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# Distinct Roles of the Adaptor Protein Shc and Focal Adhesion Kinase in Integrin Signaling to ERK*

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**It has been proposed that integrins activate ERK through the adaptor protein Shc independently of focal adhesion kinase (FAK) or through FAK acting on multiple target effectors, including Shc. We show that disruption of the actin cytoskeleton by cytochalasin D causes a complete inhibition of FAK but does not inhibit Shc signaling and activation of ERK. We have then generated primary fibroblasts carrying a targeted deletion of the segment of $\beta_1$ subunit cytoplasmic domain required for activation of FAK. Analysis of these cells indicates that FAK is not necessary for efficient tyrosine phosphorylation of Shc, association of Shc with Grb2, and activation of ERK in response to matrix adhesion. In addition, integrin-mediated activation of FAK does not appear to be required for signaling to ERK following growth factor stimulation. To examine if FAK could contribute to the activation of ERK in a cell type-specific manner through the Rap1/B-Raf pathway, we have used Swiss-3T3 cells, which in contrast to primary fibroblasts express B-Raf. Dominant negative studies indicate that Shc mediates the early phase and peak, whereas FAK, p130$^{CAS}$, Crk, and Rap1 contribute to the late phase of integrin-dependent activation of ERK in these cells. In addition, introduction of B-Raf enhances and sustains integrin-mediated activation of ERK in wild-type primary fibroblasts but not in those carrying the targeted deletion of the $\beta_1$ cytoplasmic domain. Thus, the Shc and FAK pathways are activated independently and function in a parallel fashion. Although not necessary for signaling to ERK in primary fibroblasts, FAK may enhance and prolong integrin-mediated activation of ERK through p130$^{CAS}$, Crk, and Rap1 in cells expressing B-Raf.**

The integrins bind to extracellular matrix (ECM)[1] proteins or counter-receptors on other cells and transmit mechanical and chemical signals inside the cell (1–3). The divergent effects of the ECM on the survival, proliferation, and differentiation of cells imply that subsets of integrins, and possibly individual integrins, activate specific signaling pathways. There is now considerable evidence in support of this model (4).

Integrins activate two major tyrosine kinase-dependent pathways, the FAK pathway and the Shc pathway. Whereas most integrins activate FAK (5, 6), a subset, which includes $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$, also activates the Shc pathway (7, 8). Both pathways have as their central element an Src family kinase. Accordingly, fibroblasts lacking the three Src family kinases Src, Fyn, and Yes display a profound and specific defect in integrin signaling (9).

The mechanism by which FAK is activated is poorly understood, but there is evidence indicating that Rho-mediated contractility of the actin cytoskeleton plays a part (10). Upon activation, FAK undergoes autophosphorylation at Tyr-397 and associates with the SH2 domain of Src. The Src kinase then phosphorylates paxillin and p130$^{CAS}$, which serve as scaffolds for the recruitment of various adaptors and signaling intermediates (5, 6). In addition to stimulating MAP kinase JNK (11, 12), FAK-Src signaling regulates the cytoskeleton by opposing the assembly of focal adhesions and promoting cell migration (13, 14).

The recruitment of Shc by integrins requires a series of sequential interactions. At least in primary fibroblasts and endothelial cells, the oligomeric protein caveolin-1 appears to function as a membrane adaptor, which couples the integrin $\alpha$ subunit to the Src family kinase Fyn (8). Upon integrin-mediated activation Fyn undergoes a conformational change and interacts, through its SH3 domain, with Shc. Shc is then phosphorylated predominantly at Tyr-317 and combines with the Grb2-mSOS complex (8). Yes, and possibly other palmitoylated Src family kinases (Lck and Lyn but not Src) able to interact with caveolin-1 may functionally replace Fyn in cells that do not express this kinase.[2] Biochemical and genetic evidence suggests that the Shc pathway play an important part in cell proliferation (7, 15–17).

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[1] The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; SH2, Src homology 2; SH3, Src homology 3; Crk, CT-10-regulated kinase; p130$^{CAS}$, p130 Crk-associated substrate; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; Pyk-2, proline-rich tyrosine kinase 2; HEK, human embryonic kidney; MEFs, mouse embryo fibroblasts; HA, hemagglutinin; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; PDGF, platelet-derived growth factor.

[2] K. K. Wary and F. G. Giancotti, unpublished results.


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Available models of anchorage-dependent cell growth imply that integrins and growth factors cooperate to activate the MAP kinase ERK and thereby regulate progression through the $G_1$ phase of the cell cycle (18–20). In addition, ERK plays a part in the regulation of cell migration (21, 22). Although both FAK and Shc can promote the recruitment of mSOS to the plasma membrane and thereby possibly activate Ras, the extent to which each one of the two pathways promotes the activation of ERK is unclear.

Initial studies indicated that Src phosphorylates FAK at Tyr-925 and promotes the recruitment of the Grb2-mSOS complex (23, 24). Overexpression of FAK in 293 HEK cells or Src in immortalized $Src^{-/-}$ mouse embryo fibroblasts (MEFs) enhances integrin-mediated activation of ERK. This effect, however, requires autophosphorylation of FAK at Tyr-397 and association of FAK with Src but not phosphorylation of Tyr-925 by Src and consequent recruitment of Grb2-mSOS (25, 26). In addition, a fragment of Src comprising only the SH2 and the SH3 domain augments signaling to ERK and at the same time causes increased phosphorylation of $p130^{CAS}$. Thus, FAK can signal to ERK through phosphorylation of $p130^{CAS}$ and, possibly, recruitment of Nck and thereby mSOS but probably not through phosphorylation of Tyr-925 (25). Whether specific inhibition of FAK or Src suppresses integrin-mediated activation of ERK in cells expressing physiological levels of the two kinases has not been addressed.

Biochemical and genetic evidence supports the role of Shc in integrin-mediated activation of ERK. First, the integrins that do not activate Shc are weak activators of ERK in primary fibroblasts, endothelial cells, and keratinocytes (7, 8, 15, 17). Second, a dominant negative version of Shc effectively suppresses signaling to ERK, but three different dominant negative forms of FAK do not (7, 8, 27). Third, deletion of the cytoplasmic domain of the integrin $\beta_1$ subunit prevents activation of FAK without impairing signaling to ERK (7, 27). Finally, a chimera containing the transmembrane domain of the integrin $\alpha_1$ subunit associates with caveolin-1 and causes recruitment of Shc and activation of ERK without inducing activation of FAK (8). These results suggest that the Shc pathway is necessary and sufficient for integrin-mediated activation of ERK. However, the evidence that FAK is dispensable for integrin-dependent activation of ERK comes from studies employing expression of dominant negative mutants or cross-linking of mutant integrins with antibodies (7, 8, 27). Both approaches have limitations.

