subjected to a Ras Western blot except that Blotto without azide was used in place of Blotto.

**Antibodies**

PR292 is a mouse monoclonal antibody that recognizes an epitope in the extracellular domain of the αPDGFR and was purchased from Genzyme. 80.8 is a crude rabbit polyclonal antiserum raised against a glutathione S-transferase (GST) fusion protein including a portion of the first immunoglobulin domain (residues 52–94) and was used at a 1:1000 dilution. The βPDGFR was immunoprecipitated using crude polyclonal rabbit sera (30A) raised against glutathione S-transferase fusion proteins that included the entire carboxyl terminus of the human βPDGFR subunit (3). The βPDGFR (3), RasGAP (3), and the 385 subunit of PI3K (4) were blotted as described previously. For Ras Western blot analysis, a monoclonal pan-Ras antibody (Transduction Laboratories) was used at a 1:500 dilution. To blot for SHP-2, a monoclonal PTPIP1D antibody (Transduction Laboratories) was used at a 1:1000 dilution. For anti-phosphorysreine Western blot analysis, a combination of a polyclonal pan-Erk antibody (Cell Signaling Technology, Inc.) was used, each at a 1:1000 dilution. The phospho-Erk antibody (New England Biolabs) is an affinity purified rabbit polyclonal antibody raised against a synthetic phosphorysreine peptide corresponding to amino acid residues 196–209 of human p44 Erk. It recognizes both tyrosine-phosphorylated and threonine/tyrosine doubly phosphorylated p42 and p44 Erk and was used at a 1:500 dilution. The pan-αPDGFR antibody used for Western blot analysis was a monoclonal antibody available from Zymed Laboratories Inc. and was used at a 1:2500 dilution.

**Construction of the PDGFRs**

Chimeric PDGFRs—Construction of the wild-type chimeric PDGFR was described previously (4, 24). The N2F771 chimera was constructed by subcloning a SacI/SalI (3′) end of the βPDGFR up to and including the SacI site at position 1972 fragment of the F771 PDGFR (17) into the SacI/SalI-cut wild-type chimeric PDGFR. The chimeras were subcloned into the pLXSN2 retroviral vector (4, 25); virus was generated using the 293T system (26), and the resulting virus was used to infect Ph cells. Receptor-expressing cells were selected in DME medium supplemented with 5% calf serum, and 1 mg/ml G418 was added to cultures of cells expressing the introduced chimeric constructs. The F cells were obtained from embryoid nullizygous for both the α and β PDGFRs and were kindly provided by Michelle Tallquist and Philippe Soriano (Fred Hutchinson Cancer Research Center, Seattle). They were maintained in DME supplemented with 5% fetal bovine serum.

**GST Assay**

GST was described previously (4, 24). The N2F771 chimera was constructed by subcloning a SalI fragment of the F771 PDGFR (17) into the SacI/SalI-cut wild-type chimeric PDGFR. The Ras assay is a modified version of the protocol previously described (31).

**Erk Activation**

Parental Ph cells or Ph cells expressing a chimeric PDGFR were grown to 85–90% confluence, serum-starved in DME + 0.1% calf serum for 18–24 h, exposed to 50 ng/ml PDGF AA or buffer (2 mM acetic acid and 10 mg/ml BSA), washed 2× in H/S (32), lysed, and then incubated with approximately 20–30 μg of GST-Raf fusion protein for 30 min at 4 °C. The beads were washed three times in 0.01% IgG buffer; samples from buffer (32) were used as negative controls and the beads were resolved on a 15% SDS-PAGE gel. The proteins were transferred to Immobilon in a methanol-containing transfer buffer (150 mM glycine, 25 mM NaCl, 10% CH₃OH) and subjected to a Ras Western blot except that Blotto without azide was used in place of Blotto.
a pan-Erk antibody (3). Erk immunoprecipitates representing approxi-
mately 1.5 × 10⁶ cells were subjected to an in vitro kinase assay in the
presence of 0.75 µg of myelin basic protein (Sigma). The proteins were
resolved on a 15% polyacrylamide gel, and the gel was stained to show
the relative amounts of myelin basic protein present and then exposed
to film. The phosphorylated bands were excised and counted in a scint-
illation counter.

