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Selective Desensitization of Growth Factor Signaling by Cell Adhesion to Fibronectin*[S]

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Cell adhesion to the extracellular matrix is required to execute growth factor (GF)-mediated cell behaviors, such as proliferation. A major underlying mechanism is that cell adhesion enhances GF-mediated intracellular signals, such as extracellular signal-regulated kinase (Erk). However, because GFs use distinct mechanisms to activate Ras-Erk signaling, it is unclear whether adhesion-mediated enhancement of Erk signaling is universal to all GFs. We examined this issue by quantifying the dynamics of Erk signaling induced by epidermal growth factor, basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) in NIH-3T3 fibroblasts. Adhesion to fibronectin-coated surfaces enhances Erk signaling elicited by epidermal growth factor but not by bFGF or PDGF. Unexpectedly, adhesion is not always a positive influence on GF-mediated signaling. At critical subsaturating doses of PDGF or bFGF, cell adhesion ablates Erk signaling; that is, adhesion desensitizes the cell to GF stimulation, rendering the signaling pathway unresponsive to GF. Interestingly, the timing of growth factor stimulation proved critical to the desensitization process. Erk activation significantly improved only when pre-exposure to adhesion was completely eliminated; thus, concurrent stimulation by GF and adhesion was able to partially rescue adhesion-mediated desensitization of PDGF- and bFGF-mediated Erk and Akt signaling. These findings suggest that adhesion-mediated desensitization occurs with rapid kinetics and targets a regulatory point upstream of Ras and proximal to GF receptor activation. Thus, adhesion-dependent Erk signaling is not universal to all GFs but, rather, is GF-specific with quantitative features that depend strongly on the dose and timing of GF exposure.

Cell adhesion plays a key role in regulating cellular behaviors such as gene expression, cell survival, and proliferation. Normal cells deprived of adhesion to the extracellular matrix undergo cell cycle arrest (1, 2) and programmed cell death even when soluble growth and survival cues are present (1, 3–5). This adhesion dependence is often de-regulated during cancer development, allowing transformed cells to acquire growth and survival advantages over their normal counterparts (6–8). Adhesion-independent survival and proliferation plays a role not only in the build-up of cell mass during tumor formation but also in the survival of cancer cells in foreign, secondary sites during metastasis (6, 9, 10).

Because of the physiological importance of adhesion-mediated cell regulation, significant attention has been given to uncovering the underlying signaling mechanisms. One prominent point of cross-talk between adhesion and growth factors involves the serine/threonine kinase, extracellular signal-regulated kinase (Erk).[2] Several reports have shown that growth factor-mediated Erk signaling is enhanced among cells adhered to extracellular matrix (ECM) proteins (2, 11–20). In fact, this adhesion-mediated enhancement of Erk signaling plays a crucial role in cell cycle regulation. In NIH-3T3 fibroblasts, suspended cells trigger only a transient Erk signal; however, when adhered to FN, growth factor treatment supports both a sustained Erk signal and subsequent progression through the cell cycle (11).

Erk is a major signaling protein that is activated by a wide array of stimuli, including several growth factors such as PDGF, bFGF, and EGF (12–14). It is unclear whether adhesion enhances Erk signaling in response to all of these growth factors or whether only a subset of growth factors signal in an adhesion-dependent manner. Growth factors use substantially different mechanisms to trigger Erk signaling. Unlike EGF, bFGF binding to the cell surface is mediated by two distinct families of cell surface receptors (13). After ligand binding, EGF receptors are phosphorylated on key tyrosine residues that recruit signaling proteins. In contrast, bFGF receptors phosphorylate the multidocking protein FSR2, which subsequently serves as a scaffold to trigger downstream signaling pathways. In addition to activation pathways, growth factors differ in negative feedback mechanisms that desensitize signaling (15, 16). For example, although stimulation via EGF and PDGF results in serine/threonine phosphorylation of their respective receptors, this receptor phosphorylation results in Erk inhibition only in the cells stimulated by PDGF. Interestingly, Erk activation in EGF-stimulated cells remains unaffected (15). Such differences in growth factor signaling mechanisms raise the hypothesis that growth factors may differ in the extent to which their stimulation of Erk signaling is adhesion-dependent.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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[2] The abbreviations used are: Erk, extracellular signal-regulated kinase; GF, growth factor; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; FN, fibronectin; PH, poly-HEMA; Erk_T, total Erk; ECM, extracellular matrix; ppErk, active Erk.

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To begin to test this hypothesis, we measured the effect of cell adhesion on Erk signaling by three growth factors (EGF, bFGF, and PDGF). To measure the level of adhesion dependence on both the magnitude and dynamics of the Erk signal, we implemented a quantitative protocol for Western blot imaging and analysis. This quantitative approach revealed that adhesion-dependent Erk signaling is selective to EGF in NIH-3T3 fibroblasts. Furthermore, our data reveal that adhesion is not always a positive influence on GF-mediated Erk signaling. At a critical subsaturating dose of PDGF and bFGF, cell adhesion actually thwarts Erk signaling. Our results show that adhesion desensitizes cells from subsequent GF-mediated activation of Erk; that is, adhesion renders the signaling pathway unresponsive to GF treatment. Interestingly, reducing the duration of cell adhesion before GF stimulation proved critical in the desensitization process. PDGF- and bFGF-mediated Erk signaling significantly improved among adherent cells only when pre-exposure to adhesion was completely eliminated; however, concurrent stimulation by growth factors and adhesion was only able to partially neutralize GF-mediated desensitization. Our findings suggest that adhesion dependence of Erk signaling is not universal to all GFs but, rather, is GF-specific with quantitative features that depend strongly on the dose and timing of adhesion and GF exposure.

EXPERIMENTAL PROCEDURES

Cell Maintenance—NIH-3T3 fibroblasts (ATTC) were cultured and maintained in 89% Dulbecco's modified Eagle's medium (Invitrogen), 10% Donor Calf Serum + iron (Invitrogen), 1% PenStrep (Invitrogen). After ~2 days of growth, when cells reached between 70 and 80% confluence, subconfluent cells were suspended using 0.25% trypsin, EDTA (Invitrogen) and reseeded onto tissue culture dishes.

Protein-coating Surfaces—Fibronectin (FN)-coated surfaces were prepared by incubating 2 ml of 5 μg/ml FN (Sigma) diluted in Dulbecco's phosphate-buffered saline (Sigma) in tissue culture dishes overnight at 4 °C. The dishes were gently rocked during adsorption. The dishes were then blocked with 1 mg/ml heat-inactivated bovine serum albumin in phosphate-buffered saline for 1 h at 37 °C. Poly-HEMA (PH)-coated surfaces were prepared by incubating 5 ml of a solution containing 6 mg/ml PH (Sigma) dissolved in 70% biological grade ethanol (Sigma) in uncovered tissue culture dishes overnight at room temperature.