Does FAK contribute to integrin-mediated activation of ERK and, if so, to what extent and by what mechanism? The role of Shc in signaling to ERK is now recognized (22, 28–30). In particular, a recent study has shown that integrin-mediated activation of ERK is defective in $Shc^{-/-}$ fibroblasts (31). However, three papers (28, 29, 32) have placed Shc downstream of FAK. In addition, it has been reported that dominant negative forms of FAK can inhibit integrin-mediated activation of ERK in NIH-3T3 cells (28, 33). Finally, it has been proposed that FAK is necessary for activation of ERK in response to growth factor stimulation (34). Further studies are thus needed in order to determine the relative roles of Shc and FAK in integrin signaling to ERK.

To examine if FAK contributes to the activation of ERK, and to what extent, it would be desirable to suppress FAK signaling in primary cells, possibly by using a gene targeting approach. The $FAK^{-/-}$ fibroblasts, which have been analyzed, do not display a defect in integrin-mediated activation of ERK. However, these cells display elevated levels of the FAK family kinase Pyk-2, which could compensate for the absence of FAK (29). The $Src^{-/-}$ fibroblasts also show normal activation of ERK in response to integrin ligation, but other Src family kinases may replace Src and associate with FAK in these cells (8).

To examine whether Shc signaling to ERK can proceed in the absence of activation of FAK or Pyk-2, we have used primary MEFs carrying a targeted deletion of the C-terminal segment of the $\beta_1$ cytoplasmic domain. Our results indicate that Shc is activated independently of FAK or Pyk-2 and plays a predominant role in integrin-mediated activation of ERK in primary fibroblasts. Although FAK is not required for activation of ERK in primary fibroblasts, it increases and prolongs integrin-mediated activation of ERK through $p130^{CAS}$, Crk, and the small G protein Rap1 in cells that express B-Raf.

## EXPERIMENTAL PROCEDURES

*Cells and Transfections*—A targeted deletion of the C-terminal segment of the $\beta_1$ cytoplasmic domain was generated by homologous recombination in embryonic stem cells and introduced in mice. As expected, the targeting vector integrated only at the endogenous $\beta_1$ locus. However, whereas the 3′ end of the construct correctly replaced the homologous wild-type $\beta_1$ sequences, the 5′ end integrated downstream of the expected site, leading to a duplication of exon 6. The resulting $\beta_1$ allele, defined as $\beta 1_{de6}$, encodes a $\beta_1$ subunit that is truncated after the membrane-proximal segment common to all $\beta_1$ splice variants, as intended, but it contains 11 additional unrelated amino acids at the C terminus (TVLLVPTSSQL). Whereas embryos lacking fibronectin or FAK display profound mesodermal defects at embryonic day 8.5 and deteriorate rapidly thereafter (13, 35), $\beta_{1de6}$ homozygous embryos do not display such defects and die from vascular abnormalities at embryonic days 10.5–11.5. The molecular characterization of the homozygous $\beta_{1de6}$ mutation and its phenotypic consequences on mouse development will be reported elsewhere. Primary MEFs were derived from wild-type and $\beta_{1de6}/\beta_{1de6}$ E10.5 embryos. Yolk sacs were used for genotyping by polymerase chain reaction. Upon dissociation in trypsin, cells from individual embryos were plated onto 6-cm diameter dishes and cultured in DMEM, 20% fetal calf serum. These cells were used between passages 3 and 7. Unless stated otherwise, the experiments were performed with primary MEFs or with both primary and spontaneously immortalized MEFs with similar results. NIH-3T3, Swiss-3T3, and WI-38 fibroblasts, 293 human embryonic kidney cells, Jurkat T lymphoma cells, and A431 squamous carcinoma cells were obtained from ATCC (Manassas, VA) and cultured in DMEM, 10% calf serum or fetal calf serum. Human umbilical vein endothelial cells were purchased from Clonetics (San Diego, CA) and cultured on gelatin-coated dishes in Human Endothelial-SFM (Life Technologies, Inc.) supplemented with 20% fetal calf serum (Life Technologies, Inc.), 10 ng/ml EGF, 20 ng/ml bFGF, and 1 μg/ml heparin (all from Intergen, Purchase, NY). $FAK^{-/-}$, $p53^{-/-}$, and $FAK^{+/+}$, $p53^{-/-}$ immortalized mouse fibroblasts were previously described (34). Swiss-3T3 cells and MEFs were transiently transfected by the LipofectAMINE method (Life Technologies, Inc.).

*Antibodies, Constructs, and Other Reagents*—Rabbit antisera to synthetic peptides modeled after the C termini of integrin $\alpha_1$, $\alpha_2$, $\alpha_5$, and $\alpha_v$ subunits were as described previously (36). Rabbit antibodies to the SH2 domain of Shc were affinity-purified from a previously described antiserum by affinity chromatography on a column containing the GST fusion protein used as immunogen (8). The rabbit antiserum to a GST fusion protein comprising the C-terminal domain of FAK was described previously (37). Goat or rabbit IgGs raised against synthetic peptides modeled after the N terminus of Fyn (3), Pyk-2 (N-19), and c-Src (N-16) or the C terminus of B-Raf (C-19), Shc (C-20), Grb2 (C-23), and ERK2 (C-14) and the mAb to phospho-ERK (E-4) were all from Santa Cruz Biotechnology (Santa Cruz, CA). The mAb to $p130^{CAS}$ (clone 21) was from Pharmingen/Transduction Laboratories (San Diego, CA). The anti-Tyr(P) mAb RC20-H (peroxidase-conjugated recombinant PY20) and rabbit IgGs to a GST fusion protein comprising Shc amino acid residues 366–473 were both from Transduction Laboratories (Lexington, KY). The mAb M2 to FLAG-tag was purchased from Eastman Kodak Co. The mAb 3C2 reacting with the Gag portion of v-Crk was as described previously (12). The mAb 12CA5 to the HA tag was produced and purified by the Monoclonal Antibody Core Facility of Memorial Sloan-Kettering Cancer Center.

Cytomegalovirus promoter-based vectors encoding HA-tagged ERK and FLAG-tagged Shc-Y317F (7, 8) and $Rap\text{-}1^{Asn\text{-}17}$(38) were described previously. The Moloney leukemia virus-long terminal repeat-based pMEXneo vector encoding v-Crk D386DRHAD (SH3 insertional mutant) and the pEBG vector encoding a substrate region deleted form of rat $p130^{CAS}$ (SD, Δ213–514) were also described previously (12). The

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human B-Raf cDNA was obtained from Andrew Chan (Mount Sinai School of Medicine, New York, NY) and subcloned in pRK5.

Fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose, as described previously (39). [$\gamma$-$^{32}$P]ATP (>4, 500 Ci/mmol) was from ICN Pharmaceuticals, Inc. (Costa Mesa, CA).