**Immunoprecipitation and Western Blot Analysis**

For studies involving cells plated on plastic, Ph cells expressing the
chimeric PDGFR were grown to 85–90% confluence, incubated in DME
+ 0.1% calf serum for 18–24 h, and stimulated with 50 ng/ml PDGFAA
for the times indicated. For studies involving cells plated on polylysine
or an ECM, Ph cells were grown to 90% confluence, trypsinized brie-
lly, and plated on 5 mg/ml polylysine (Sigma)- or fibronectin (Falcon)-
coated dishes. The cells were incubated for 30 min at 37 °C, washed
twice in PBS, and incubated in DME alone for 12 h at 37 °C and 5%
CO₂. Under both plating conditions, the cells were then stimulated
with buffer (10 mM acetic acid + 2 mg/ml BSA) or 40 ng/ml PDGFB BB for
the times indicated, washed, and lysed in EB (32), and the βPDGFRs were
immunoprecipitated using 30A (3). The immunoprecipitates were
bound to formalin-fixed *Staphylococcus aureus* membranes and washed
as described previously (33). The resulting gel was transferred to Im-
mobilon and subjected to Western blot analysis. Proteins were detected
using ECL (Amersham Pharmacia Biotech).

**In Vitro Kinase Assay**

The intrinsic tyrosine kinase activity of the PDGFR was analyzed
using receptor immunoprecipitates from approximately 2 × 10⁶ cells as
described previously (4).

**³HThymidine Uptake**

PDGF-stimulated [³H]thymidine uptake was assayed as follows. Cells
were plated at 3 × 10⁶ cells/ml in DME + 2% FBS in 24-well dishes
and incubated at 37 °C for 1 h at which time they were washed
2× in PBS and placed in DME containing 0.1% FBS. Cultures were
incubated at 37 °C and 5% CO₂ for 48 h at which time they were washed
2× in PBS and incubated for an additional 24 h at 37 °C in DME
containing 2 mg/ml BSA. Buffer (10 mM acetic acid and 2 mg/ml BSA),
PDGFB BB (5 or 40 ng/ml), or 10% FBS was added; the cultures were
incubated for 22 h and the media replaced by DME + 5% FBS and 0.8
µCi of [³H]thymidine per ml, and the incubation was prolonged for 4 h.
The cells were then harvested as described previously (24).

**RESULTS**

We have previously found that artificially altering the com-
oposition of signaling molecules that are recruited to the
βPDGFR profoundly changes the nature of the signaling cas-
ses initiated by PDGFR (3, 11, 12). Since extracellular changes
such as cell density or ECM alter the ability of PDGF to initiate
biological responses (21, 22, 34), we postulated that the basis of
the altered responsiveness to PDGF is due to a change in
signaling enzymes that associate with the receptor. In this
study, we examined whether changing the composition of the
signal enzymes which associated with the wild type βPDGFR
was the mechanism by which integrins can modulate growth
factor receptor function. Consequently, we focused on cell lines
naturally expressing the wild type βPDGFR, and we compared
RasGAP co-immunoprecipitation with the βPDGFR when the
cells were cultured on polylysine or fibronectin. Ph cells were
grown to 90% confluence, trypsinized, resuspended in DME +
2% calf serum, and then plated in dishes coated with polylysine
or fibronectin. Once the cells had adhered (after 30 min), the
cultures were washed twice with PBS and then incubated in
serum-free DME at 37 °C for 1–2 h. Pilot experiments indi-
cated that Erk activity was elevated soon after plating and
gradually returned to basal levels by 12–14 h (data not shown).
The cells were either left resting or stimulated with 40 ng/ml
PDGFB BB for 5–50 min and lysed; the βPDGFR was immuno-
precipitated, and the amount of co-immunoprecipitating Ras-
GAP was assessed by Western blotting (Fig. 1A). In cells cul-
tured on polylysine, the level of receptor-associated RasGAP
was similar from 5 to 20 min, and less RasGAP was present in