Cell Adhesion Experiments—NIH-3T3 cells were suspended using 0.25% trypsin-EDTA, reseeded, and grown until 70–80% confluent. Subconfluent dishes were starved in completely serum-free media (99% Dulbecco's modified Eagle's medium (Invitrogen), 1% PenStrep (Invitrogen), 1 mg/ml bovine serum albumin (Sigma)) for 20 h to bring adhesion signals back to basal levels. Serum-starved cells were suspended using 0.05% trypsin. Trypsin activity was quenched by adding soybean trypsin inhibitor (Sigma) to a final concentration of 0.5 mg/ml. Cells were re-suspended in serum-free media to a concentration of 5×10^5 cells/ml and were either plated onto FN-coated dishes or PH-coated dishes; PH-coated dishes were rocked to prevent cell aggregation.

After cells acclimated to the surface for varying lengths of time (either 2.5, 1, or 0 h, as described in the figure legends), cells were stimulated with the indicated amount of either PDGF (Sigma), bFGF (Sigma), or EGF (Peprotech). Cells were lysed in buffer containing 50 mM Tris (pH 7.5), 150 mM sodium chloride, 50 mM β-glycerophosphate (pH 7.3), 10 mM sodium pyrophosphate, 30 mM sodium fluoride, 1% Triton X-100, 1 mM benzamidine, 2 mM EGTA, 100 μM sodium orthovanadate, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated in lysis buffer for 15 min on ice before centrifugation and collection of the supernatant. Micro-BCA protein assay kit (Pierce) was used to determine the total protein concentration.

Immunoblotting—Whole cell lysates were resolved by 10% SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane. Blots were probed using either an antibody against dually phosphorylated Erk (Cell Signaling), Ser-473-phosphorylated Akt (Cell Signaling), Erk2 (Santa Cruz Biotechnology, Inc.), Sos (Santa Cruz), or caspase 3 (Upstate Biotechnology, Inc.). In the cases of Sos and caspase 3, 7 and 15% gels were used to better resolve high and low molecular weight proteins, respectively. Blots were imaged and quantified as described under "Results" and "Discussion."

RESULTS

Quantitative Measurement of Erk Signaling—To quantify the extent to which different GFs induce adhesion-dependent Erk signaling, we developed a systematic, quantitative Western blotting protocol. The methodology is based on digital imaging using a cooled CCD camera that has a theoretically wider linear dynamic range than standard film-based imaging (17, 18). However, even when signals are within the detection limit of the imaging system, data points do not always conform to the expected linear trend (supplemental Fig. 1). We expect that factors such as antigen saturation may contribute to the observed non-linearity.

To address these sources of non-linearity that may be specific to each blot, we developed a quantitative Western blotting protocol that employs standard samples to establish the linear dynamic range of each blot. The standards are a set of dilutions of a positive control lysate as illustrated for an anti-Erk Western blot in Fig. 1*A*. The band intensities from the standard lanes are quantified, and the working linear range is established empirically for each blot (Fig. 1*B*). Band intensities from the lanes loaded with lysates of interest are then confirmed to fall within the linear dynamic range (Fig. 1*B*); any band intensities that fall outside the linear dynamic range are discarded. The sole exceptions to this requirement are samples whose basal signal cannot be distinguished from background noise; a typical example is the initial time point after serum starvation. This approach ensures that the measurements of Erk expression levels lie within the linear dynamic range of each Western blot. A similar approach is applied to quantify phospho-Erk and phospho-Akt. To adjust for unequal loading, the band intensity associated with a phosphoprotein (*e.g.* phospho-Erk) is normalized to the band intensity of an equal-loading control, such as total Erk (Erk_T).

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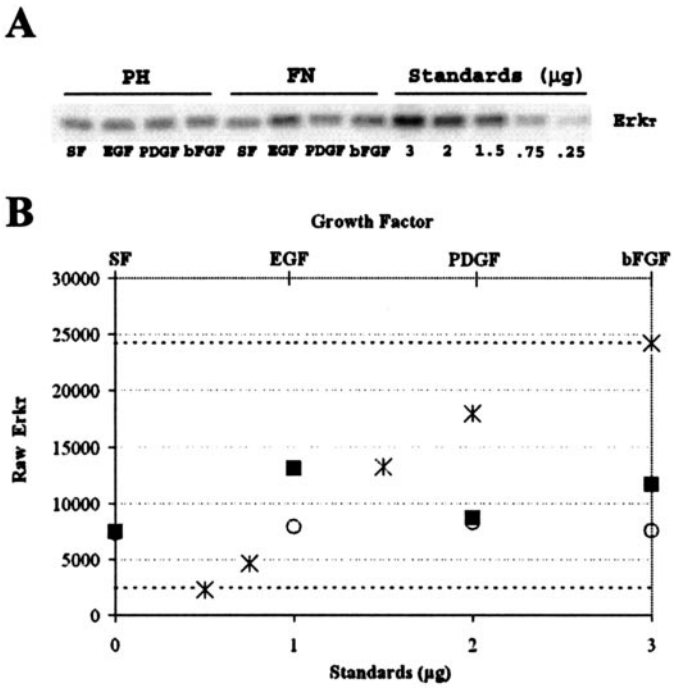

FIGURE 1. **Quantitative Western blot imaging and analysis.** *A*, a representative anti-Erk2 Western blot of NIH-3T3 cell lysate. *Lanes 1–4* contain lysates from cells that have been held in suspension for 2.5 h followed by stimulation with 1 of 3 different growth factors for 12 min. *Lanes 5–8* represent cells that were adhered on FN-coated dishes for 2.5 h before stimulation for 12 min by 1 of 3 growth factors. Different relative amounts of the standard lysate were loaded in *lanes 9–13*. In this test case the standard lysate was derived from cells adhered on FN-coated dishes for 2.5 h before stimulation with a mixture of 4 growth factors (800 pM EGF, 800 pM PDGF, 1000 pM bFGF, 1 μM insulin) for 40 min. SF, serum-free medium. *B*, quantification of Erk2 band intensities and the identification of the linear dynamic range. The Erk2 band intensities were quantified for the blot depicted in *panel A*. The intensities of the standard bands (*asterisks*) are plotted as a function of the relative amounts of the standard sample (*bottom x* axis). The upper and lower bounds (*dashed lines*) of the empirically verified linear dynamic range are indicated. The intensities of the total Erk bands for the cells adhered to fibronectin (*solid squares*) and cells held in suspension (*open circles*) are plotted as a function of the treatment condition (*top x* axis).

TABLE 1

Summary of growth factor properties including the critical concentration at which adhesion-mediated desensitization occurs

NA, not applicable.

Growth factor	M_r	K_d	Critical concentration, $[GF]_c$
		pM	pM
EGF	6.20	670 (Ref. 43)	NA
PDGF	24.6	100–1000 (Refs. 44–50)	8.1
bFGF	16.4	30 (Refs. 37 and 51)[a]	1.2

[a] At low concentrations, bFGF will bind almost exclusively to high affinity sites (51); thus, the reported K_d corresponds to bFGF interaction with its high affinity receptor.