*Biochemical Methods*—To examine the integrin repertoire of wild-type and mutant MEFs, the cells were detached by incubation with 5 mM EDTA and subjected to cell surface biotinylation, immunoprecipitation, and immunoblotting, as described previously (40). To monitor integrin signaling, cells were deprived of growth factors for 24 h, detached, washed with DMEM three times, and kept in suspension for 45 min. When indicated, 1 $\mu$M cytochalasin D was added to the cells during the last 15 min of incubation in suspension. The cells were then either lysed immediately, or they were plated on dishes coated with 15 $\mu$g/ml fibronectin for the indicated times and then lysed. Extraction buffer consisted of 25 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM $Na_3VO_4$, 10 mM NaF, 1 mM $Na_4P_2O_7$, and 1 mM EDTA. Immunoprecipitation and immunoblotting were performed as described previously (7, 8). To co-immunoprecipitate FAK and Src, the anti-FAK antibody was covalently linked to CNBr-activated Sepharose. After immunoprecipitation, the beads carrying the immune complexes were washed and boiled in non-reducing sample buffer. $\beta$-Mercaptoethanol was added to the sample buffer only after the beads were removed by centrifugation. ERK immune complex kinase assays were performed as described previously (7). For Fyn immune complex kinase assays, cells were extracted with 50 mM HEPES, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium fluoride, 1 mM pyrophosphate, 1 mM $Na_3VO_4$, 10% glycerol, and protease inhibitors. Immune complexes were washed and incubated in 30 $\mu$l of 20 $\mu$M HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 150 mM NaCl, 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP for 30 min at 30 °C.

*Adhesion Assay*—Microtiter plates were coated with fibronectin at the indicated concentrations and saturated with 0.1% bovine serum albumin. Cells were detached with 0.02% trypsin, 1 mM EDTA, resuspended in DMEM, 10% calf serum, washed three times with DMEM, and plated at $5 \times 10^4$/100 $\mu$l in DMEM. After 1 h at 37 °C adherent cells were washed twice with phosphate-buffered saline, fixed with 3% paraformaldehyde, stained with Coomassie Blue, and counted under the microscope.

## RESULTS

*Cytochalasin D Blocks FAK but Amplifies Shc Signaling to ERK*—The observation that cytochalasin D both inhibits FAK and prevents signaling to ERK in cells adhering to ECM (41–43) has been interpreted as evidence in support of the role of FAK in activation of ERK (28). Cytochalasin D caps actin filaments and stimulates ATP hydrolysis on G actin leading to a very rapid dissolution of the actin cytoskeleton (44). As the actin filament system supports a variety of distinct cellular functions, the effect of cytochalasin D on any two such functions cannot be interpreted as a proof of their epistatic relationship.

We wanted to examine if integrin-dependent Shc signaling and activation of ERK required an intact actin cytoskeleton. To this end, we conducted preliminary experiments to identify the lower dose of cytochalasin D which could totally disrupt the actin cytoskeleton and prevent FAK activation in NIH-3T3 fibroblasts. Staining with fluorescein isothiocyanate-labeled phalloidin, which binds only to F-actin, revealed that 1 $\mu$M cytochalasin D was the lower dose capable of disrupting the actin cytoskeleton in NIH-3T3 cells adhering to fibronectin (not shown). Immunoblotting with anti-phosphotyrosine antibodies indicated that the same dose of cytochalasin D was able to suppress the tyrosine phosphorylation of FAK induced by adhesion to fibronectin completely (Fig. 1*A*).

Notably, cytochalasin D did not inhibit but rather amplified the tyrosine phosphorylation of Shc, the association of Shc with Grb2, and the activation of ERK caused by adhesion to fibronectin (Fig. 1*A*). A previous study has shown that cytochalasin D can activate Src in NIH-3T3 cells (45). We have observed that, at the dose used in this study, cytochalasin D activates also Fyn in NIH-3T3 cells (Fig. 1*B*). It is thus possible that cytochalasin D enhances Shc signaling to ERK at least in part by enhancing the activation of Src family kinases.

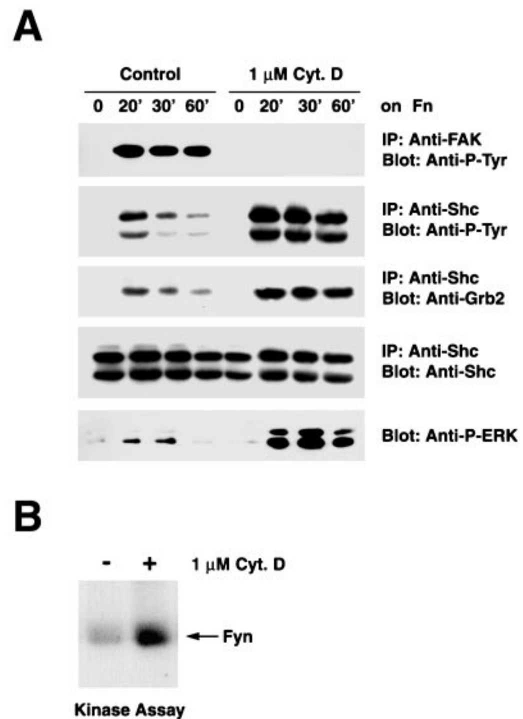


FIG. 1. **Cytochalasin D inhibits FAK but amplifies Shc signaling to ERK.** *A*, NIH-3T3 cells were detached and then plated on fibronectin (*Fn*)-coated plates either in the absence or presence of 1 $\mu$M cytochalasin D. Equal amounts of total proteins were immunoprecipitated (*IP*) with antibodies to FAK or Shc and immunoblotted with antibodies to phosphotyrosine (*anti-P-Tyr*), Grb2, or Shc. Total proteins were immunoblotted with antibodies to phospho-ERK (*anti-P-ERK*). This experiment was repeated three times with similar results. *B*, NIH-3T3 cells were treated with 1 mM cytochalasin D (*Cyt. D*) for 15 min or left untreated. Fyn was immunoprecipitated and subjected to kinase assay. This experiment was repeated twice with similar results.

These results indicate that disruption of the actin cytoskeleton inhibits FAK without suppressing Shc signaling to ERK.

*Fibroblasts Carrying a Targeted Deletion of the C-terminal Portion of the $\beta_1$ Cytoplasmic Tail Attach to Fibronectin at a Normal Rate and Do Not Express Pyk-2*—Because cytochalasin D enhances the activation of Src kinases, it cannot be used to determine with accuracy the relative roles of FAK and Shc in integrin-mediated activation of ERK. We have thus used primary fibroblasts derived from mice carrying a targeted deletion of the C-terminal segment of the $\beta_1$ cytoplasmic domain. This portion of the $\beta$ subunit tail is thought to be necessary for activation of FAK (46, 47). The original mutation was generated by homologous recombination in embryonic stem cells and introduced in mice. The resulting $\beta_1$ allele, defined as $\beta_{1de6}$, encodes a $\beta_1$ subunit that is truncated after the membrane-proximal segment common to all $\beta_1$ splice variants, as intended, but contains 11 additional unrelated amino acids at the C terminus. The structure of wild-type $\beta_1$ 65 and the truncated $\beta_1$ subunit encoded by the $\beta_{1de6}$ allele are illustrated schematically in Fig. 2*A*. The molecular characterization of the homozygous mutation and its phenotypic consequences on mouse embryo development will be reported elsewhere.