**FIG. 1. Comparison of RasGAP binding. A, Ph cells were
trypsinized, resuspended in DME + 2% calf serum, and seeded onto
plates coated with fibronectin or polylysine. Thirty minutes after pla-
ting, both sets of cells had adhered and begun to spread. The cells were
washed twice with PBS and then incubated in serum-free DME for 12 h.
The cells were left resting (−) or stimulated with 40 ng/ml PDGFB BB for
the times indicated and then lysed, and the receptor was immunopre-

cipitated with an antibody against the tail of the βPDGFR. Receptor
immunoprecipitates representing approximately 1.0 × 10⁶ cells were
resolved by SDS-PAGE, transferred to Immobilon, and immunoblotted
with the indicated antibodies. In three experiments, similar amounts of
RasGAP associated with the receptor at the earliest time points,
whereas at the later time points (20 and 30 min) less RasGAP co-
immunoprecipitated with receptor from cells cultured on fibronectin.
B, the PDGFR immunoprecipitates shown in A were subjected to an anti-
phosphotyrosine Western blot analysis. C, the βPDGFR immuno-
precipitates were subjected to an in vitro kinase assay in the presence of an
exogenous substrate, GST-PLCγ; the proteins were resolved by SDS-
PAGE, and the gel was subjected to autoradiography.

the 30-min sample. The decrease in the amount of co-precipit-
ating RasGAP was seen earlier in cells cultured on fibronectin
(at the 20-min time point), and by 30 min only a trace amount of
RasGAP was present. Thus RasGAP associates with the
receptor under both sets of conditions, but in the cells cultured
on fibronectin, RasGAP binding is more transient than in the
cell controls.

Since binding of RasGAP depends on tyrosine phosphoryla-
tion of the βPDGFR, it is possible that the differences in Ras-
GAP binding reflect an ECM-dependent alteration in the ex-
tent and/or the duration of receptor phosphorylation and/or
kinase activity. To investigate this possibility, receptors were
immunoprecipitated from cells that were treated as described in
Fig. 1A, and the extent of receptor phosphorylation was
examined by Western blot analysis (Fig. 1B). We found that

**Effect of ECM on Pathways Downstream of RasGAP—We
next examined whether pathways downstream of RasGAP
were affected by culturing cells on an ECM, and we focused on
PDGF-dependent Ras and Erk activation. Ph cells were plated,
starved, and stimulated as described above and lysed, and
active GTP-bound Ras was recovered from the lysate with a
GST fusion protein that includes the Ras binding domain of Raf. The samples were subjected to Western blot analysis with
a pan-Ras antibody; the signal was quantitated, and the fold activation was plotted as a function of time. Fig. 2A shows that PDGF-induced Ras activation comparably at the earliest time points under either culture conditions but that the levels of active Ras persisted longer in the cells cultured on fibronectin. Furthermore, the difference in Ras activation between the two culture conditions was most apparent at the times when RasGAP binding diminished. It is not obvious why receptors that bind RasGAP are able to activate Ras at the earliest time point (Fig. 2A). The arrows in A indicate the times at which RasGAP binding is reduced in PDGF-stimulated cells plated on fibronectin (Fig. 1). B, the cells were cultured, stimulated, and lysed as described in Fig. 1. Thirty μg of clarified Triton X-100 soluble lysate was subjected to Western blot analysis with antibodies that recognize phospho-Erk (top panel). The bottom panel of each pair is a Western blot of the same samples and indicates the levels of protein (lysate standard).