We note that the same standards are not used in every blot. Rather, dilutions of a positive control lysates are used as standards. This approach ensures that for a particular blot, the band intensities of the standards will encompass nearly the entire linear range of the blot. Such an approach increases the likelihood that the band intensities of the lysates of interest will fall within the linear range. To ensure that data collected from two distinct blots can be compared with one another, we included a common reference point in each blot. This reference is a sample generated under the same stimulation conditions. The data are then always analyzed and reported relative to this reference point.

EGF, but Not PDGF and bFGF, Induces Adhesion-dependent Erk Activation—Using this quantitative Western blotting protocol, we measured the Erk signaling response to growth factor treatment of cells adhered on FN or held in suspension. Our initial experiments used growth factor concentrations well above the dissociation constant (K_d) (Table 1). At these saturating growth factor concentrations, EGF-mediated Erk signaling is enhanced by cell adhesion. Cells adhered on FN exhibit ~3-fold greater Erk activation than cells held in suspension in response to treatment with EGF for 12 min (Fig. 2*A*). Meanwhile, neither bFGF- nor PDGF-mediated Erk signaling at a single early time point are adhesion-dependent (Fig. 2*A*). These results suggest that some GFs (EGF) signal better via the Erk pathway when in an adhesive setting, whereas other factors (PDGF, bFGF) promote Erk signaling in an adhesion-independent manner.

These observations are based on a single, early time point. However, others have shown that GF-mediated Erk signaling may exhibit different dynamical features in adhered *versus* non-adhered cells (11, 19–21). For example, in both adhered and suspended cells, PDGF stimulates Erk equivalently at early times, but only the adhered cells maintain a sustained Erk signal (21). To examine whether adhesion affects the dynamics of GF-mediated Erk signaling, we measured a full time-course of Erk signaling in response to each of the three GFs. For EGF-stimulated cells, the early phase of Erk activation (<1 h) is adhesion-dependent, whereas the late phase of the signal reaches a nearly equivalent, basal signal for both adhered and suspended cells (Fig. 2*B*). Furthermore, both the adherent and suspended cells reach maximum signal intensity after only 6 min of stimulation. Thus, the kinetics of EGF-induced Erk signaling is similar in both adherent and suspended cells, although signal magnitude is clearly adhesion-dependent.

In contrast, Erk activation in cells stimulated with either PDGF (Fig. 2*C*) or bFGF (supplemental Fig. 2) was adhesion-independent. For both growth factors, the Erk signal reached a similar maximum after ~30 min of stimulation. Furthermore, in the case of PDGF, the Erk signal decays with similar kinetics for both suspended and adhered cells. In the case of bFGF, however, the Erk signal is sustained at near-maximum levels in both adhered and suspended cells. Thus, measurements of the complete dynamics of Erk signaling show that EGF, but not PDGF or bFGF, induces Erk signaling in an adhesion-dependent manner.

Subsaturating Doses of PDGF or bFGF Reveal Negative Adhesion-GF Synergism—The apparent lack of adhesion dependence in Erk signaling for bFGF and PDGF may be linked to the fact that high concentrations of the growth factors were used. In this concentration regime, excessive GF signaling may overcome the need for cell adhesion. Thus, we hypothesized that for PDGF and bFGF, Erk signaling may be adhesion-dependent if concentrations are near or less than K_d (Table 1) were used. To test this possibility, we measured GF-mediated Erk signaling across a broad range of GF concentrations.

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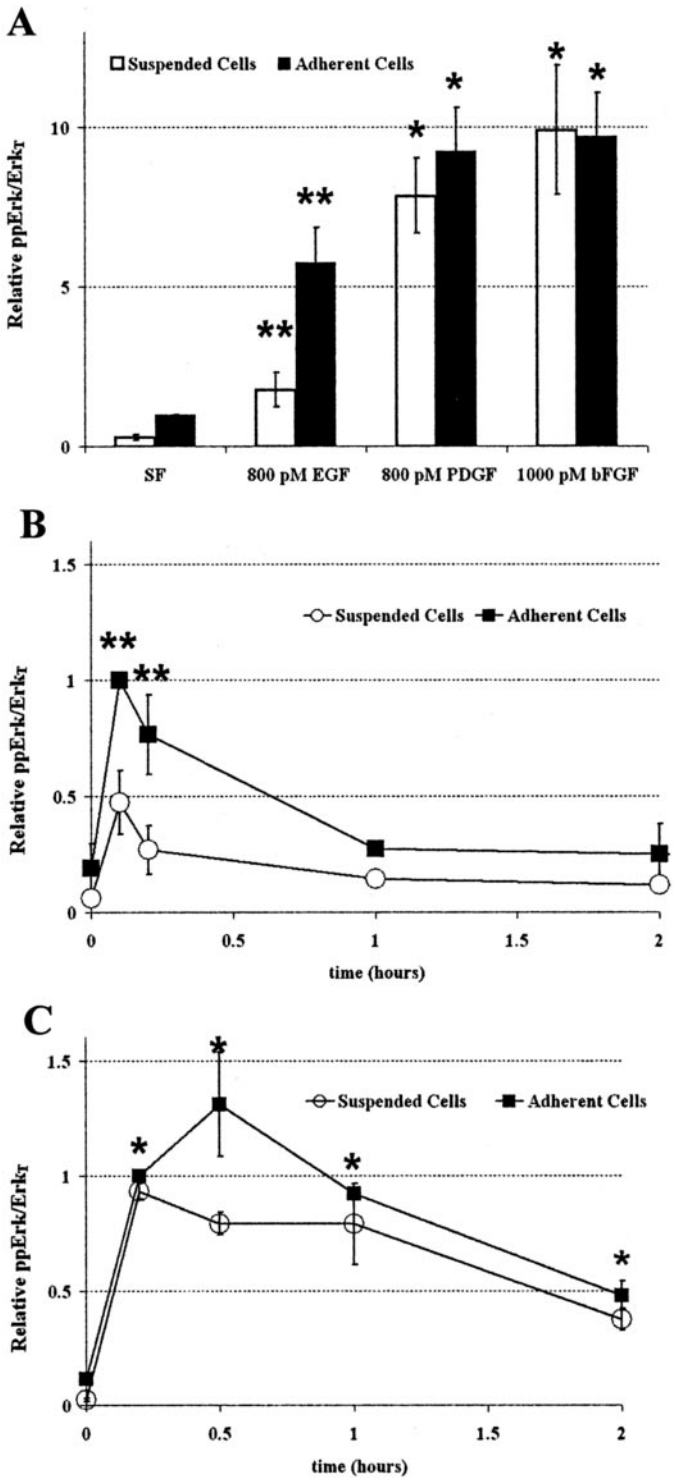