Primary fibroblasts derived from $\beta_{1de6}$ homozygous embryos were found to express cell surface levels of mutant $\beta_1$ comparable to those of wild-type $\beta_1$ in normal MEFs. As expected, the mutant $\beta_1$ subunit encoded by the $\beta_{1de6}$ allele associated normally with companion $\alpha$ subunits and could be immunoprecipitated by antibodies to the ectodomain of $\beta_1$ but not by antibod-

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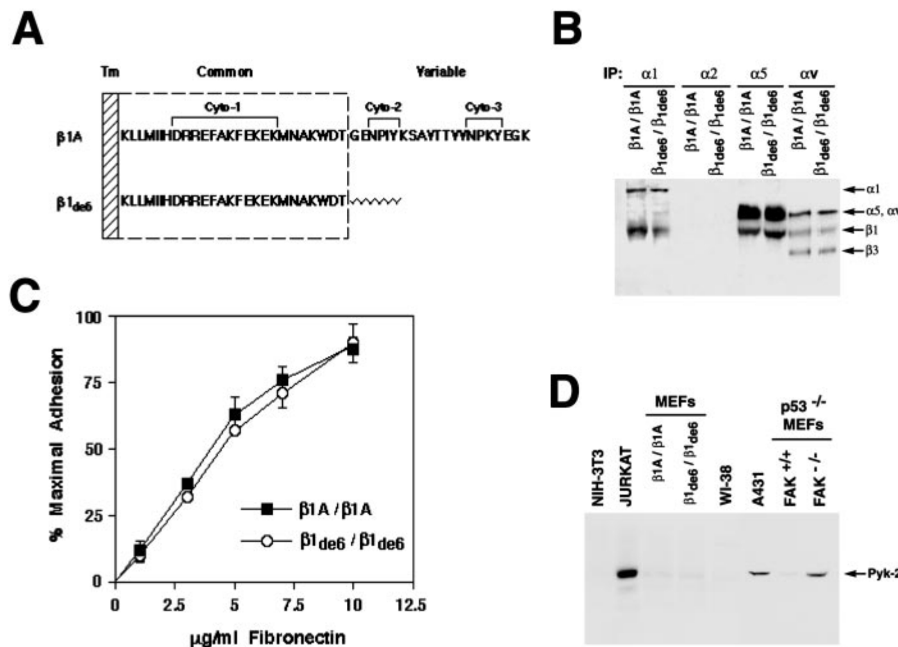


FIG. 2. **Analysis of MEFs carrying a targeted mutation of the C-terminal segment of the $\beta_1$ cytoplasmic domain.** *A,* schematic representation of the cytoplasmic domain of $\beta_{1A}$ and $\beta_{1de6}$. Normal MEFs express only the canonical form of $\beta_1$, $\beta_{1A}$. The cytoplasmic domain of $\beta_{1A}$ consists of a common region, which includes the Cyto-1 motif, and a variable region, which is composed of two NPXY motifs (Cyto-2 and -3). MEFs derived from mice homozygous at the $\beta_{1de6}$ allele express a mutant $\beta_1$ subunit, which contains only the common region of the cytoplasmic domain. The variable region is replaced by 11 unrelated amino acids. *B,* surface biotinylated proteins from wild-type ($\beta_{1A}/\beta_{1A}$) and mutant MEFs ($\beta_{1de6}/\beta_{1de6}$) were immunoprecipitated (*IP*) with rabbit antibodies to the indicated integrin $\alpha$ subunits and probed by immunoblotting with peroxidase-streptavidin. This experiment was repeated twice with similar results. *C,* wild-type ($\beta_{1A}/\beta_{1A}$) and mutant ($\beta_{1de6}/\beta_{1de6}$) MEFs were plated on microtiter wells coated with the indicated concentrations of fibronectin for 60 min. This experiment was repeated three times with similar results. *D,* expression of Pyk-2. Total lysates from the indicated cells were subjected to immunoblotting with antibodies to Pyk-2. This experiment was repeated twice with similar results.

ies to the C-terminal segment of the $\beta_1$ cytoplasmic domain. Cell surface biotinylation followed by immunoprecipitation indicated that wild-type and mutant MEFs did not express $\alpha_2\beta_1$ and had similar levels of $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ at the cell surface (Fig. 2*B*). In accordance with the notion that FAK/Src signaling promotes cell spreading (48), the mutant MEFs showed slower spreading (data not shown) but attached to fibronectin at a normal rate (Fig. 2*C*). Interestingly, the mutant MEFs developed focal adhesions larger and stress fibers more prominent than wild-type cells (not shown). This result is consistent with the role of FAK-Src signaling in disassembly of focal adhesions (13, 14). In addition, it suggests that the membrane-proximal portion of the $\beta$ subunit tail, which can interact with the talin head domain (49, 50), is sufficient for nucleation of focal adhesions in MEFs. Thus, despite the reduced rate of spreading, mutant MEFs do not have a disorganized cytoskeleton.

It is known that immortalized $FAK^{-/-}$ fibroblasts express elevated levels of Pyk-2, which could compensate, at least in part, for the absence of FAK in these cells (29). We thus wanted to examine if fibroblasts derived from $\beta_{1de6}$ homozygous embryos expressed Pyk-2 and at what level. Immunoblotting experiments indicated that MEFs derived from $\beta_{1de6}$ homozygous embryos, like wild-type MEFs, do not express detectable levels of Pyk-2 (Fig. 2*D*). We also observed no expression of Pyk-2 in WI-38 human fibroblasts and NIH-3T3 mouse fibroblasts and a low level of expression in A431 squamous carcinoma cells. Whereas immortalized $FAK^{+/+}$ fibroblasts do not express Pyk-2, their $FAK^{-/-}$ counterparts express low but clearly detectable levels of Pyk-2. In comparison, Jurkat lymphoma cells express relatively high levels of Pyk-2 (Fig. 2*D*). These results are consistent with the reported cell type distribution of Pyk-2 (51) and indicate that mutation of the segment of $\beta_1$ involved in activation of FAK does not lead to compensatory up-regulation of Pyk-2 in primary MEFs.

*FAK/Src Signaling Is Defective in Fibroblasts Carrying a Targeted Deletion of the C-terminal Portion of the $\beta_1$ Cytotail*—To examine the role of the C-terminal segment of the $\beta_1$ cytoplasmic domain in FAK/Src signaling, we plated primary MEFs, derived from either wild-type or homozygous $\beta_{1de6}$ embryos, on fibronectin for various times and performed immunoprecipitation and immunoblotting experiments. As shown in Fig. 3*A*, adhesion of wild-type MEFs to fibronectin caused tyrosine phosphorylation of FAK and association of FAK with Src. By contrast, adhesion of mutant MEFs to fibronectin induced only negligible tyrosine phosphorylation of FAK and no detectable association of FAK with Src. These results indicate that the C-terminal segment of the $\beta_1$ cytoplasmic domain is necessary for activation of FAK and association of FAK with Src in response to integrin binding to ECM.