To assess whether the ECM-dependent extension of Ras activation affected downstream signaling events, we examined PDGF-dependent Erk activation under different culture conditions. Ph cells were cultured, stimulated, and lysed as described in Fig. 1, and 30 μg of Triton X-100 soluble cell lysate was subjected to Western blot analysis with antibodies that recognize phosphorylated p42Erk and p44Erk. Regardless of the culture conditions, the cells exhibited a PDGF-dependent increase in Erk phosphorylation that was greatest at the 5-min time point (Fig. 2B). For cells plated on polylysine, Erk activity returned to basal levels at approximately 30 min post-PDGF stimulation. In contrast, phospho-Erk persisted for at least 30 min in cells plated on fibronectin (Fig. 2B). These findings suggest that culturing cells on fibronectin decreases RasGAP recruitment to the receptor and regulates the duration of Ras and Erk activation.

**RasGAP Regulation of Ras and Erk Using Receptor Mutants**—The findings presented above showed that at times greater than 10 min post-PDGF, there was an inverse correlation between binding of RasGAP and the levels of active Ras. This suggests that at early times after exposure to PDGF, RasGAP plays little role in regulating the level of active Ras, and it is at the later time points when the presence of RasGAP results in a decrease in RasGTP levels. To investigate further the role of RasGAP in the kinetics of Ras activation, we compared the timing of Ras and Erk activation in cells expressing either WT or mutant βPDGFRs that do not bind RasGAP. Since Ph cells express the βPDGFR, we used α/β chimeric receptors, which can be selectively activated with PDGF AA (4). We constructed a mutant PDGFR chimera, N²F²771, in which the tyrosine required for binding of RasGAP was mutated to phenylalanine. The wild-type (N³WT) and N²F²771 chimeras were expressed in Ph cells to comparable levels (data not shown). As expected, the N²F²771 receptor was not able to associate with RasGAP (data not shown). Additional characterization of the receptors indicated that they had comparable PDGF-stimulated kinase activity toward an exogenous substrate (data not shown). To compare the ability of N³WT and N²F²771 chimera to activate Ras, cells were grown to 85–90% confluence, arrested by serum deprivation in DMEM + 0.1% calf serum, and left resting or stimulated with PDGF AA for the times indicated. Active Ras was recovered with the GST-Raf fusion protein and quantitated as described in Fig. 2. At the 5-min time point, Ras was activated to a similar level in both cell types, indicating that the ability of RasGAP to associate with the receptor does not play a major role in the early accumulation of active Ras (Fig. 3A). At the later time points, the levels of active Ras diminished quickly in the N³WT receptor-expressing cells but persisted in the mutant receptor-expressing cells. Thus the ability to associate with RasGAP has the greatest effect on the levels of active Ras only at times greater than 5 min post-PDGF. We also measured Erk activation and found that like Ras activation, the differences between the two receptors became apparent at later time points (Fig. 3, B and C). In this series of studies, we obtained similar results using a phospho-Erk Western blot or monitoring kinase activity of Erk immunoprecipitates, strongly suggesting that either assay was an accurate measure of Erk activation. These studies indicate that binding of RasGAP to the βPDGFR does indeed alter PDGF-dependent activation of Ras and that the effect is greatest after the initial accumulation of active Ras has occurred. Furthermore, preventing the association of RasGAP with the βPDGFR specifically at late time points post-PDGF appears to be the mechanism by which culturing cells on fibronectin prolongs the PDGF-dependent activation of Ras and Erk.