FIGURE 2. **Adhesion dependence of Erk signaling at saturating concentrations of growth factors.** Serum-starved cells were either held in suspension via PH-coated dishes or allowed to adhere on FN-coated dishes for 2.5 h before stimulation with serum-free medium containing a single growth factor at the indicated concentrations. After the desired time of exposure to growth factor, cells were lysed, and lysates were analyzed via Western blot with the anti-phospho-Erk and anti-Erk antibodies. The relative amount of active Erk (*ppErk*) normalized to the equal-loading control, total Erk (Erk_T), is reported for the different treatment conditions. *A*, adhesion enhances Erk signaling in response to EGF, but not bFGF or PDGF, stimulation. Cells held in suspension (*empty*) and those adhered to FN (*filled*) were stimulated with the indicated GF-containing medium or with serum-free medium (*SF*) and were lysed after 12 min of stimulation. *Error bars* represent sample S.E. ($n = 2–9$). The *single asterisk* denotes that Erk activation in the suspended and adherent cells is statistically similar. The *double asterisk* denotes that ERK activation in the suspended and adherent cells is statistically different ($p < 0.01$) using Student's *t* test. *B*, adhesion enhances EGF-mediated Erk signaling over the entire time course. Cells held in suspension (*empty circles*) or allowed to adhere on FN (*solid square*) were stimulated with 800 pM EGF for the indicated times. *Error bars* represent sample S.E. ($n = 2–4$). The *double asterisk* denotes that ERK activation in the suspended and adherent cells is statistically different with $p < 0.05$ (6 min) and $p < 0.09$ (12 min). All *p* values were computed using Student's *t* test. *C*, PDGF activates Erk in an adhesion-independent manner over the entire time course. Cells held in suspension (*empty circles*) or allowed to adhere on FN (*solid squares*) were stimulated with 800 pM PDGF for the indicated times. *Error bars* represent sample S.E. ($n = 2–4$). The *single asterisk* denotes that ERK activation in the suspended and adherent cells is not statistically different using Student's *t* test.

In the case of EGF, varying its concentration over 3 orders of magnitude did not affect the observed adhesion-mediated enhancement in Erk signaling (Fig. 3*A*). Regardless of its concentration, EGF stimulated an ~3-fold greater Erk response among adherent cells than among suspended cells (Fig. 3*A*). In contrast, experiments with different PDGF and bFGF concentrations revealed an unexpected response (Fig. 3, *B* and *C*). At a critical growth factor concentration (8 pM PDGF or 1 pM bFGF), cells in suspension induced Erk signaling to a significantly greater extent than adherent cells. Above the critical PDGF and bFGF concentration, adhered and suspended cells responded equivalently (Fig. 3, *B* and *C*). At the critical PDGF and bFGF concentration, the suspended cells responded 7- and 13-fold better, respectively, than their adherent counterparts (Fig. 3, *B* and *C*). These results reveal a negative synergism between adhesion and GF stimulation; adhesion thwarts Erk activation at critically low doses of PDGF and bFGF.

Because this negative synergy was observed at a specific time point in Erk signaling, we investigated the dynamics of Erk signaling more completely at the critical PDGF concentrations (Fig. 4) and bFGF concentrations (supplemental Fig. 3). At the critical PDGF concentration, adherent cells were unable to induce Erk signaling during the entire time course (Fig. 4). In contrast, Erk signaling in suspended cells was substantial throughout the entire time course (Fig. 4). Thus, the observed negative synergism between adhesion and GF stimulation is not an artifact of selecting a specific time point; rather, the entire dynamics of PDGF-mediated Erk signaling is suppressed among adherent cells at the critical PDGF concentration. Similar results were observed for bFGF as adhesion completely ablated bFGF-mediated Erk activation (supplemental Fig. 3).

Adhesion Desensitizes PDGF- and bFGF-mediated Erk Signaling—The observed negative synergy reveals that cell adhesion to FN selectively abrogates PDGF- and bFGF-mediated Erk signaling when these growth factors are present at low concentrations. These observations raise the possibility that adhesion desensitizes subsequent Erk activation. Desensitization refers to a state in which a signaling pathway becomes unresponsive to stimuli. This desensitized or refractory state may occur when a signal triggers negative feedback mechanisms that persist and prevent re-activation of the signal in response to new stimuli. Published reports have shown that growth factor stimulation inhibits Erk signaling in response to a second challenge of growth factor (22–24). Our results suggest that adhesion to FN may also desensitize Erk signaling to a select subset of growth factors (PDGF and bFGF).

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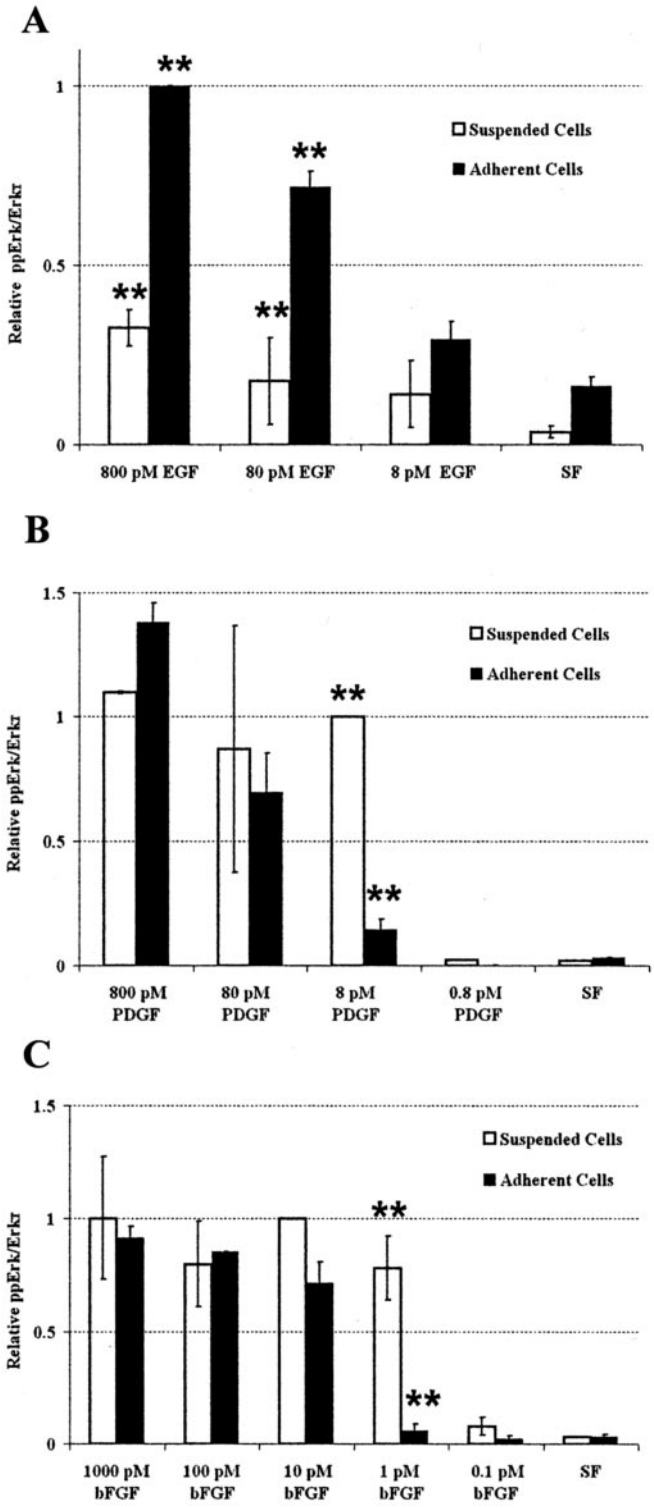