$p130^{CAS}$ is a major target effector of the FAK-Src complex and, upon phosphorylation, combines with the adaptor proteins Crk and Nck (25, 52). Since both Crk and Nck can promote signaling to ERK, we examined if $p130^{CAS}$ signaling was defective in MEFs derived from homozygous $\beta_{1de6}$ embryos. Fig. 3*B* shows that adhesion to fibronectin caused tyrosine phosphorylation of $p130^{CAS}$ in wild-type MEFs. By contrast, adhesion of mutant MEFs to fibronectin did not induce tyrosine phosphorylation of $p130^{CAS}$. These results indicate that the C-terminal portion of the $\beta_1$ cytoplasmic domain is required for integrin-mediated $p130^{CAS}$ signaling.

Taken together, the observations described above indicate that MEFs derived from homozygous $\beta_{1de6}$ embryos display a defect in FAK-Src signaling.

*Shc Signaling to ERK Proceeds Normally in Fibroblasts Carrying a Targeted Deletion of the C-terminal Portion of the $\beta_1$ Cytotail*—We next compared integrin-mediated Shc signaling in wild-type and mutant MEFs. As shown in Fig. 4*A*, immunoprecipitation with anti-Shc followed by immunoblotting with

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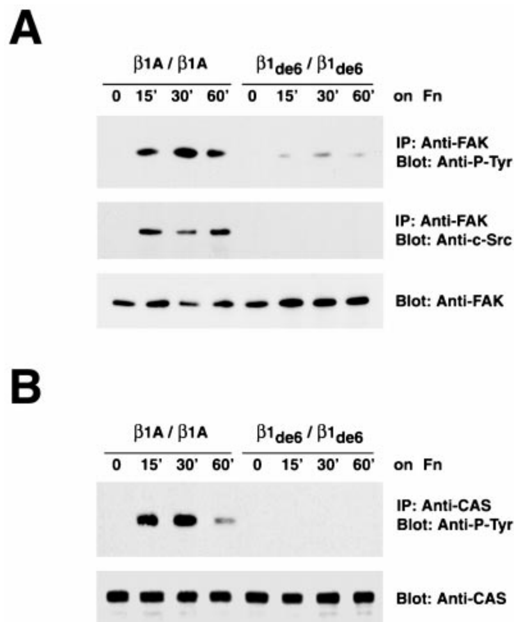


FIG. 3. **MEFs carrying a targeted deletion of the C-terminal segment of the $\beta_1$ cytoplasmic domain display defective FAK/Src signaling.** *A,* wild-type ($\beta_{1A}/\beta_{1A}$) and mutant ($\beta_{1de6}/\beta_{1de6}$) MEFs were detached and replated on fibronectin (*Fn*) coated dishes. Equal amounts of total proteins were immunoprecipitated (*IP*) with antibodies to FAK and immunoblotted with antibodies to phosphotyrosine (*anti-P-Tyr*) or c-Src. Aliquots of total lysates were immunoblotted with antibodies to FAK. *B,* wild-type ($\beta_{1A}/\beta_{1A}$) and mutant ($\beta_{1de6}/\beta_{1de6}$) MEFs were plated on fibronectin and lysed as above. Equal amounts of total proteins were immunoprecipitated with antibodies to p130$^{CAS}$ (*anti-CAS*) and immunoblotted with anti-Tyr(P) antibodies. Aliquots of total lysates were immunoblotted with antibodies to p130$^{CAS}$. These experiments were repeated three times with similar results.

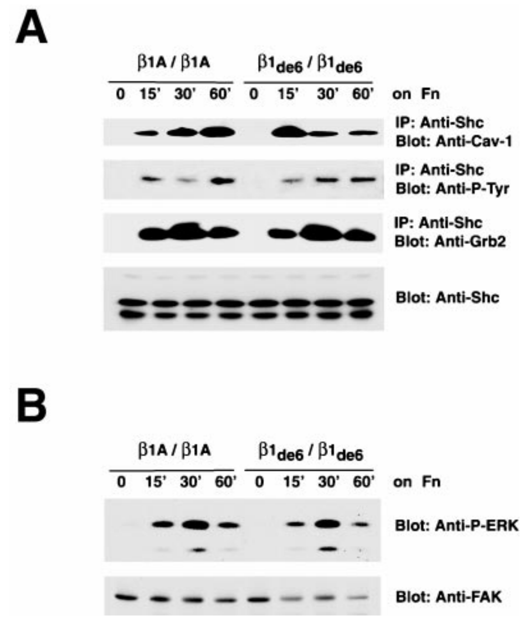


FIG. 4. **Shc signaling to ERK proceeds normally in MEFs carrying a targeted deletion of the C-terminal segment of $\beta_1$.** *A,* wild-type ($\beta_{1A}/\beta_{1A}$) and mutant ($\beta_{1de6}/\beta_{1de6}$) MEFs were detached and replated on fibronectin (*Fn*)-coated dishes. Equal amounts of total proteins were immunoprecipitated (*IP*) with antibodies to Shc and immunoblotted with antibodies to caveolin-1, phosphotyrosine, or Grb2. Aliquots of total lysates were immunoblotted with antibodies to Shc. This experiment was repeated three times with similar results. *B,* equal amounts of total proteins from cells treated as in *A* were separated by SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose. The bottom portion of the blot was subjected to immunoblotting with antibodies to phospho-ERK. To control for equal loading while avoiding stripping and reprobing, which may cause artifacts, the top portion of the blot was probed with antibodies to FAK. This experiment was repeated five times with similar results.

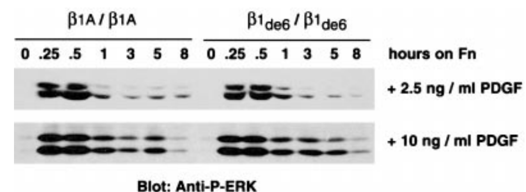


FIG. 5. **Integrin-mediated activation of FAK is not required for efficient signaling to ERK in response to PDGF.** Wild-type ($\beta_{1A}/\beta_{1A}$) and mutant ($\beta_{1de6}/\beta_{1de6}$) MEFs were serum-starved, detached, and replated on fibronectin (*Fn*)-coated dishes in the presence of either 2.5 or 10 ng/ml PDGF. Equal amounts of total proteins were immunoblotted with antibodies to phospho-ERK. Immunoblotting with anti-FAK was used to control for equal loading (not shown). This experiment was repeated four times with similar results.

anti-caveolin-1 antibodies indicated that Shc combined with caveolin-1 in response to adhesion to fibronectin in both wild-type and mutant MEFs. However, the kinetics of the association was faster in mutant than in wild-type MEFs. Immunoblotting with anti-phosphotyrosine antibodies showed that the 52-kDa isoform of Shc was phosphorylated on tyrosine residues to a similar extent in both wild-type and mutant MEFs plated on fibronectin but with a faster kinetics in mutant than in wild-type MEFs. The 46-kDa isoform of Shc, which is expressed at lower levels in MEFs, was not phosphorylated on tyrosine in response to integrin ligation in both cell types. The mechanism underlying the faster association of caveolin with Shc and total tyrosine phosphorylation of Shc in mutant than in wild-type MEFs was not explored. Finally, Shc combined with Grb2 in response to adhesion to fibronectin to similar extent and with similar kinetics in both wild-type and mutant MEFs (Fig. 4*A*). The observation that Shc signaling is not inhibited in mutant MEFs is in accordance with the hypothesis that this pathway is activated through the integrin $\alpha$ subunit independently of the $\beta_1$ cytoplasmic domain and FAK/Src signaling (7, 8).