**Molecular Basis for Decreased RasGAP Recruitment in Cells Cultured on Fibronectin**—We next examined the molecular basis for the decreased recruitment of RasGAP to the βPDGFR in cells cultured on fibronectin. One possible explanation for the ECM effect is the action of protein tyrosine phosphatases, and a good candidate is SHP-2 which associates with the βPDGFR and selectively dephosphorylates photophosphorysine 771 and 751 (35). To determine whether binding of SHP-2 to the βPDGFR was affected by the ECM of the cells, we com-
pared the amount of SHP-2 that co-precipitated with the βPDGFR in cells that had been cultured on either polylysine or fibronectin. We found that PDGF stimulated SHP-2 binding to the βPDGFR and that 2–3 times more SHP-2 was recovered in samples from cells that had been cultured on fibronectin (Fig. 4A). This effect appeared to be specific for SHP-2 since PDGF-dependent binding of the p85 subunit of PI3K to the βPDGFR was comparable in cells cultured on polylysine or fibronectin (Fig. 4A). Despite the increased amount of SHP-2 that associated with the receptor at the earliest time points, a decrease in RasGAP binding was observed only at the later time points. It may be that the initial phase of PDGF-stimulated receptor kinase activity outweighs the initial phase of protein tyrosine phosphatase activity. Thus the results in Figs. 1A and 4 indicate that culturing cells on fibronectin selectively alters the proteins that are recruited to the βPDGFR. Furthermore, an increased association of SHP-2 correlates with the decreased binding of RasGAP. Consequently, we postulate that the ECM effect involves an increase in SHP-2 binding to the βPDGFR, which then decreases the time that RasGAP stays associated with the receptor, leading to prolonged Ras and Erk activity.

Mutation of the SHP-2-binding Site Negates the Effect of Plating Cells on Fibronectin—The experiment presented in Fig. 4 suggests that the ECM effects are mediated at least in part by SHP-2. If this is indeed the case, then cells expressing a βPDGFR that is unable to bind SHP-2 should be insensitive to the ECM. To test this idea, we compared the effect of ECM on PDGF-dependent Erk activation in cells expressing the F1009 βPDGFR. This receptor has a tyrosine to phenylalanine substitution at 1009 and does not bind SHP-2 effectively (27, 36). This experiment could not be done in the Ph cells since they express the endogenous βPDGFR. Consequently, we chose F cells, which are nullizygous for both PDGFRs. The wild-type and F1009 βPDGFRs were each expressed to the level that is typically seen in fibroblasts (approximately 1 × 10⁵ receptors/cell; data not shown). We first examined the ability of the WT and F1009 receptors to associate with SHP-2. Confluent, quiescent F cells expressing the WT or F1009 receptors were left resting or stimulated with 40 ng/ml PDGF BB for 5 min at 37 °C. The cells were lysed, and the βPDGFR was immunoprecipitated with a receptor-specific antibody, and SHP-2 association was assayed by Western blot analysis. In response to the PDGF stimulation, the wild-type receptor bound SHP-2 in a PDGF-dependent manner, whereas SHP-2 was unable to efficiently associate with the mutant receptor (Fig. 5B). Thus the F1009 βPDGFR was unable to bind SHP-2 when expressed in F cells, which is what we and others (3, 27, 36) have observed in several cell types.

We next examined if in this cell type, fibronectin has the same effect on RasGAP recruitment to the βPDGFR as in Ph cells. We immunoprecipitated the βPDGFR from F cells cultured on polylysine or fibronectin and examined RasGAP binding as described in Fig. 1A. Similar to what we observed in the Ph cells, RasGAP recruitment to the βPDGFR was decreased at 20 and 30 min post-PDGF stimulation in cells plated on fibronectin as compared with those cultured on polylysine (Fig. 5B). We then hypothesized that if the ECM effect requires SHP-2 binding to the βPDGFR, then cells expressing the F1009 receptor will be insensitive to the extracellular ECM. Consistent with our previous findings (36), we found that the F1009 receptor bound RasGAP slightly better than the WT βPDGFR. In addition, recruitment of RasGAP to the βPDGFR was similar in the F1009-expressing cells regardless of whether they were cultured on polylysine or fibronectin (Fig. 5C). Hence, mutation of the binding site for SHP-2 on the βPDGFR negates the effect of culturing cells on fibronectin. These findings