FIGURE 3. **Adhesion dependence of Erk signaling across a wide range of growth factor doses.** NIH-3T3 cells were treated as described in the legend to Fig. 2*A*, except that cells were stimulated with serum-free medium containing different doses of EGF for 12 min (*A*), PDGF for 30 min (*B*), or bFGF for 30 min (*C*). The response of cells held in suspension (*empty*) is compared with cells adhered on FN (*filled*). *A*, *error bars* represent sample S.E. ($n = 2–4$). The *double asterisk* denotes that ERK activation in suspended and adherent cells is statistically different with $p < 0.001$ (800 pm) and $p < 0.07$ (80 pm). *B*, *error bars* represent sample S.E. ($n = 2–4$). The *double asterisk* denotes that ERK activation in suspended and adherent cells is statistically different with $p < 0.04$. *C*, *error bars* represent sample S.E. ($n = 2–4$). The *double asterisk* denotes that ERK activation in the suspended cells is statically different with $p < 0.07$. All *p* values were computed using Student's *t* test. *ppErk*, active Erk; *SF*, serum-free medium.

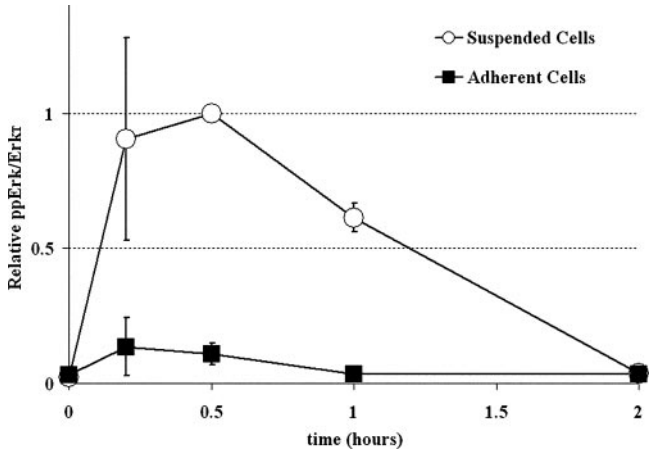

FIGURE 4. **Time course of adhesion-dependent Erk signaling at the critical PDGF concentration.** Serum-starved NIH-3T3 cells were held in suspension (*empty circles*) or adhered on FN (*solid squares*) as described in the legend to Fig. 2. Cells were stimulated with serum-free medium containing 8 pM PDGF and lysed at the indicated times. The relative amount of active Erk normalized to total Erk is reported. *Error bars* represent sample S.E. ($n = 2–4$).

Consistent with this hypothesis of adhesion-mediated desensitization, cell adhesion to FN in the absence of growth factors promotes Erk activation (Fig. 5). Adhesion rapidly stimulates the Erk pathway with maximal activation occurring by ~12 min after cell seeding. This adhesion-mediated Erk signaling may trigger negative feedback loops that desensitize cells to subsequent Erk signaling by PDGF and bFGF. If desensitization is responsible for reduced PDGF- and bFGF-mediated Erk signaling, then reducing the duration of cell adhesion before growth factor stimulation might alleviate this suppression. Because cells were seeded 2.5 h before stimulation in all previous experiments, we tested this hypothesis by measuring PDGF-mediated Erk signaling among cells that were exposed to FN-coated surfaces for shorter times, specifically 1 and 0 h (Fig. 6, *A* and *B*, respectively). In the 0-h case, cells were concurrently stimulated with growth factor and plated onto FN-coated dishes.

Reducing the duration of adhesion significantly enhanced PDGF-mediated Erk signaling among adherent cells. Although Erk signaling was severely attenuated among cells that had adhered for 2.5 h (Fig. 4), reducing adhesion time to 1 h only slightly improved Erk signaling (Fig. 6*A*). However, eliminating pre-exposure to adhesion altogether by concurrent stimulation with PDGF significantly improved Erk signaling among adherent cells (Fig. 6*B*). To quantify the enhancement in PDGF-mediated Erk signaling in response to decreasing the duration of adhesion, we integrated the time course of Erk signaling for cells held in suspension or adhered on FN for 0, 1, and 2.5 h (Fig. 6*C*). When the pre-exposure time to adhesion was reduced from 2.5 to 0 h, the integrated Erk signal increased ~5-fold. Notably, even concurrent stimulation was unable to rescue PDGF-mediated Erk activation to the same level as that observed in suspended cells, suggesting that adhesion-mediated desensitization occurs rapidly. The rapid timescale of adhesion-mediated desensitization is consistent with the fact that cell adhesion to FN significantly activates Erk within 12 min of cell seeding (Fig. 5).

Mechanisms Underlying Adhesion-mediated Desensitization—The hyperphosphorylation of Sos is a prominent mechanism in

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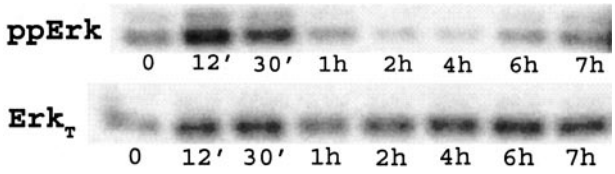

FIGURE 5. **Adhesion-mediated Erk activation.** Serum-starved NIH-3T3 cells were suspended and re-plated on FN-coated plates as described in the legend of Fig. 2. Cells were lysed at the indicated times after plating without growth factor stimulation. Lysates were analyzed by SDS-PAGE and Western blotting with an anti-phospho-Erk antibody (*top panel*) and an anti-Erk antibody (*bottom panel*) as an equal loading control.

GF-mediated desensitization of Erk (25–29). To determine whether cell adhesion to FN desensitizes the Erk pathway in a similar manner, we measured the effect of cell adhesion on the hyperphosphorylation of Sos using a gel retardation assay (Fig. 7). The mobility of Sos did not change among cells that were plated on FN-coated substrates in the absence of GF (Fig. 7, *0–240 min lanes*). In contrast, a positive-control treatment with PDGF induces a clear and significant retardation in Sos mobility (Fig. 7, *PC lane*). Thus, adhesion-mediated desensitization of Erk signaling does not involve hyperphosphorylation of Sos.