To examine the relative roles of FAK and Shc in integrin-mediated activation of ERK, wild-type and mutant MEFs were either kept in suspension or plated on fibronectin for various times. Total lysates were analyzed by immunoblotting with anti-phospho-ERK antibodies. As shown in Fig. 4*B*, ERK was activated to a very similar extent in both wild-type and mutant MEFs plated on fibronectin. These findings indicate that the FAK and Shc pathways are activated independently and proceed largely in a parallel fashion. They also show that, at least in primary fibroblasts, FAK/Src signaling does not contribute to a significant extent to the activation of ERK.

*Exposure to Mitogenic Growth Factors Causes Efficient Activation of ERK in Fibroblasts Carrying a Targeted Deletion of the C-terminal Portion of the $\beta_1$ Cytotail*—There is evidence suggesting that integrin-mediated FAK/Src signaling contributes to ERK activation in response to growth factor stimulation (34). In this model, FAK would not necessarily be required for integrin-mediated activation of ERK, but it would play a crucial part in growth factor-mediated activation of ERK.

To examine the role of integrin-mediated FAK/Src signaling in the growth factor response of primary fibroblasts, we plated MEFs, derived from either wild-type or $\beta_{1de6}$ homozygous embryos, on fibronectin for various times up to 8 h in the presence of two distinct concentrations of PDGF. As revealed by immunoblotting with anti-phospho-ERK antibodies, adhesion to fibronectin in the presence of 2.5 ng/ml PDGF caused a transient activation of ERK in both wild-type and mutant MEFs. The extent of ERK activation was similar in wild-type and mutant

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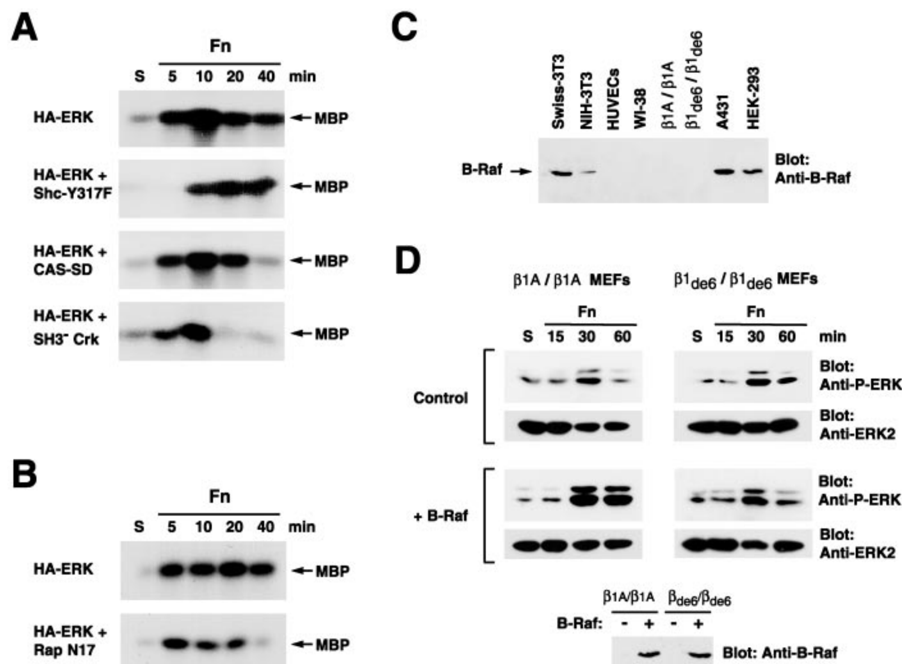


FIG. 6. **p130$^{CAS}$/Crk signaling enhances and prolongs ERK activation in cells expressing B-Raf.** *A,* Swiss-3T3 cells were transiently transfected with 1 μg of vector encoding HA-tagged ERK, alone or in combination with 10 μg of vectors encoding the dominant negative forms of Shc (FLAG-tagged *Shc-Y317F*), p130$^{CAS}$ (*CAS-SD*), or Crk (*SH3$^-$ Crk*). The cells were detached and plated on fibronectin (*Fn*)-coated dishes. HA-ERK was immunoprecipitated from equal amounts of total proteins and subjected to kinase assay with myelin basic protein (*MBP*) as a substrate. Expression levels were verified by immunoblotting total lysates with antibodies recognizing the HA tag, the FLAG-tag, p130$^{CAS}$, and the Gag portion of v-Crk (not shown). *B,* Swiss-3T3 cells were transiently transfected with 1 μg of vector encoding HA-tagged ERK, alone or in combination with 10 μg of vector encoding a dominant negative version of Rap-1 (Rap-1$^{Asn-17}$), and analyzed as above. Peak activation of ERK occurred at different times in different experiments presumably as a consequence of differences in the rate of adhesion (compare for example *A* and *B*). These experiments were repeated three times with similar results. *C,* equal amounts of total proteins from the indicated cells were probed by immunoblotting with antibodies to B-Raf. This experiment was repeated twice with similar results. *D,* spontaneously immortalized wild-type ($\beta_{1A}/\beta_{1A}$) and mutant ($\beta_{1de6}/\beta_{1de6}$) MEFs were transfected with 10 μg of vector encoding B-Raf or left untreated. Cells were detached and replated on fibronectin for the indicated times. Equal amounts of total proteins were immunoblotted with antibodies to phospho-ERK or ERK2. Immunoblotting was used to control for equal expression of B-Raf after transfection of the two cell types. This experiment was repeated twice with similar results.

MEFs (Fig. 5). In the presence of 10 ng/ml PDGF, the activation of ERK became significantly more sustained. Even at this higher dose of PDGF, which is sufficient to induce mitogenesis in MEFs (data not shown), we observed similar levels of ERK activation in wild-type and mutant MEFs (Fig. 5*A*).

These findings suggest that the C-terminal segments of the cytoplasmic domain of $\beta_1$ and, by inference, integrin-mediated FAK/Src signaling are not necessary for activation of ERK in adherent primary fibroblasts exposed to PDGF.