**Fig. 3.** Receptors that cannot associate with RasGAP are better able to activate Ras and Erk. Ph cells expressing the N²WT or N²F771 receptors were grown to 95% confluence, starved overnight in DME + 0.1% calf serum, and left resting (–) or stimulated with 25 (B and C) or 50 ng/ml PDGF AA for the times indicated. A, Ras activation. Active Ras was recovered and detected as described in Fig. 2. B, Erk activation assessed by Western analysis described in Fig. 2. C, Erk activation assessed by an in vitro kinase assay. The cells were washed and lysed, and Erk was immunoprecipitated. Erk immunoprecipitates representing approximately 4.0 × 10⁴ cells were subjected to an in vitro kinase assay in the presence of 0.75 μg of myelin basic protein, and the proteins were resolved by SDS-PAGE, and the resulting gel was exposed to film. The extent of substrate phosphorylation was quantitated by excising the bands and counting them in a scintillation counter. The resulting data, calculated as fold activation relative to the time 0 (ordinate), are plotted as a function of time.
indicated by an arrow on the right-hand side of the blot. Binding of SHP-2 was 3.3, 2.0, 1.9, and 2.3-fold greater at 5, 10, 20, and 30 min post-PDGF, respectively, in cells plated on fibronectin than on polylysine. In 3 independent experiments, we consistently observed that binding of SHP-2 was increased in cells cultured on fibronectin by 1.7–3.5-fold over the cells cultured on polylysine, whereas the amount of p85 that coupled with the βPDGFR was not affected by the ECM.  

strongly suggest that SHP-2 is a key regulator of changes in PDGFR signaling resulting from culturing cells on fibronectin.

Consequences of Decreased RasGAP Recruitment on PDGFR-Dependent Biological Responses—We next determined if changes in PDGFR signaling had biological consequences. Previous reports have suggested that integrin engagement increased PDGFR-dependent mitogenicity and chemotaxis (21, 22). We tested the effect of ECM on PDGFR-dependent responses in F cells. PDGFR stimulated S phase entry in cells expressing the WT βPDGFR, and the response was modestly and reproducibly enhanced when cultured on fibronectin (Fig. 6A). At low and high doses of PDGFR, the cells responded 61 and 67%, respectively, better on fibronectin than on polylysine. The ability of the F1009-expressing F cells to initiate DNA synthesis was also assessed. We found at low doses of PDGFR, F1009 was less able than the WT-expressing cells to drive DNA synthesis, whereas at high doses of PDGFR comparable responses are initiated (Fig. 6B). Importantly, culturing F1009-expressing cells on fibronectin did not improve the response, as it did with the WT βPDGFR. Collectively, these findings suggest that association of SHP-2 is required for cells to respond to the ECM and are consistent with the idea that SHP-2 is the liaison between integrins and βPDGFR signaling.

**DISCUSSION**

To test if culturing cells on ECMs alters PDGFR signaling, we compared the amount of signal relay enzymes that associate with the βPDGFR. Indeed, we found that the composition of the complex of receptor/signaling enzymes was sensitive to the ECM. RasGAP recruitment to the βPDGFR was decreased in cells cultured on fibronectin, and the net effect was an increase in the duration of Ras activation and pathways downstream of Ras, such as Erk. Furthermore, decreased RasGAP binding to the βPDGFR appeared to be a consequence of increased recruitment of SHP-2 to the βPDGFR. Finally cells expressing receptors that did not associate with SHP-2 were not affected by the ECM. Hence, integrins change signaling initiated by the βPDGFR by altering the ability of the receptor to recruit signal relay enzymes.  