To determine whether adhesion-mediated suppression of PDGF and bFGF signaling was specific to the Erk pathway, we measured Akt signaling under similar conditions. PDGF- and bFGF-mediated Akt phosphorylation was also significantly diminished among adherent cells (Fig. 8 and supplemental Fig. 4, respectively). In addition, PDGF-mediated Akt activation among adherent cells significantly improved as adhesion time on FN was decreased (Fig. 8). Although reducing the duration of adhesion from 2.5 to 1 h only slightly improved Akt signaling (compare Figs. 8, *A* and *B*), concurrent stimulation significantly improved Akt signaling among adherent cells (Fig. 8*C*). Indeed, the integrated Akt signal shows a trend identical to that of the integrated Erk signal. Although the integrated Akt signal for suspended cells remains constant for adherent cells, the signal clearly increases as the duration of adhesion on FN is reduced (Fig. 8*D*). Because adhesion suppresses both Erk and Akt signaling, it suggests that adhesion-mediated desensitization of PDGF and bFGF signaling may occur at or above the level of Ras activation but independent of Sos regulation.

DISCUSSION

This study demonstrates that cell adhesion has quantitatively intricate effects on GF-mediated Erk signaling. We report that the effect of cell adhesion is specific to the type of growth factor, its dose, and the timing of stimulation. Our system exclusively uses NIH-3T3 fibroblasts that are stimulated in defined medium. We find that adhesion to FN selectively enhances Erk signaling elicited by EGF but has no effect on bFGF- or PDGF-mediated Erk activation. Unexpectedly at concentrations of PDGF and bFGF (GF_c) that are significantly less than K_d (Table 1), cell adhesion severely attenuates GF-mediated Erk signaling. Thus, adhesion not only enhances cell response to specific growth factors but also filters out potentially noisy signals from low levels of growth factor. This aspect of adhesion-GF cross-talk may play an important role in buffering cell response to noisy background levels of GF stimulation. These results reveal

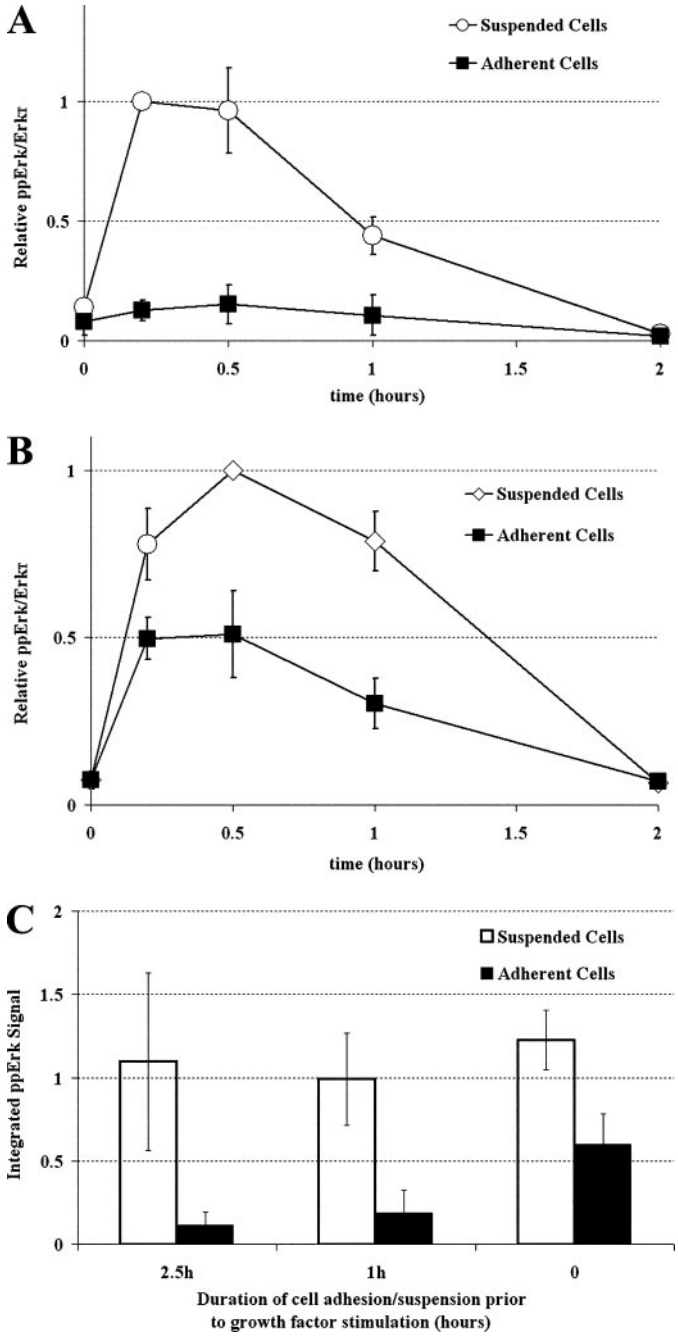

FIGURE 6. **The dependence of PDGF-mediated ERK signaling on the duration of pre-exposure to FN-coated surfaces.** Serum-starved NIH-3T3 cells were held in suspension (*empty circles*) or adhered on FN-coated plates (*solid squares*) as described in the legend to Fig. 2. The duration cells spent in suspension or adhered to FN before stimulation with 8 pM PDGF was reduced from 2.5 h to 1 h (*A*) or 0 h (*B*). The integral of the ERK time-course for all three acclimation times are shown in *C*. For *A* and *B*, *error bars* represent sample S.E. ($n = 3–6$). For *C*, the error bars represent propagated error when the trapezoid rule is used to calculate the integrated signal.

that the cross-talk between adhesion and growth factor signaling has intricate quantitative features, consistent with the extensive connectivity between adhesion and growth factor signaling pathways (14, 30, 31).

Our observation that adhesion to FN enhances EGF-mediated Erk signaling is consistent with other reports (32). Our results further demonstrate that adhesion does not enhance

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either PDGF- or bFGF-mediated Erk signaling in NIH-3T3 cells, a finding that is contrary to some reports (11, 19–21). In one such report bFGF treatment was found to induce sustained Erk signaling that supports cell cycle progression of NIH-3T3 fibroblasts seeded on FN-coated surfaces (33). However, the 3T3 cells used express exogenous human $\alpha_5\beta_1$ integrin, whereas our cell system expresses only endogenous integrin adhesion receptors. Furthermore, both the aforementioned study and others using NIH-3T3 cells supplement the growth factor-containing medium with serum (11, 19). This serum supplement is essential to maintain long-term cell viability, a clear requirement for studying cell cycle progression. Our studies, in contrast, employ serum-free medium supplemented with specific growth factors. We have carefully assayed cell death under serum-free conditions by trypan blue staining and by Western blotting for caspase 3 cleavage (data not shown). Our measurements show that cells held in suspension or adhered on FN-coated plates remain viable for 4–5 h in serum-free conditions. Thus, all reported results are gathered in this time window and offer a clear indication of how Erk signaling by each growth factor is influenced by adhesion without confounding contributions from serum.