*FAK/Src Signaling Enhances and Prolongs Integrin-mediated Activation of ERK in Cells Expressing B-Raf*—Our current and previous observations indicate that integrin signaling to ERK proceeds in large part independently of FAK/Src signaling in primary fibroblasts and endothelial cells. There is, however, evidence suggesting that FAK contributes to ERK activation in NIH-3T3 fibroblasts (28, 33). Is it possible that FAK plays a part in ERK activation in a cell type-specific manner? Recent studies have indicated that B-Raf, which is expressed only in certain cell types, may be able to couple p130$^{CAS}$/Crk signaling to ERK. In this pathway, the Crk-associated exchange factor C3G activates the Ras-related small G protein Rap-1, which in turn acts on B-Raf and thereby MEK (38, 53–55). *In vivo* B-Raf is expressed in neural tissues and in the gonads (56), but established fibroblastic cell lines, such as the Swiss-3T3 and NIH-3T3 cells, also express B-Raf (57, 58).

A dominant negative approach was used to examine the relative roles of FAK and Shc in signaling to ERK in cells expressing B-Raf. We used Swiss-3T3 fibroblasts because they express levels of B-Raf higher than the NIH-3T3 cells (see below). The cells were transiently transfected with a vector encoding an influenza hemagglutinin (HA)-tagged form of ERK, alone or in combination with a vector encoding a dominant negative version of Shc (Shc-Y317F), a dominant negative version of p130$^{CAS}$ (CAS-SD), or a dominant negative version of Crk (SH3$^-$ Crk). Shc-Y317F carries a phenylalanine permutation at the tyrosine residue that is phosphorylated and binds to Grb2 in response to integrin-mediated adhesion *in vivo* (8). CAS-SD lacks the entire substrate region, which is composed of the nine tyrosine phosphorylation sites involved in the recruitment of Crk. Finally, SH3$^-$ Crk is a membrane-anchored form of Crk carrying a mutation in the SH3 domain, which is required for interaction with target effectors, such as C3G (12). Upon transfection, the cells were plated on fibronectin for various times and subjected to immunoprecipitation with anti-HA antibodies followed by immune complex kinase assay. As shown in Fig. 6*A*, expression of dominant negative Shc suppressed the early phase and the peak of integrin-mediated activation of ERK in Swiss-3T3 cells, at 5 and 10 min of adhesion, respectively. Dominant negative Shc, however, had no effect on the late phase of ERK activation in these cells. By contrast, dominant negative p130$^{CAS}$ and, even more effectively, dominant negative Crk suppressed the late phase of ERK activation, at 20 and 40 min of adhesion, without exerting any significant effect on the early phase and the peak. The effect of dominant negative constructs was dose-dependent, as lower doses inhibited to a lower extent (data not shown). The more profound effect of dominant negative Crk relative to dominant negative p130$^{CAS}$ may be a consequence of its ability to inhibit the binding of Crk to both p130$^{CAS}$ and paxillin. We finally tested the effect of a dominant negative form of Rap-1

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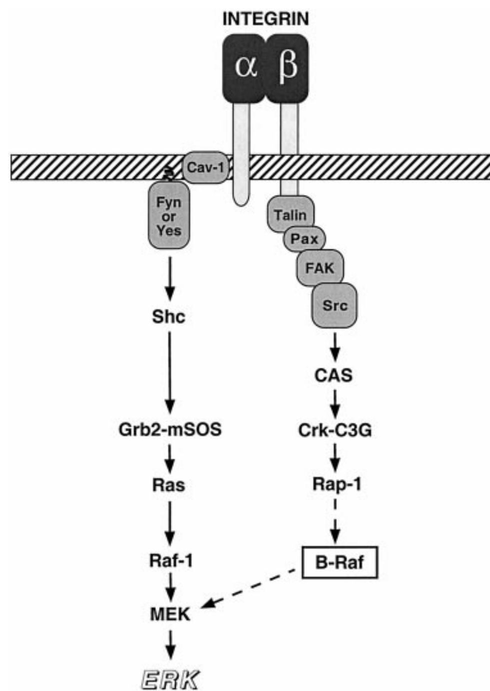


FIG. 7. **Hypothetical model of integrin signaling to ERK.** The Shc pathway, which is activated by a subset of integrins through their $\alpha$ subunits, plays a predominant role in the activation of ERK in most primary cells. The FAK pathway, which is activated by most integrins and contributes to the activation of JNK and regulation of the actin cytoskeleton, enhances and sustains the activation of ERK in cells expressing B-Raf (*dotted lines*).

($Rap^{Asn-17}$). As shown in Fig. 6*B*, expression of this mutant protein inhibited the late phase of integrin-mediated ERK activation without significantly affecting the early phase (Fig. 6*B*).

Immunoblotting experiments indicated that the major 95-kDa molecular mass isoform of B-Raf is expressed in Swiss-3T3 fibroblasts, in 293 HEK cells, and in A431 cells, and at lower levels in NIH-3T3 cells. It is, however, not expressed in primary fibroblasts derived from either wild-type or $\beta_{1de6}$ homozygous mouse embryos, in primary human umbilical vein endothelial cells, or in primary human fibroblasts (WI-38) (Fig. 6*C*). None of the cells examined expressed the minor 68-kDa isoform of B-Raf. These observations suggest that most of the previous studies supporting the role of FAK in signaling to ERK were performed in cells expressing B-Raf. They also raise the possibility that the expression of B-Raf in fibroblasts is a consequence of immortalization.

We thus asked if introduction of B-Raf in B-Raf-negative cells enhanced integrin-mediated activation of ERK. As shown in Fig. 6*D*, transient transfection of a vector encoding B-Raf caused an increase in the level and duration of ERK activity in wild-type MEFs plated on fibronectin. The effect observed was relatively large, considering that only approximately 25% MEFs are transfected under our experimental conditions. By contrast, B-Raf did not amplify integrin-mediated activation of ERK in MEFs carrying the targeted deletion of the $\beta_1$ cytoplasmic domain. Thus, B-Raf augments integrin signaling to ERK, and this process requires the C-terminal segment of the $\beta_1$ cytoplasmic domain and, by inference, FAK/Src signaling.

Taken together, these findings suggest that $p130^{CAS}$/Crk signaling can augment and prolong integrin-mediated activation of ERK in cells expressing B-Raf. The involvement of Crk and the requirement for B-Raf suggest that the late phase of ERK activation in Swiss-3T3 cells is mediated by the Rap-1/B-Raf pathway.

## DISCUSSION

In this paper we have used a number of complementary approaches to probe specifically the role of FAK in integrin-mediated activation of ERK. Our results indicate that Shc signaling to ERK proceeds independently of FAK and do not support the hypothesis that places Shc downstream of FAK (28, 32). They also indicate that FAK/Src signaling does not play a part in integrin-mediated activation of ERK in primary fibroblasts. However, it may contribute to enhance and sustain the activation of ERK initiated by Shc in immortalized Swiss-3T3 fibroblasts, which express B-Raf. A similar cooperation between Shc and FAK may occur in those neurons and gonadal cells that physiologically express B-Raf (56).