These studies strongly suggest that ECM modulates the wild-type receptor via SHP-2, which dephosphorylates the βPDGFR at phosphotyrosine 771 in the RasGAP-binding site. Previous studies with mutant PDGFRs support the idea that this is one of the functions of SHP-2 (35) and are consistent with the findings of investigators studying Torso signal relay (15). Corkscrew (the *Drosophila* homolog of SHP-2) selectively dephosphorylates the Torso receptor at the tyrosine required for binding of v-RasGAP (15). Furthermore, dephosphorylation at the v-RasGAP-binding site modulates pathways downstream of GAP and the strength of Torso signaling (15, 16). Hence, SHP-2 appears to play an important role in modulating receptor tyrosine kinase signaling via dephosphorylation of the tyrosine required for binding of RasGAP.  

In addition to decreasing the duration of RasGAP binding to the βPDGFR, SHP-2 may also promote Ras activation via its ability to recruit Grb2 (37, 38). Tyrosine phosphorylation of SHP-2 has been shown to enable SHP-2 to associate with Grb2. Furthermore, permitting βPDGFR mutants to associate with SHP-2 enhances activation of Ras (3). However, the timing of ECM-dependent enhancement of Ras activation correlated more strongly with a decrease in RasGAP binding than an
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The findings that SHP-2 mediates the ECM effect initiate the question of how plating cells on fibronectin leads to increased association of SHP-2 with the βPDGFR. Integrin engagement has been shown to increase phosphorylation of the PDGFR, in a PDGF-independent manner (39), and perhaps the receptor is phosphorylated better in cells plated on fibronectin. However, we did not detect any tyrosine phosphorylation of the receptor is phosphorylated better in cells plated on fibronectin. Thus the difference appears to be a post-PDGF event and involves recruitment of SHP-2 to the plasma membrane. The findings presented here raise a number of important questions about the nature of βPDGFR signaling. It is somewhat curious that whereas there is noticeably more SHP-2 associated with the βPDGFR at 5 min post-PDGF stimulation, the decrease in the amount of RasGAP bound to the βPDGFR is visible only at 30 min post-PDGF stimulation. This could arise because the steady state level of phosphorylation of tyrosine 771 (binding site for RasGAP) is a balance of phosphatase and kinase activity. At early time points (5 min) post-PDGF, SHP-2 could be dephosphorylating the phosphotyrosine at position 771, but the kinase activity of the receptor rephosphorylates it. At later time points (20 and 30 min), the kinase activity of the receptor begins to decrease, and the balance is shifted in favor of the phosphatase activity. The effect is that the βPDGFR kinase is no longer able to re-phosphorylate the RasGAP-binding site, and a decrease is observed in the amount of RasGAP that associates with the βPDGFR. Consistent with this hypothesis, we have found that the kinase activity of the βPDGFR begins to decrease 30 min post-PDGF stimulation (Fig. 1C).

Hence, during the first 30 min of PDGF stimulation, the nature of the signaling events are dynamic and can be influenced by the ECM of the cell. It is possible that multiple extracellular signals are integrated at these early times. Alternatively, we and others (31)2 have detected signaling events that occur in mid to late G1, and therefore, it is possible that the ECM also influences these events. Identification of the times during G1 progression that key signaling events occur, as well as the identity of the signaling enzymes involved, will facilitate future studies focused on how multiple environmental cues are coordinated and impact a cellular response.

Acknowledgments—We thank Dan Bowen-Pope for the Ph cells; Michelle Tallquist and Philippe Soriano for the F cells; Gen-Sheng Feng for the pan-Erk antibody; Charlie Hart for the PDGF AA; Amon for the PDGF BB; and Joe Avruch for the GST-Raf construct. We also thank Alex Toker, Amy Bernard, Steven Jones, Yasushi Ikuno, Mark Nickas, Nader Rahimi, and Stephan Rosenkranz for comments on this manuscript.

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J. Biol. Chem. 1999, 274:19551-19558.
doi: 10.1074/jbc.274.28.19551

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