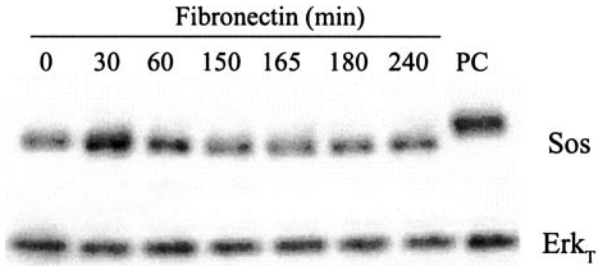

FIGURE 7. **The effect of cell adhesion on Sos hyperphosphorylation.** Serum-starved NIH-3T3 cells were suspended and re-plated on FN-coated plates as described in the legend of Fig. 2. Cells were lysed at the indicated times after plating without growth factor stimulation. Lysates were analyzed by SDS-PAGE and Western blotting with an anti-Sos antibody. A shift in total Sos indicates presence of the hyperphosphorylated form of Sos. The *lane marked PC* represents the positive control for the hyperphosphorylated form of Sos and contains cells that have been adhered to fibronectin for 2.5 h before stimulation by 800 pM PDGF for 12 min.

In addition to serum, cell type differences may also contribute to apparent differences in adhesion dependence of Erk signaling. Kazlauskas and co-workers (21) showed that PDGF treatment of mouse embryo fibroblasts adhered on FN induces sustained Erk activation, whereas cells seeded on poly-lysine support only a transient Erk signal. The difference between our results and those of Kazlauskas and co-workers (21) may be due to the use of mouse embryo fibroblast *versus* NIH-3T3 cells. Moreover, the mouse embryo fibroblast strain used in the study lacks PDGF receptor α and expresses endogenous PDGF recep-

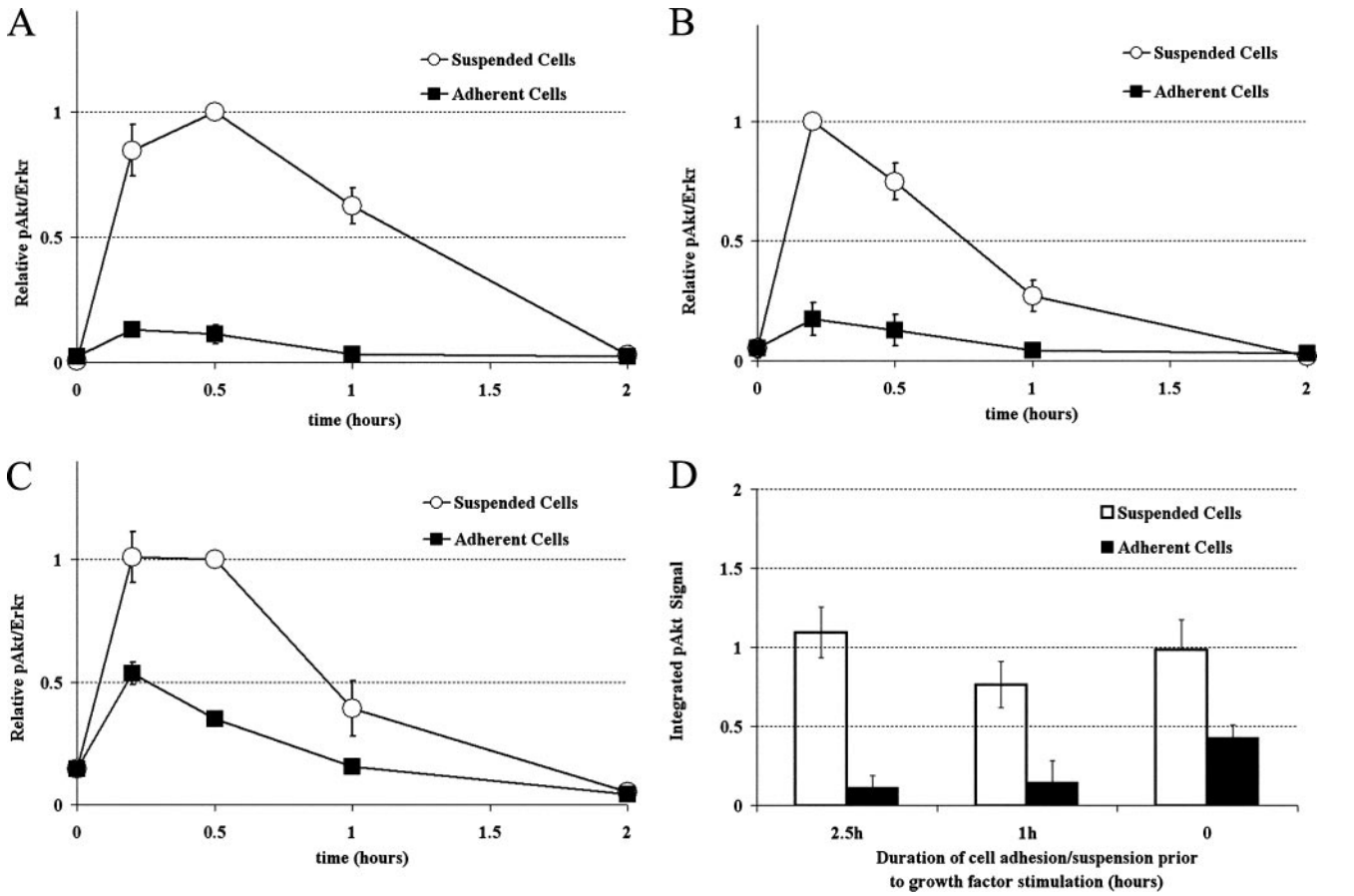

FIGURE 8. **Adhesion dependence of PDGF-mediated Akt signaling.** Serum-starved NIH-3T3 cells were held in suspension (*empty circles*) or adhered on FN (*solid squares*) for 2.5 h as described in the legend to Fig. 2. Cells were then treated with PDGF and lysed at the indicated time points. The level of phosphorylated Akt (*pAkt*) was quantified and normalized to the amount of total cellular Erk (*Erk$_T$*). The duration for which cells were either held in suspension or adhered on FN was reduced from 2.5 h (*A*) to 1 h (*B*) or 0 h(*C*). The integrated area for all three acclimation times is shown in *D*. For *A* and *B*, *error bars* represent the sample S.E. ($n = 3–6$). For *D*, the *error bars* represent propagated S.E. when the trapezoid rule is used to approximate the integrated signal.