We have used cytochalasin D, which disrupts the actin cytoskeleton and prevents activation of FAK, because previous results had suggested that it also inhibits integrin-mediated activation of ERK (28, 41–43). We find that, at the minimal dose required to suppress FAK completely, cytochalasin D does not inhibit, but rather enhances, both integrin-dependent Shc signaling and activation of ERK in NIH-3T3 cells. Most previous studies had employed a prolonged pretreatment with cytochalasin D, a higher dose of the drug, or different cells. We suspect that, at an intracellular concentration higher than that achieved in our study, cytochalasin D may prevent ERK activation by a mechanism distinct from inhibition of FAK. Indeed, doses of cytochalasin D higher than 2 $\mu$M inhibit integrin-mediated activation of ERK also under our experimental protocol. Our observations with cytochalasin D suggest that integrin-mediated Shc signaling does not require an intact actin cytoskeleton and that it can effectively promote activation of ERK in the absence of functional FAK. However, cytochalasin D is not an adequate tool to address the relative roles of FAK and Shc in activation of ERK because it enhances Shc signaling, presumably by activating Fyn, and thus it may obscure a potential contribution of FAK to the activation of ERK.

We have thus examined primary MEFs derived from mice carrying a targeted deletion of the C-terminal portion of the $\beta_1$ subunit cytoplasmic domain, which previous studies had implicated in the activation of FAK (46, 47). This approach has three advantages. First, it affords a genetic test of the role of FAK in integrin-mediated signaling. Second, it focuses on the analysis of primary, non-immortalized cells. Third, it is not limited by compensatory up-regulation of Pyk-2 or functional redundancy among Src family kinases. As expected, adhesion of mutant MEFs to fibronectin did not cause tyrosine phosphorylation of FAK, association of FAK with Src, and tyrosine phosphorylation of $p130^{CAS}$. By contrast, Shc signaling proceeded normally, and ERK was activated to the same level as in normal MEFs. These findings provide strong genetic evidence that Shc and FAK largely function in parallel pathways and that FAK does not contribute to integrin-mediated activation of ERK in primary fibroblasts.

Does FAK contribute to the activation of ERK caused by growth factor stimulation? This hypothesis is suggested by the observation that overexpression of an activated version of FAK or of an interleukin 2-$\beta_1$ chimera, which activates FAK independently of adhesion, rescues growth factor-mediated activation of ERK in suspended cells (34). Our results are not fully consistent with this hypothesis because they show that treatment with growth factors results in a similar level of ERK activation in both mutant and normal MEFs. However, it remains possible that FAK contributes to the activation of ERK in an integrin-independent manner, as this mechanism would not be revealed in our studies or that it does so only in certain cell types. Based on prior observations (reviewed in Refs. 18–20) and our current results, we posit that integrin and growth

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factor-dependent signals converge at various levels to activate ERK completely. In primary fibroblasts, the coordinate regulation of ERK by integrins and growth factor receptors does not appear to involve FAK.

How do we reconcile the primary role of Shc in integrin-mediated activation of ERK implied by our studies with the observation by others (28, 33) that dominant negative forms of FAK can partially inhibit integrin signaling to ERK? A major difference may lie in the choice of cells and the temporal phase of adhesion examined. Most of the studies supporting the role of FAK have employed immortalized cells, especially NIH-3T3 fibroblasts, plated on fibronectin for 30 min or more. As shown here, primary human and mouse fibroblasts do not express B-Raf, but two mouse fibroblast cell lines immortalized by the 3T3 protocol, the NIH-3T3 and Swiss-3T3 cells, express this kinase. This is of interest because B-Raf is part of a recently identified pathway, which potentially links Crk to ERK independently of Ras. In this pathway, Crk associates with the exchange factor C3G, which in turn acts on the small G protein Rap1. Rap1 then activates B-Raf and thereby MEK and ERK (38, 53–55).

Bearing in mind the existence of the Rap1/B-Raf connection, we have tested the relative roles of Shc and FAK in integrin-mediated activation of ERK in Swiss-3T3 cells, which express B-Raf. Our results indicate that a dominant negative form of Shc blocks the early phase and the peak of integrin-mediated activation of ERK, whereas dominant negative versions of $p130^{CAS}$ and Crk suppress the late phase in these cells. These observations suggest that FAK/Src signaling, acting through $p130^{CAS}$ and Crk, can enhance and sustain the activation of ERK initiated by Shc signaling, at least in cells expressing B-Raf. Several observations are consistent with the hypothesis that Crk acts through C3G and Rap1 to activate B-Raf. First, a previous study has provided evidence that the late phase of ERK activation by integrins is independent of Ras in NIH-3T3 cells (59). Second, there is evidence that integrin-mediated adhesion causes tyrosine phosphorylation, and thereby activation, of C3G (60, 61). Third, overexpression of B-Raf allows for anchorage-independent activation of ERK (62). Fourth, we have observed that a dominant negative form of Rap1 suppresses the late phase of integrin-mediated activation of ERK in Swiss-3T3 cells. Finally, introduction of B-Raf enhances and prolongs integrin signaling to ERK in wild-type primary fibroblasts, but not in those carrying a targeted deletion of the C-terminal segment of $\beta_1$. It remains possible, however, that Crk activates ERK also through mSOS (52). Although this second pathway does not naturally explain the cell type specificity of the contribution of FAK to the activation of ERK, more studies are necessary to address whether it contributes to the overall response. Whatever is the precise mechanism, our results suggest that FAK can regulate the late phase of ERK activation in a cell type-specific manner.

What is the physiological significance of the more protracted integrin-mediated activation of ERK observed in cells expressing B-Raf? PC12 cells, which express B-Raf, are induced to differentiate to sympathetic neurons and extend neurites when plated on laminin or collagen in the presence of nerve growth factor (63). There is evidence that this process requires a sustained activation of ERK (52, 64). It is tempting to speculate that integrins and the nerve growth factor receptor cooperate to activate Rap1 and thereby mediate sustained activation of ERK in differentiating neuronal cells.

The findings in this paper suggest that significant differences in integrin signaling exist not only among cell types but also between primary and immortalized cells, thus providing a potential explanation for a number of previous, apparently discrepant results. Our results provide further evidence that integrins activate Shc signaling to ERK independently of FAK in primary cells. FAK/Src signaling, however, can sustain the activation of ERK initiated by Shc in cells expressing B-Raf. In this model, B-Raf acts as a switch to connect Crk signaling to ERK (Fig. 7). Thus, integrins not only activate multiple signaling pathways, but they do so in a cell type-specific and temporally distinct manner. In retrospect, this added level of complexity in integrin signaling is not surprising, considering that integrins form relatively large aggregates at the plasma membrane and interact not only with many cytoskeletal and signaling components but also with several other transmembrane proteins (4). Future studies will undoubtedly shed more light into the features that distinguish integrins from other signaling receptors and the mechanisms by which integrins cooperate with growth factor receptors to regulate cell behavior.

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**Distinct Roles of the Adaptor Protein Shc and Focal Adhesion Kinase in Integrin Signaling to ERK**

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