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tor β; our NIH-3T3 cells express both isoforms. Thus, the complement of homo- and heterodimer receptors available for binding PDGF-BB and for intracellular signaling are likely to be different in these two cell types.

Because of both the differences in cell types and receptor expression profiles as well as the potentially confounding contributions from serum, it remains unclear whether adhesion-dependent Erk signaling is specific to particular growth factors. We sought to address this issue by developing a system that utilizes only NIH-3T3 fibroblast cells expressing endogenous integrins. Furthermore, growth factor stimulation was limited to use of only one growth factor in defined media; thus, no serum was used during the course of the experiments. Therefore, our data begin to provide a systematic comparison of the cross-talk between adhesion and three different growth factors. We show that adhesion to FN enhances Erk signaling elicited by EGF but not by bFGF and PDGF. Our observation that adhesion-mediated enhancement of Erk signaling is not a universal property of all growth factors is consistent with our previous findings in Chinese hamster ovary cells. In these cells the dynamics and magnitude of insulin-mediated Erk signaling is unaffected by cell adhesion to FN (34). Rather, adhesion and insulin synergistically affected IRS-1 phosphorylation en route to co-regulating cell cycle activity.

The specificity of adhesion dependence might be explained by intrinsic differences in how the receptors of these growth factors signal to Erk. Although all three growth factors employ the canonical Ras-mitogen-activated protein kinase cascade to activate Erk, there are significant differences in the upstream machinery that connect to the core Ras/mitogen-activated protein kinase signaling module. Although EGF receptors recruit the necessary signaling proteins mostly by themselves (13), bFGF receptors rely on the formation of a multidocking signaling protein complex to recruit the majority of signaling components (35). In addition, bFGF binds to two distinct families of cell surface receptors, the first being the bFGF receptor-tyrosine kinase and the second being heparin sulfate proteoglycans (36, 37). The binding to and signaling from two distinct receptor families provide additional layers of control and complexity to bFGF-mediated signaling to Erk (38).

In addition to utilizing different mechanisms for activating the Ras/mitogen-activated protein kinase module, growth factor receptors differ in their susceptibility to negative regulatory mechanisms. For example, serine/threonine phosphorylation of EGF and PDGF receptors has been shown to affect the two receptors differently. G protein-coupled receptor kinase 2-mediated serine/threonine phosphorylation of the PDGF receptor results in a decrease in PDGF receptor tyrosine phosphorylation, which correlates to an observed decrease in Erk activation by PDGF stimulation (15). In contrast, G protein-coupled receptor kinase 2-mediated serine/threonine phosphorylation has no effect on the tyrosine phosphorylation of the EGF receptor, and subsequent Erk activation is also not affected. In summary, there are distinct pathways by which growth factors activate the Ras/mitogen-activated protein kinase module as well as differences in growth factor receptor sensitivity to negative regulatory mechanisms. Cell adhesion may also couple to pathways unique to EGF, thereby selectively enhancing EGF-mediated Erk signaling.

Although adhesion selectively enhances EGF-mediated Erk signaling at saturating growth factor concentrations, an intriguing feature of adhesion dependence was found at low, subsaturating doses of growth factors. PDGF- and bFGF-mediated Erk signaling is substantially attenuated among cells adhered on FN. This adhesion-mediated suppression of growth factor-induced Erk signaling is alleviated if the duration of cell adhesion is reduced. These observations suggest that cell adhesion rapidly triggers mechanisms that desensitize Erk signaling by low concentrations of PDGF and bFGF.

Desensitization of the ERK signaling pathway has been reported in response to growth factor stimulation. Growth hormone (GH) induces Erk activation in HA cells; however, re-exposure to GH in cells that have been pretreated with this GH for 3 h fails to stimulate ERK (23). Similar desensitization of Erk activation has been shown in insulin-treated Chinese hamster ovary cells expressing insulin receptors (CHO/IR). Although Erk activation occurs upon initial insulin exposure, a second exposure to insulin fails to induce Erk signaling (24). Comparable insulin-mediated desensitization to three independent Gα-coupled ligands has been observed in 3T3-LI adipocyte cells (22). However, in these cells, insulin pretreatment does not desensitize EGF-mediated ERK activation. In contrast to insulin and EGF, heterologous desensitization has been observed between EGF and PDGF (39). Swiss-3T3 cells first exposed to PDGF fail to induce Erk activation upon a subsequent treatment with either PDGF or EGF. The converse is also observed; initial exposure to EGF inhibits subsequent stimulation of ERK signaling by PDGF or EGF treatment. Hence, there is precedent for desensitization to selectively affect a subset of growth factors.

A prominent mechanism by which GF desensitizes ERK signaling in response to subsequent GF stimulation involves Sos hyperphosphorylation (25–28). However, our results demonstrate that adhesion to FN does not induce Sos hyperphosphorylation, suggesting that adhesion-mediated desensitization does not occur at the level of Sos regulation.

Our results suggest that the time scale of desensitization is remarkably rapid. Thus, although Sos is not the target of desensitization, another signal extremely proximal to growth factor detection must be involved. Consistent with this possibility, our results show that both Akt and Erk signaling are subject to adhesion-mediated desensitization, suggesting that Ras or some other common upstream element is the point of desensitization. In fact, several growth factor receptors directly interact with adhesion receptors (40). Although the association of growth factor receptors with adhesion receptors has been predominantly correlated with positive synergism, it may also sequester and inhibit the activity of low levels of ligand-bound growth factor receptors. Indeed, such heterologous desensitization by receptor sequestration has been demonstrated for EGFR and PDGF receptor (39). Another possible mechanism of growth factor desensitization may involve direct interactions between growth factors and ECM proteins. Sequestration of transforming growth factor β, vascular endothelial growth factor, and hepatocyte growth factor by ECM is well documented

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(41). In fact, vascular endothelial growth factor has recently been shown to bind specific sites on FN, suggesting potential sequestration in more basic reconstituted systems. Thus, ECM protein-mediated sequestration may play a role in diminishing GF-mediated signaling on FN-coated dishes, especially in systems employing low doses of GF (42). In fact, because NIH-3T3 cells deposit their own matrix, it remains a possibility that ECM proteins other than the adsorbed FN may be responsible for the observed desensitization, possibly via GF sequestration.

Although elucidating the precise role of these mechanisms is the subject of ongoing work in our laboratory, it is especially intriguing that adhesion-mediated desensitization occurs selectively at low growth factor concentrations. Thus, adhesion may play an important role in buffering cell response to noisy, background levels of growth factor stimulation. Combined with the ability to enhance signaling for select growth factors, adhesion may have a net positive effect on the signal:noise ratio of detecting and responding to growth factors. Deciphering these and other quantitatively intricate ways in which cell adhesion influences growth factor signaling will be crucial to developing a better understanding of how the adhesive microenvironment "primes" cell behaviors. Such quantitative insight will be important in designing synthetic microenvironments for applications such as tissue engineering and regenerative medicine.

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